Investigating the effects of mobile phone radiation and heating on spermatogenesis and sperm function

Brendan James Houston

B. Biotech (Hons Class I)

University of Newcastle

Thesis submitted to the Faculty of Science and Information Technology, The University of Newcastle, Australia in fulfilment of the requirement of the degree of the Doctor of Philosophy

Date: 30th June, 2017

Declaration

Originality statement

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository**, subject to the provisions of the Copyright Act 1968. **Unless an Embargo has been approved for a determined period.

Statement of Authorship

I hereby certify that the work embodied in this thesis contains a published paper(s) / scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publication(s) / scholarly work.

Signed

Brendan James Houston

Acknowledgements

My biological journey started quite a few years ago now, when I chose to study a bachelor of biotechnology. In the first year of this program I was exposed to two amazing scientists, who I was lucky enough to have secured as my PhD supervisors. John hooked in everyone from his first talk and Brett was incomparable as a lecturer. It was in my third year that I met another great scientist, Geoff, who has helped to shape me extensively over the years. To John, thank you for your guidance, advice and verve. You are a truly remarkable scientist. To Brett, I'm so glad to have been able to involve you more in my PhD. You remained positive, and supported and encouraged me continually; I am very thankful for this. To Geoff, I'm grateful of all the assistance and insight you've given me, and your calm and friendly nature. I'm glad we were able to reinvigorate the EMR project and thank you for all of your support. To Bruce, thank you for your help with the EMR project and addressing all my questions and problems. I've learnt so much from all of you over my four and a half years here in the repro lab and I am very grateful for all your support, words of advice and colossal wealth of knowledge.

I wouldn't have made it through those less motivated times without the support of all the office crew. First of all, a massive thanks to my science partners in crime. Jakinta, you've been there for me throughout it all and we've had tonnes of fun in between that too. You keep me scientifically level-headed and I am deeply grateful for the friendship we've forged. Bettina, we've been friends since undergrad, have spent the longest time together and I'm thankful of our happy and fun times. Of course there's also Aimee, the determined; Sally, with the midas touch; Liz, the angel; Alle, the creative; Nicole, the dedicated; Tessa, the accomplished; Taylor, the friendly; Nat, the proficient and Jess, the enthusiastic. Thanks to my biotech pals Michael, Adam and Grace who have also stuck it out in the PhD realm, as well as my peers Chamila, Wei, Jinwei, Caitlin, Brandan, Emma, Nikki, Matt and Claire and everyone else. We've become an expanded family, set to colonise all the corners of the world and spread our scientific knowledge. Thank you everyone for all your help, support and, most importantly, friendship.

I have the utmost respect for our research assistants, who work tirelessly to conduct many, many experiments. Haley and Sara, you have helped me out extensively over my time here and I'm so appreciative of everything you've done for me. Kristen, I am very thankful of your help with my

experiments, you have made my time easier with your assistance during my days of multiple mice. To Amanda, Mona, Ilana, Sarah, Kasey, Barb, Louise and all the other RAs who have helped me over my time here, thank you for showing me the way, and for lending me science tips from your banks of knowledge. Throughout my PhD I was also fortunate enough to receive other forms of mentoring and advice from some great scientists; Shaun, Zamira, Jessie and Kate, and I'm quite thankful for these experiences and friendships. To Angela, who has always looked out for me and has had time to chat. You've ensured that I was able to see John at many crucial times and are an important pillar of the group. Remember that I'm your favourite and Jacinta is number 2.

Of course I could not go without acknowledging my favourite ROS molecule, hydrogen peroxide - keep up the good work as a positive control.

Dealing with multiple mice is never a simple task, but was made easy with the enormous efforts of Hayley and Alecia in the animal house. I am greatly appreciative of your help with any little enquiry or modification (and we all know I had many, elaborate requests).

To my oldest friends, most who are sort of unsure what I do, thank you for your support over the years. To my greatest friend, Angela, I cherish our friendship, synchronicity and laughs, and will forever. To Michelle, Emma, Nick, Peter, and everyone else, you're an amazing part of my life and a great foundation. To my rover buddies, thanks for your friendship and relating to the overnight shift experiences.

Finally, to all my family, thanks for the support through my 'IVF studies' and being excited when I was published or got to attend exciting conferences. To my parents, thanks for being supportive and proud of my accomplishments, and to my Nan, thanks for putting up with my eccentric and crazy self and for helping to make my life easier over the last 7.5 years.

EDIT 13-1-18

My move to the O'Bryan lab at Monash University has been comfortable and easy thanks to the kindness and support of everyone. Thanks to Moira, Anne, Jo, Christiane, Avi, Jess, Hide and Jinghua. Special shout-out to Vu for being an absolute champion and great friend.

Publications and awards arising from work in this thesis

Publications

CHAPTER 1

Houston, B. J., Nixon, B., King, B. V., De Iuliis, G. N. and Aitken, R. J. 2016. The effects of radiofrequency electromagnetic radiation on sperm function. *Reproduction*. 152(*6*), R263-276.

CHAPTER 2

Submitted *Frontiers in Public Health*

CHAPTER 4

Houston, B. J., Nixon, B., Martin, J. H., De Iuliis, G. N., Bromfield, E. G., McEwan, K. E. and Aitken, R. J. 2018. Heat exposure induces oxidative stress and DNA damage in the male germ line. *Biology of Reproduction*. Accepted 13/1/18.

Statements of contribution

I attest that the Research Higher Degree candidate Brendan Houston has contributed upward of 50% towards data collection/analysis and manuscript preparation for all the publications included in this thesis for which I am a co-author.

L. Prof John Aitken

Date:

Prof. Brett Nixon

Date:

Dr. Geoffry De Iuliis

Date:

Prof. Bruce King

Date:

Jacinta Martin

Date:

Kristen McEwan

Date:

Dr. Elizabeth Bromfield

Date:

Conference proceedings related to this thesis

Houston, B.J, King, B.V. and Aitken, R.J. Radiofrequency electromagnetic radiation and the male reproductive tract. 12th International Symposium on Spermatology. Newcastle, Australia. August 2014 (Poster).

Houston, B.J, King, B.V. and Aitken, R.J. Radiofrequency electromagnetic radiation and the male germ line. 45th Annual Meeting for the Society for Reproductive Biology. Melbourne, Australia. August 2014 (Poster).

Houston, B.J, King, B.V. and Aitken, R.J. Radiofrequency-electromagnetic radiation and male reproduction. 19th Annual University of Newcastle SELS RHD conference. Newcastle, Australia. December 2014 (Oral).

Houston, B.J, King, B.V. and Aitken, R.J. Radiofrequency electromagnetic radiation and the male reproductive tract. Australian Society for Medical Research Newcastle Satellite Scientific Meeting. Newcastle, Australia. March 2015 (Poster).

Houston, B.J, Nixon, B., King, B.V., De Iuliis, G.N and Aitken, R.J. The effects of radiofrequency-electromagnetic radiation on the male germ line. 46th Annual Meeting for the Society for Reproductive Biology. Adelaide, Australia. August 2015 (Oral).

Houston, B.J, Nixon, B., King, B.V., De Iuliis, G.N and Aitken, R.J. Whole body exposure to RF-EMR induces DNA damage in mouse spermatozoa. Australian Society for Medical Research (ASMR) Newcastle Satellite Scientific Meeting. Newcastle, Australia. April 2016 (Oral).

Houston, B.J, Nixon, B., King, B.V., De Iuliis, G.N and Aitken, R.J. Whole body exposure to radiofrequency electromagnetic radiation induces DNA damage in mouse spermatozoa. 47th Annual Meeting for the Society for Reproductive Biology. Gold Coast, Australia. August 2016 (Oral).

Houston, B.J, Nixon, B., King, B.V., De Iuliis, G.N and Aitken, R.J. Whole body exposure to radiofrequency electromagnetic radiation induces DNA damage in mouse spermatozoa. IBS

2016, the 17th International Biotechnology Symposium and Exhibition. Melbourne, Australia. October 2016 (Poster).

Houston, B.J, Nixon, B., King, B.V., De Iuliis, G.N and Aitken, R.J. Whole body exposure to radiofrequency electromagnetic radiation induces DNA damage in mouse spermatozoa. 21st Annual University of Newcastle SELS RHD conference. Newcastle, Australia. December 2016 (Oral).

Houston, B.J, Nixon, B., De Iuliis, G.N and Aitken, R.J. Whole-body heating induces DNA fragmentation and impairs motility in mouse spermatozoa. ICA, the 11th International Congress of Andrology. Copenhagen, Denmark. May 2017 (Poster).

Additional publications

Houston, B. J., Curry, B. and Aitken, R. J. 2015. Human spermatozoa possess an IL4I1 l-amino acid oxidase with a potential role in sperm function. *Reproduction*. 149(*6*), 587-96

Awards

Oozoa Award finalist for best student presentation, Society for Reproductive Biology national conference (2015, 2016).

Second place, best student presentation, Australian Society for Medical Research Newcastle satellite meeting (2016).

International Society of Andrology travel grant, Denmark International Congress of Andrology meeting (2017).

Faculty of Science and Information Technology International Travel Grant, University of Newcastle, Australia (2017).

TABLE OF CONTENTS

Abstract

Male infertility is a common issue affecting 7% of the population, and recent emphasis has been placed on the involvement of environmental pressures in eliciting or exacerbating this condition. We live in a world where we are ubiquitously exposed to radiofrequency electromagnetic radiation (RF-EMR), emitted from a variety of electronic devices for the purpose of communication. The biological effects of this radiation are under active debate due to the absence of a widely supported cellular mechanism of action that could account for the inconsistent experimental outcomes documented in response to RF-EMR exposure. However, a growing body of evidence suggests that RF-EMR may act as a stimulator of cellular oxidative stress. In a similar context, we are now also experiencing warmer climates in line with a state of global warming, and it is known that heat is capable of negatively affecting the sensitive physiological environment of the mammalian male reproductive system. While oxidative stress has similarly been implicated in this pathology, the complete reproductive consequences of heating the male reproductive system are not fully understood.

The collective studies described in this thesis were formulated to address the overarching hypothesis that environmental exposures are capable of eliciting a state of oxidative stress within the male reproductive system, particularly affecting the spermatozoa produced under these conditions and leading to impaired male reproductive health. Furthermore, it was hypothesized that the physical factors investigated in this thesis were acting upon the mitochondria of male germ cells and spermatozoa to induce this stress. Accordingly, my specific aims were to dissect the mechanisms by which RF-EMR and environmental heating affect sperm quality, and particularly to advance our understanding of how RF-EMR interacts with biological systems and cells.

Through studying the effects of RF-EMR and ambient temperature heating on the male reproductive tract, we add weight to the concept that the male reproductive system is sensitive to such a state of oxidative stress. We have identified important mechanistic features accounting for the pathology of these stresses. Here, we exposed isolated murine male germ cells and spermatozoa to RF-EMR, which supported our hypothesis that this insult targets the mitochondria of these cells, resulting in the generation of mitochondrial ROS, a subsequent state of oxidative

stress and reduced sperm quality. Similarly, when whole body heating was used as the insult, spermatozoa were compromised in their motility and membrane integrity in association with elevated ROS originating from the mitochondria. Furthermore, with both heating and RF-EMR exposure, sperm oxidative DNA damage was significantly elevated.

We have also generated evidence to support Complex III of the mitochondrial electron transport chain as a likely target of RF-EMR in male germ cells. Our understanding of this mechanism will direct future studies, where it will be important to focus on multigenerational approaches conducted at real-life exposure levels, to gain further understanding of the clinical effects of RF-EMR. With respect to ambient temperature heating, the round spermatids were particularly susceptible, but also spermatocytes, proliferating spermatogonia and spermatozoa exhibited susceptibility to this form of stress; whereas this was not the case for spermatogonial stem cells. However, the spermatozoa produced under both insults were readily capable of undergoing capacitation and fertilizing oocytes in an *in vitro* setting. Furthermore, the resulting embryos were not overtly inhibited in their early embryonic development. Nevertheless, such findings do not discount the possibility that damaged spermatozoa may have been excluded from the fertilization cascade in favour of their undamaged counterparts. Alternatively, it is possible that the consequences of these insults will present themselves at a later point in development, thus providing a clear imperative to conduct detailed exploration of the genetic and epigenetic integrity of the resultant embryos as well as the health of offspring in future studies.

In conclusion, the detrimental impacts elicited by environmental factors such as electromagnetic radiation and heat on the male reproductive tract, has identified that generating a state of oxidative stress is a key susceptibility of this biological system.

Foreword

A majority of this thesis focused on studies investigating the effects of electromagnetic radiation (RF-EMR) on spermatogenesis and sperm function, and the potential molecular mechanisms that may be involved. A final study included the effects of heating on spermatogenesis and sperm function, which is produced as a secondary effect of RF-EMR exposure at higher wave intensities (>~4 W/kg). These external insults both generate a state of oxidative stress in the male reproductive tract, a common vulnerability of this system. Initially, a literature review was conducted, analyzing published data to identify the effects of RF-EMR on sperm function. In this paper, the number of studies demonstrating clear effects induced by RF-EMR was weighed against those which did not. Furthermore, a biological mechanism to explain the effects was proposed, which was subsequently tested through two approaches. All chapters following this are presented in the form of a manuscript, as they will be submitted to be considered for publication. First, an *in vitro* study was undergone to explore the outcomes of RF-EMR exposure on cells within the male germ line, including mouse spermatogonial and spermatocyte-like cultured germ cells, and spermatozoa. Here, we unveiled evidence to support a mechanism of oxidative stress in these cell types and susceptibility of male germ cells and spermatozoa to RF-EMR, revolving around interference with the electron transport chain. This paper is currently under second revisions with *Andrology*. Next, the response of the male productive system to RF-EMR was characterized using a mouse whole body, *in vivo*, exposure. This study allowed us to further verify our proposed mechanism, whereby oxidative stress was again observed in the spermatozoa collected from these mice. Finally, the secondary effect of RF-EMR exposure at elevated doses was investigated, in the form of environmental heating. This is additionally relevant due to the state of global warming the world is experiencing. This conclusory study involved elevating ambient temperature and assessing the effects of this treatment on key germ cell maturation stages. As with RF-EMR, heat stress resulted in the propagation of oxidative stress throughout the testicular germ cells and epididymal spermatozoa.

CHAPTER 1: LITERATURE REVIEW

The effects of radiofrequency electromagnetic radiation on sperm function

Published: Reproduction (2016).152(*6*), R263-R276

Authors: Brendan J Houston[1], Brett Nixon[1], Bruce V King[2], Geoffry N De Iuliis[1]* and R John Aitken[1]*

[1] Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

[2] School of Mathematical and Physical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

* Co-senior author

Chapter 1: Overview

The purpose of this review was to consolidate the currently published studies regarding the effects of radiofrequency-electromagnetic radiation (RF-EMR) on sperm function in order to further understand how this form of radiation affects biological systems. One hindrance to the growth of this field is the contradictory results generated across the variety of cell and tissues types exposed to RF-EMR. Therefore, to characterize the subtle effects of this radiation, an emphasis was placed on spermatozoa as a model cell type. This review extends on the literature by proposing a mechanism of mitochondrial impairment resulting from RF-EMR to explain these changes.

As part of the first section of this paper, all studies investigating the effects of RF-EMR on the male reproductive system were compiled. Here, we identified a common result in many of the studies to be an elevation in the generation of reactive oxygen species in spermatozoa, losses to sperm motility and induction of sperm DNA damage. In light of this, it was concluded that RF-EMR has the capacity to induce a state of oxidative stress in this cell type. This was supported further by the observation that antioxidant co-supplementation was capable of reversing these hallmarks. This manuscript next examined the underlying reason for these molecular changes, through the proposal of a mechanism. Here, we broadened our scope to identify pathways commonly stimulated across other cell types. As part of a two-step mechanism, we proposed that RF-EMR induces mitochondrial dysfunction which leads to a state of oxidative stress.

Finally, a literature review was conducted to determine the effects of another prominent environmental factor, ambient temperature heating, on the male reproductive system and ultimately sperm function. Through this, it was highlighted that heating is also likely to induce a state of oxidative stress within the testis and epididymis, leading to reduced semen quality and poor embryonic development. Furthermore, this may be dependent on germ cells within the testis that are susceptible to this insult.

REPRODUCTION
REVIEW

The effects of radiofrequency electromagnetic radiation on sperm function

B J Houston[1], B Nixon[1], B V King[2], G N De Iuliis[1,*] and R J Aitken[1,*]

[1]*Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences and*
[2]*School of Mathematical and Physical Sciences, University of Newcastle, Callaghan, New South Wales, Australia*

Correspondence should be addressed to B J Houston; Email: brendan.houston@uon.edu.au

*(G N De Iuliis and R J Aitken contributed equally to this work).

Abstract

Mobile phone usage has become an integral part of our lives. However, the effects of the radiofrequency electromagnetic radiation (RF-EMR) emitted by these devices on biological systems and specifically the reproductive systems are currently under active debate. A fundamental hindrance to the current debate is that there is no clear mechanism of how such non-ionising radiation influences biological systems. Therefore, we explored the documented impacts of RF-EMR on the male reproductive system and considered any common observations that could provide insights on a potential mechanism. Among a total of 27 studies investigating the effects of RF-EMR on the male reproductive system, negative consequences of exposure were reported in 21. Within these 21 studies, 11 of the 15 that investigated sperm motility reported significant declines, 7 of 7 that measured the production of reactive oxygen species (ROS) documented elevated levels and 4 of 5 studies that probed for DNA damage highlighted increased damage due to RF-EMR exposure. Associated with this, RF-EMR treatment reduced the antioxidant levels in 6 of 6 studies that discussed this phenomenon, whereas consequences of RF-EMR were successfully ameliorated with the supplementation of antioxidants in all 3 studies that carried out these experiments. In light of this, we envisage a two-step mechanism whereby RF-EMR is able to induce mitochondrial dysfunction leading to elevated ROS production. A continued focus on research, which aims to shed light on the biological effects of RF-EMR will allow us to test and assess this proposed mechanism in a variety of cell types.

Reproduction (2016) **152** R263–R276

Introduction

Over the past 20 years, the use of mobile phones has increased exponentially (Gorpinchenko *et al*. 2014), with a current estimate of more than one billion users worldwide (French *et al*. 2001, Meral *et al*. 2007). In the United States, there is approximately one device in use per person, and well above more than one person in European countries such as Germany, Denmark and Italy (U.S. Census Bureau, 2012). Furthermore, the number of devices in service is rising at an estimated rate of 3% annually (ACMA 2013). Accordingly, the exposure of humans to radiofrequency electromagnetic radiation (RF-EMR) emitted from these devices has also increased substantially, with an average talk time of 30 min per day spent talking on mobile phones (CTIA 2011). The effect of this radiation on human health remains to be fully elucidated with current literature detailing an array of apparently contradictory results. Indeed, although some studies have identified pronounced deleterious effects of RF-EMR on a variety of cell types (Balode 1996, d'Ambrosio *et al*. 2002, Bilgici *et al*. 2013, Furtado-Filho *et al*. 2014, Hou *et al*. 2015, Kahya *et al*. 2014, Dasdag *et al*. 2015), others have reported only very subtle or no significant effects (Marchionni *et al*. 2006, Masuda *et al*. 2006, Dasdag *et al*, 2009, Demirel *et al*. 2012, Khalil *et al*. 2014). A confounding factor in these studies involves the use of differing RF intensity, frequency, exposure length and method of administration, which discounts the possibility of direct and robust study-to-study comparisons. Such variation attempts to simulate elevated levels of exposure in certain studies and real-life mobile phone exposure in others, which is extremely hard to model given the variability that exists in each of these parameters of intensity and frequency (Lerchl 2013). For instance, the intensity of RF-EMR emitted from mobile phones varies from ~0.1–4 W/kg (Fejes *et al*. 2005, Guney *et al*. 2007, La Vignera *et al*. 2012), whereas mechanistic studies have involved intensities as high as 27.5 W/kg (De Iuliis *et al*. 2009*a*). Regardless of these differences, the balance of evidence supports the principle that RF-EMR has the ability to induce cellular damage (Adams *et al*. 2014). In light of this conclusion

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI:10.1530/REP-16-0126
Online version via www.reproduction-online.org

and to work towards identifying real clinical risks, it is imperative that we develop an understanding of the mechanism(s) by which this form of radiation affects different biological systems.

Physical parameters of RF-EMR

Radiofrequency electromagnetic radiation is a form of microwave radiation. Its important properties include the frequency at which it is generated, measured in megahertz (MHz) or gigahertz (GHz), and the intensity of the waves, or the specific absorption rates (SAR), which is a measure of the rate of energy transfer from the electromagnetic field to particles in an absorber, defined at a particular point in the absorber (Durney 1986). The frequency of RF-EMR emitted by mobile phone devices is in the range of 900–1800 MHz, and the intensity of this radiation is generally restricted to a local limit of <2 W/kg and whole-body limit of 0.08 W/kg (Durney 1986, Chen 2007) to enforce safe exposure levels in humans. Meanwhile, the ability of RF-EMR itself to penetrate into the skin and body is dependent on the permittivity and conductivity of the irradiated tissue, as well as the wavelength of the radiation, which is inversely related to the wave frequency (Fig. 1). Therefore, at lower frequencies, the penetration of the RF-EMR is higher and devices operating in the 900 MHz range will irradiate the body more; approximately 25% of the body in humans compared with 20% penetration

Cell phone mode	Intensity (W/kg; 0, 10, 30 cm distance)
Standby	0.001
Talk (900 MHz)	0.011, 0.002, 0.003
Talk (1800 MHz)	0.008, 0.0009, 0.0002

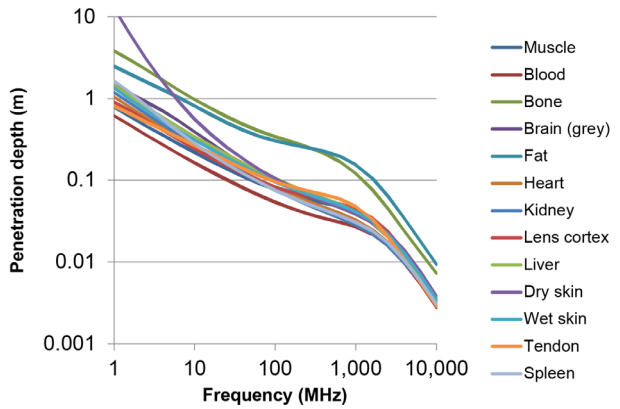


Figure 1 Physical aspects of radiofrequency electromagnetic radiation. A table identifying the estimated intensity of radiation emitted from devices in talk mode of either 900 or 1800 MHz (Durney 1986, Panagopoulos *et al*. 2010, Liu *et al*. 2013*a*) and plot of penetration depth of this radiation in different tissue types over the MHz-GHz ranges (Gabriel *et al*. 1996).

at 1800 MHz (Durney 1986). However, it is possible that the penetration of RF-EMR into the testis may be more pronounced than other tissues, due to the fact that this organ is less protected by tissue in comparison to others. Mobile phone communications uses a variety of different frequency ranges, with the most common utilising the 880–915 MHz range for the global system for mobile communications (GSM) 900 uplink (from mobile phone to base station), 925–960 MHz for the GSM900 downlink (from base station to mobile phone), 1710–1785 MHz for the DCS1800 uplink, 1805–1880 MHz for the GSM1800 downlink, 1920–1980 MHz for the universal mobile telecommunications system (UTMS) data uplink and 2110–2170 MHz for the UTMS data downlink (Bolte & Eikelboom 2012). Of particular interest is this radiofrequency range, in which a majority of studies have used exposure frequencies of 900–1800 MHz. This in turn forms the basis of studies selected for this review.

Review focus

For the purpose of this review, we shall focus on an analysis of the effects of RF-EMR on the male reproductive system, a site that may be uniquely vulnerable to chronic EMR exposure from devices stored in the vicinity of the testes that are held in 'standby mode' and, more importantly, at the initiation of a call or when hands-free mode is in use. Our specific interest is to draw a consensus regarding the impact of RF-EMR on the male germline, with an emphasis on frequencies that equate to analog/digital signals (900/1800 MHz (Irmak *et al*. 2002)) and with specific absorption rates (SAR) of up to 4 W/kg. We imposed strict search criteria, which gives this review focus on probing a potential mechanism of action, independent of its clinical significance. To source the appropriate studies, we used the following search terms: 'rf-emr spermatozoa'; 'radiofrequency electromagnetic radiation spermatozoa' and 'cell phone radiation + spermatozoa' in the PubMed database. Of those studies identified, we elected to review those reporting exposure at the RF range of between ~900 and 1800 MHz and those that focused on the male reproductive tract/spermatozoa. Such criteria were imposed to reflect the intensity of radiation emitted from the devices. This narrowed the list of articles to those summarised in Table 1. Largely independent of clinical significance, the unique cell biology of spermatozoa provides an ideal model in which the specific physical and chemical responses to EMR can be observed. These cells provide a sensitive model as (Aitken 2013, Aitken *et al*. 2014) (i) they are sensitive to damage by environmental factors including free radicals, (ii) they can be maintained for 48–72 h *in vitro* in simple, defined culture media, (iii) their motility provides a readily assessable means of monitoring adverse biological

Table 1 Review of studies investigating the effect of RF-EMR on the spermatozoa and male reproductive system of mice, rats and humans.

Reference	Species	Frequency (MHz)	Duration of exposure	Specific absorption rate (W/kg)	Motility	Vitality	ROS	DNA damage	Main outcomes
No effects									
Dasdag *et al.* (2003)	Sprague–Dawley rat	900	20 min per day, 4 weeks	0.52	NA	NA	NA	NA	No effects on testicular structure or sperm morphology
Imai *et al.* (2011)	Sprague–Dawley rat	1950	5 h per day, 5 weeks	0.4	NA	NA	NA	NA	No changes to epididymal or testis weights, increased sperm production with EMR treatment
Ozlem Nisbet *et al.* (2012)	Wistar rat	900/1800	2 h per day, 90 days	1.2–3/0.01–0.05 (900/1800)	–	NA	NA	NA	Increased sperm motility and morphology with EMR treatment
Sommer *et al.* (2009)	C57BL mouse	1966	24 h per day, 4 generations	0.08–2.34	NA	NA	NA	NA	No changes to sperm morphology, count, testis or epididymal weights
Trosic *et al.* (2013)	Wistar rat	915	1 h per day, 2 weeks	0.6	–	NA	NA	NA	No changes to motility, morphology or counts with EMR treatment
Tumkaya *et al.* (2013)	Sprague–Dawley rat	900	1 h per day, 45 days	0.48	NA	NA	NA	NA	No effects on testicular size, histology or spermatogenesis
Effects of RF-EMR									
Liu *et al.* (2013*a*)	Cultured mouse spermatocyte	1800	1 min per 20 min, 24 h	0.13	NA	NA	NA	+	Increased DNA single-strand breaks with radiation intensity which was prevented with antioxidant pre-treatment
Agarwal *et al.* (2009)	Human spermatozoa	850	1 h	1.46	+	+	+	–	Healthy semen donors and infertility patients both experienced a loss in motility, vitality coupled with increases in ROS production. Infertility patients experienced a decreased total antioxidant status
De Iuliis *et al.* (2009*a*)	Human spermatozoa	1800	16 h	1	+	+	+	+	Dose-dependent effects for all parameters. At 1 W/kg significant decreases in motility and vitality, increases in ROS and DNA damage
Erogul *et al.* (2006)	Human spermatozoa	900	5 m	NA	+	NA	NA	NA	Reduced rapid and slow progressive sperm motility
Falzone *et al.* (2011)	Human spermatozoa	900	1 h	2	NA	NA	NA	NA	Morphological impacts: reduced acrosome and total sperm head sizes as well as zona binding
Fejes *et al.* (2005)	Human spermatozoa	NA	NA	NA	+	NA	NA	NA	Questionnaire for mobile phone usage, duration of mobile phone usage correlated negatively with progressive motility
Gorpinchenko *et al.* (2014)	Human spermatozoa	900/1800	5 h	NA	+	–	NA	+	Reduced progressive sperm motility, increased DNA fragmentation

(Continued)

Table 1 *Continued*.

Reference	Species	Frequency (MHz)	Duration of exposure	Specific absorption rate (W/kg)	Motility	Vitality	ROS	DNA damage	Main outcomes
Wdowiak *et al*. (2007)	Human spermatozoa	NA	0–2 years use of phone	NA	+	NA	NA	NA	Reduced sperm motility and increased irregular morphology
Zalata *et al*. (2015)	Human spermatozoa	850	60 min	NA	+	NA	NA	+	Significant reductions to sperm motility of men with astheno-spermia and oligospermia, significant induction of DNA damage in sperm from healthy and sub-fertile semen profiles
Liu *et al*. (2015)	Sprague–Dawley rat	900	2 h per day, 50 days	0.66	NA	NA	+	NA	Decreased epipidymis:body weight ratio, sperm count and total antioxidant capacity. Increased ROS concentration, apoptosis and ultrastructural neck deformations
Yan *et al*. (2007)	Sprague–Dawley rat	1900	6 h per day, 18 weeks	1.8	+	+	NA	NA	Significantly reduced sperm motility and vitality, abnormal sperm clumping
Aitken *et al*. (2005)	Swiss mouse	900	12 h per day, 7 days	0.09	–	–	NA	NA	No changes to motility, vitality, concentration or morphology with low SAR and duration. However, degradation to sperm mitochondrial genome
Al-Damegh (2012)	Wistar rat	900/1800	60 min per day, 14 days	0.9	NA	NA	+	NA	Antioxidant treatment prevented seminiferous tubule widening and reduced the lipid peroxidation onset by EMR treatment
Bin-Meferij and El-kott (2015)	Wistar rat	900	1 h per day, 8 weeks	NA	+	+	+	NA	Antioxidant treatment ameliorated a reduction in sperm motility, vitality, count, lipid peroxidation and morphological abnormalities observed with EMR exposure
Dasdag *et al*. (1999)	Wistar rat	900	3 min per day, 4 weeks	0.141	NA	NA	NA	NA	Thinning of seminiferous tubules, decreased progression of spermatogenesis. However, potential temperature influences
Ghanbari *et al*. (2013)	Wistar rat	915–950	8 h per day, 2–3 weeks	NA	+	+	+	NA	Time-dependent decreases to motility, vitality and antioxidant capacity
Kesari *et al*. (2011)	Wistar rat	900	2 h per day, 5 weeks	0.9	NA	NA	+	NA	Decreased glutathione peroxidase, superoxide dismutase, histone kinase expression; increased ROS, lipid peroxidation and apoptosis

Kesari and Behari (2012)	Wistar rat	900	2 h per day, 45 days	0.9	NA	NA	NA	Increased caspase activity, morphological abnormalities; decreased testosterone levels, progeny weight and number
Mailankot *et al.* (2009)	Wistar rat	900/1800	1 h per day, 4 weeks	NA	+	NA	NA	Reduced sperm motility, but not sperm count; increased MDA and decreased glutathione content of the testis and epididymis
Ozorak *et al.* (2013)	Wistar rat	900/1800	1 h per day, 4–6 weeks	0.18	NA	NA	NA	Significantly lower lipid peroxidation and total antioxidant status in the testis with 4-week EMR treatment. This change was a significant increase with EMR treatment after 6 weeks
Tas *et al.* (2014)	Wistar rat	900	3 h per day, 1 year	0.04	–	NA	NA	Increased morphological defects: tunica albuginea thinning, impaired spermatogenesis. No effects on sperm motility or concentration

NA, not mentioned or conducted in study; + negative effects documented; – no effects documented. Table arranged by model species used in study. EMR, electromagnetic radiation; MDA, malondialdehyde; ROS, reactive oxygen species; SAR, specific absorption rate.

effects and (iv) they are clinically important because DNA damage in spermatozoa has the potential to influence the health and wellbeing of the offspring. As a consequence of the information summarised in this review, we propose a mechanism for the negative effects of RF-EMR on the male germline. Given the unique susceptibility of spermatozoa to subtle oxidative insults, which may arise from RF-EMR exposure, the translation towards clinical significance, especially involving other cell types, should not be made. However, given that spermatozoa may be acutely sensitive to stressors such as RF-EMR, we propose that a clear hypothesis for a mechanism of action can be developed using this model, which can then be applied for testing in other cell types.

The impact of RF-EMR on semen quality

Mobile phone use is becoming increasingly popular worldwide, with specific population groups, including businessmen and adolescents, estimated to spend as much as half of their day in close proximity to mobile phones held in either active or standby modes (Redmayne *et al.* 2011, Roberts *et al.* 2014). Owing to the common practice of storing mobile phones in close proximity to the testes, these individuals may be unintentionally exposing their reproductive system to relatively high levels of RF-EMR. It is therefore of considerable concern that the use of mobile phones (Fejes *et al.* 2005, Yan *et al.* 2007, Agarwal *et al.* 2009, Gorpinchenko *et al.* 2014, Zalata *et al.* 2015), or exposure to RF-EMR emitted by these devices (De Iuliis *et al.* 2009*a*, Al-Damegh 2012, Ghanbari *et al.* 2013), has been linked to negative impacts on semen quality. Notwithstanding considerable controversy regarding the timing and nature of such exposures (Dasdag *et al.* 2003, Imai *et al.* 2011, Tumkaya *et al.* 2013), the principle that RF-EMR can elicit a detrimental effect on sperm function is supported by a growing number of studies (Fejes *et al.* 2005, Agarwal *et al.* 2009, Mailankot *et al.* 2009, De Iuliis *et al.* 2009*a*, Liu *et al.* 2013*a*,*b*, Gorpinchenko *et al.* 2014). In general, these data lend support to the notion that RF-EMR can significantly impair key aspects of sperm function including the motility and vitality of these cells and the integrity of their DNA (Table 1), suggesting a direct effect on mature spermatozoa. However, there is less compelling evidence to suggest an additional role at the level of spermatogenesis in reducing sperm counts *in vivo* (Imai *et al.* 2011, Tas *et al.* 2014). Indeed, a chronic, multigenerational study demonstrated RF-EMR to have no effects on sperm production and testicular or epididymal weight (Sommer *et al.* 2009).

Direct effects of RF-EMR on spermatozoa

In one of the earliest studies on the impact of RF-EMR on sperm quality, Wdowiak and coworkers (2007) demonstrated that males who use mobile phones exhibit

increased rates of abnormal sperm morphology and decreased motility compared with counterparts who did not use these devices. Furthermore, these effects were exacerbated with longer exposure to this form of radiation (Wdowiak *et al.* 2007). Since this report, additional studies have replicated the adverse impact of RF-EMR treatment on human sperm motility using a model waveguide device capable of emitting finely tuned electromagnetic radiation to mimic that emitted by mobile phones (Gajda *et al.* 2002, De Iuliis *et al.* 2009*a*). The waveguide approach improves control of exposure as well as replicates the use of a mobile phone held in talk mode (Agarwal *et al.* 2009).

Males experiencing subfertility, for example asthenozoospermia and oligozoospermia, appear to be particularly vulnerable to RF-EMR as highlighted by a marked decline in sperm motility after the exposure of semen samples to a mobile device for just 10 min (Zalata *et al.* 2015). Similar pronounced effects have also been documented after *in vivo* exposure of whole animals to a mobile phone operating in talk mode (Yan *et al.* 2007, Mailankot *et al.* 2009). In terms of the nature of the impaired motility, RF-EMR appears to primarily influence the capacity of spermatozoa to sustain forward progressive motility. Indeed, a study by Erogul and coworkers (2006) confirmed that the exposure of human spermatozoa to RF-EMR compromised their ability to sustain both rapid and slow progressive motility after an alarmingly brief exposure time of only five minutes. Although other studies have required longer exposure times (hours or days) to generate significant reductions in sperm motility, impaired progressive motility (involving a decrease in the percentage of cells displaying rapid progressive motility and a corresponding increase in cells expressing slow progressive motility) appears to be a common consequence arising from RF-EMR exposure (Fejes *et al.* 2005, Gorpinchenko *et al.* 2014) and was observed in 11/15 studies, as presented in Table 1.

Nevertheless, these studies must be considered alongside others in which the presence of RF-EMR had no overt effect on either progressive (Tas *et al.* 2014) or overall sperm motility (Aitken *et al.* 2005, Imai *et al.* 2011, Trosic *et al.* 2013). A possible explanation for such inconsistencies in the effects of RF-EMR on sperm motility rests with the use of different exposure conditions. Indeed, in a majority of studies reporting negative impacts of RF-EMR on sperm motility (64%), the study design featured the use of isolated human spermatozoa that were exposed to RF-EMR via a mobile phone device. In contrast, at least half of the instances in which no effect was recorded on sperm motility, the studies involved whole-body animal exposure using a signal generator to produce the RF-EMR (Aitken *et al.* 2005, Trosic *et al.* 2013, Tas *et al.* 2014). Although these data further lend support to our proposal of spermatozoa as a sensitive model, they also highlight that *in vivo,* the body may be capable of absorbing some of this radiation (Fig. 1), thus, diminishing the level of exposure experienced by spermatozoa within the reproductive system.

Effects of RF-EMR on spermatogenesis

In addition to the studies indicating that the RF-EMR can have detrimental effects on sperm function, there are sporadic reports that this type of radiation can also affect the testes. It has been demonstrated that a 60-minute exposure of male rats to RF-EMR daily for two weeks can cause widening of the seminiferous tubules (Al-Damegh 2012). In contrast, Dasdag and coworkers (1999) documented a thinning of seminiferous tubules in response to an intermittent mobile phone exposure of three minutes (on and off) for 2 h per day in active talk mode every day for one month. To add further difficulty to the interpretation of these data, a subsequent study by the same authors (Dasdag *et al.* 2003), reported no changes to testis structure after a similar RF-EMR exposure time of 20 min every day for one month. In addition to potential impacts on the diameter of the seminiferous tubules, chronic exposure (3 h per day for one year) of rats to RF-EMR reportedly elicited a reduction in the thickness of the tunica albuginea (Tas *et al.* 2014). Prolonged exposures (6 h daily over a 100-day period) have also been associated with patterns of sperm aggregation that were absent from unexposed rats and independent of any impact on sperm morphology (Yan *et al.* 2007). Nevertheless, abnormal sperm morphology arising from RF-EMR exposure has been documented (Wdowiak *et al.* 2007). In humans, these abnormalities have primarily been associated with the sperm head leading to a reduced capacity to engage in interactions with the oocyte (Falzone *et al.* 2011). Curiously however, Ozlem Nisbet and coworkers (2012) suggest that this form of insult appears to have no effect on the head morphology of rat spermatozoa at a frequency of 900 MHz, but instead alleviates the incidence of tail abnormalities and promotes a suite of positive functional outcomes, including increased testosterone levels and superior progressive motility. Furthermore, this group observed better formed seminiferous epithelia with 1800 MHz exposure that was not seen in 900 MHz or unexposed treatments. Moreover, another study involving exposure during pubertal development documented RF-EMR to induce no changes to the spermatogenic cycle or testicular morphology (Tumkaya *et al.* 2013).

Notwithstanding the conflicting nature of the data documented above, recent meta-analyses performed by Adams and coworkers (2014) and Liu and coworkers (2014) have concluded that RF-EMR has two major negative impacts on sperm function: significant reductions in motility and loss of viability. In line with the recent studies by Mailankot and coworkers (2009) and Trosic and coworkers (2013), this analysis confirmed that sperm concentration is not significantly

influenced by RF-EMR treatment. Although these data suggest that RF-EMR is not capable of causing major disruptions to the spermatogenic cycle, in line with Sommer and coworkers (2009), they do nonetheless highlight an effect on the functional attributes of spermatozoa. Such findings are particularly concerning given that they are attributed, at least in part, to studies involving human spermatozoa and therefore bring into question whether RF-EMR may be having any negative impact on fertility in our species. Collectively, the uncertainty surrounding the effects of RF-EMR on the male germline presents a challenge for interpretation, which is further exacerbated by the lack of any consolidated, mechanistic explanation for the effects of such low-energy radiation on biological systems.

Molecular mechanisms of RF-EMR action

Here, we focus on studies documenting the effects of RF-EMR on biology, with the purpose of identifying common pathways that may direct our understanding of how this factor influences biological systems. Furthermore, unveiling a mechanism to explain the biological stresses of RF-EMR will allow us to then rationally assess the clinical relevance of certain exposure conditions.

Generation of oxidative stress

It has previously been hypothesised that the biological effects of EMR could be attributed solely to heat stress, which is induced at the higher intensities of approximately ≥4 W/kg radiation used in some studies (Hossmann & Hermann 2003, Li *et al.* 2007). However, through the use of various 'intermittent' exposure systems (e.g. 5 min on/10 min off), it has been demonstrated that the effects of bulk heat stress are likely to be negligible at the intensities of radiation generated during typical RF-EMR exposure (Liu *et al.* 2013*a*). Such results have subsequently been verified in the transformed GC2 mouse spermatocyte cell line, in which it was shown that such transient exposure patterns are capable of inducing DNA fragmentation and oxidised base adduct formation (Liu *et al.* 2013*b*, Duan *et al.* 2015) in the absence of a significant impact on temperature.

RF-EMR treatment is known to have the capacity to induce oxidative stress, characterised by excessive generation of reactive oxygen species (ROS) that overwhelm the intrinsic cellular antioxidant capacity, in a variety of tissue types. Indeed, this phenomenon has been documented after RF-EMR treatment in whole-body and ovarian tissue models of *Drosophila* (Manta *et al.* 2014), mouse fibroblasts (Hou *et al.* 2015), cultured breast cancer cells (Kahya *et al.* 2014), rat heart tissue (Ozguner *et al.* 2005), human lens epithelial cells (Yao *et al.* 2008) and mammalian spermatozoa (Agarwal *et al.* 2009, De Iuliis *et al.* 2009*a*, Kesari *et al.* 2011). We have also replicated this response using transformed male spermatogonial and spermatocyte germ cell lines, documenting an increase in ROS of mitochondrial origin (B Houston & R J Aitken 2015, unpublished observations). Furthermore, of the 27 RF-EMR exposure studies summarised in Table 1, at least 21 of these (78%) document negative effects of RF-EMR on one or more parameters of sperm function and/or testicular histology that are characteristic of responses elicited by oxidative stress, such as lipid peroxidation, impaired motility and the formation of oxidative DNA damage.

Such pronounced effects on the male germline may stem from the fact that spermatozoa are uniquely susceptible to oxidative stress. This vulnerability arises due to the highly specialised structure of the spermatozoon, featuring limited protective antioxidant capacity due to a diminutive cytoplasmic volume and, at the same time, an abundance of substrates for free radical attack including DNA, thiol-rich proteins and polyunsaturated fatty acids (PUFAs) (Aitken *et al.* 2012*a*). The latter are of critical importance to the spermatozoon and are required to generate the membrane fluidity needed to support both motility and the membrane-fusion events associated with fertilisation (Lenzi *et al.* 2000). Yet when peroxidised, PUFAs elicit the formation of small molecular mass, electrophilic aldehydes that perpetuate a state of oxidative stress (Aitken *et al.* 2012*a*) as detailed in Fig. 2.

Human spermatozoa exposed to RF-EMR exhibit significant increases in mitochondrial and cytosolic superoxide formation (Agarwal *et al.* 2009, De Iuliis *et al.* 2009*a*), as well as a significant reduction in sperm motility (Fejes *et al.* 2005, Gorpinchenko *et al.* 2014). The causative link between excess ROS production and sperm motility loss is a well-established paradigm in sperm biology (Fig. 2). This is commonly attributed to increased lipid peroxidation and the ensuing formation of electrophilic aldehydes such as malondialdehyde, 4-hydroxynonenal (4HNE) and acrolein, which are capable of covalently binding to proteins, thus compromising their function (Jones *et al.* 1979, Koppers *et al.* 2008, 2010, Aitken *et al.* 2012*a*,*b*, Moazamian *et al.* 2015). In the case of sperm motility, these compounds appear to alkylate sperm axonemal proteins that regulate sperm motility, particularly dynein heavy chain (Baker *et al.* 2015, Moazamian *et al.* 2015). In addition, electrophiles such as 4HNE are also known to promote oxidative stress by stimulating ROS generation through the sperm mitochondria (Fig. 2). This situation arises because another group of proteins alkylated by 4HNE is the constituents of the mitochondrial electron transport chain (ETC), particularly succinic acid dehydrogenase (Aitken *et al.* 2012*b*). When these proteins become adducted by 4HNE, it promotes the leakage of electrons from the ETC, which are then

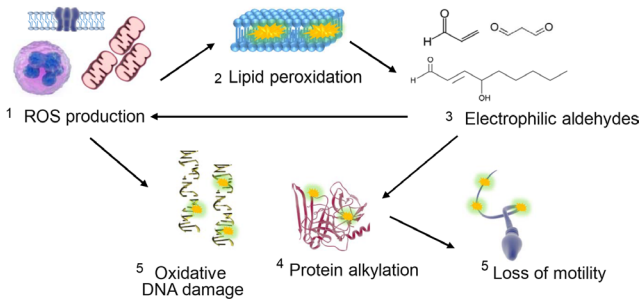


Figure 2 Oxidative stress cascade within the spermatozoon. ROS is formed within the cell from a variety of possible sources including mitochondrial dysfunction, plasma membrane NADPH oxidase activity, infiltrating leukocytes and environmental factors such as electromagnetic radiation. In the event these ROS outweigh the poor antioxidant capacity of the cell, or a deficiency in this protection exists, a state of oxidative stress ensues. ROS, particularly hydrogen peroxide, attack the lipid membranes which are richly bestowed with polyunsaturated fatty acids that are susceptible to oxidative attack, resulting in the formation of small, reactive aldehydes – acrolein, malondialdehyde and 4-hydroxynonenal. Although these aldehydes differ in their reactivity (Moazamian *et al.* 2015), they each target a specific subset of protein centres, typically thiol constituents, as a form of nucleophilic attack. One major consequence of this is impairment of protein function, such as key proteins involved in sperm motility. Succinate dehydrogenase, a protein complex within the mitochondria, is a predominantly vulnerable target of these electrophilic aldehydes, and alkylation of this complex results in the disruption of redox-regulated metabolism within the mitochondria, forcing electron flow to oxygen and thus forming yet more superoxide anion. Furthermore, this imbalance of ROS leads to oxidative DNA damage as hydrogen peroxide migrates to the sperm head and preferentially targets guanine residues within the sperm DNA, highlighted by significant increases in the oxidised base product 8-hydroxy-2′-deoxyguanosine.

consumed by the universal electron acceptor, oxygen, to generate superoxide anion (Aitken *et al.* 2012*b*). By such mechanisms, even slight increases in ROS induced by RF-EMR have the potential to become amplified through the mediation of the mitochondria. In support of this mechanism, it has been revealed that RF-EMR-induced ROS production does encourage lipid peroxidation in spermatozoa (Kesari *et al.* 2011, Al-Damegh 2012). Moreover, lipid peroxidation has also been localised within the testicular and epididymal microenvironments after RF-EMR treatment *in vivo*, and this has, in turn, been associated with a loss of sperm motility (Mailankot *et al.* 2009).

If RF-EMR is responsible for the induction of oxidative stress, we should see evidence of ROS overwhelming the sperm cell's antioxidant defences under these conditions (Gharagozloo & Aitken 2011). Indeed, intracellular concentrations of glutathione peroxidase and superoxide dismutase have been shown to be compromised in the spermatozoa of RF-EMR-exposed rats (Kesari *et al.* 2011). Furthermore, the addition of exogenous antioxidants such as vitamin C or E has been shown to significantly diminish RF-EMR-induced lipid peroxidation, while simultaneously leading to a partial restoration of the glutathione content of the testis in RF-EMR-exposed rats (Al-Damegh 2012). As an extension of this work, both spermatozoa (Kesari *et al.* 2011) and testes (Al-Damegh 2012) respond by increasing catalase activity after exposure to EMR. This potentially represents a physiological response aimed at counteracting increases in hydrogen peroxide and other ROS formation induced by RF-EMR stress. Interestingly, it has been suggested that RF-EMR may have more pronounced effects in poor quality spermatozoa as revealed in studies where only a proportion of the sperm population was found to respond to RF-EMR treatment (De Iuliis *et al.* 2009*a*). If this was the case, then the increased ROS production generated in these highly vulnerable cells could reasonably be expected to impose an oxidative stress environment upon the remainder of the sperm population (Tosic & Walton 1950).

Downstream of lipid peroxidation, oxidative stress is known to culminate in oxidative damage to sperm DNA (Fig. 2). This has been characterised by elevated levels of the DNA damage marker, 8-hydroxy, 2′-deoxyguanosine (8OHdG; Aitken *et al.* 2012*b*,*c*, Aitken *et al.* 2014). Accordingly, RF-EMR exposure has been shown to elicit a significant increase in the staining intensity for this marker in human spermatozoa (De Iuliis *et al.* 2009*a*). RF-EMR has also been correlated with DNA strand breakage in spermatozoa (Zalata *et al.* 2015), cultured spermatogonia (B Houston & R J Aitken 2015, unpublished observations) and spermatocyte cells (Liu *et al.* 2013*a*). In the latter cell type, the DNA damage was successfully ameliorated by co-incubation of the cells with the antioxidant, melatonin (Liu *et al.* 2013*a*). Meanwhile, the observation that RF-EMR has the potential to generate sperm DNA damage is especially concerning due to the fact that these cells are capable of harbouring a considerable oxidative DNA damage load independent of any pronounced effects on motility (Aitken *et al.* 1998). These spermatozoa therefore have potential to participate in fertilisation, whereupon the oocyte would bear the responsibility for repairing the DNA before the initiation of S-phase of the first mitotic division. The fact that oocytes are relatively deficient in the first enzyme in the base excision repair pathway, OGG1 (Lord & Aitken 2015), means that any 8OHdG brought into the egg by the fertilising spermatozoon are likely to persist into the first cleavage division. As 8OHdG lesions are potentially mutagenic, these considerations may carry implications for the mutational load subsequently carried by the offspring, if the father's germline has been oxidatively damaged by RF-EMR.

The ability of RF-EMR to induce damage, which leads to negative biological outcomes is yet to reach consensus; nevertheless, biological effects of RF-EMR are more strongly demonstrated in the literature and are likely to depend on the properties of the affected macromolecule. With respect to proteins, it is expected that this form of damage could be resolved upon

turnover or degradation. However, in the case of long-lived molecules such as DNA, the impact of such damage could be far more insidious. This is particularly the case in the male germline where the integrity of the paternal genome has direct implications for future generations. Of particular concern is the potential for the damage to be acquired in post-meiotic germcells, which have limited DNA repair mechanisms and are therefore unequipped to resolve the damage. This has been shown previously in spermatozoa, by the existence of dominant lethal mutations (Singer *et al.* 2006), which indicate the possibility of these mutations to be transferred through one generation. Given the strong paradigm for oxidative stress as a key mediator of sperm quality and that published data support the conclusion that RF-EMR can drive ROS production in the male germline, understanding how RF-EMR induces ROS is therefore of key importance.

Metabolic pathways activated by RF-EMR

It has been demonstrated that RF-EMR has the ability to stimulate signalling pathways in somatic cells, such as those associated with the extracellular signal-regulated kinase (ERK) cascade (Friedman *et al.* 2007) or heat-shock protein response (Di Carlo *et al.* 2002, Li *et al.* 2007, Valbonesi *et al.* 2014). As both of these pathways are known to be redox regulated, it is possible that RF-EMR activates these signal transduction cascades as a secondary consequence of ROS production (Christman *et al.* 1985, Polla *et al.* 1996, Nahomi *et al.* 2015). As indicated previously, the major site of intracellular ROS generation observed after RF-EMR exposure is the mitochondria.

There are several lines of evidence that point to the mitochondria being the major mediator of RF-EMR action of biological systems. Thus, in pancreatic cancer cells, it has been shown that EMR has the ability to induce extensive changes to the morphology of the mitochondria, stimulating a loss of their membrane potential and significantly increasing production of ROS (Curley *et al.* 2014). This effect is mirrored across a variety of additional somatic cell types including rat hippocampal slices where EMR evokes substantial changes to mitochondrial morphology (Zhao *et al.* 2012) and membrane potentials (Tattersall *et al.* 2001), and human peripheral blood monocytes where it induces a transient decrease in mitochondrial membrane potential that is accompanied by increased ROS production and caspase activation; the latter of which are hallmarks of an apoptotic cascade (Lu *et al.* 2012). As indicated previously, there is also very clear evidence that RF-EMR activates mitochondrial ROS generation in spermatozoa (De Iuliis *et al.* 2009*a*).

Although such effects of RF-EMR have been recorded at radiofrequency levels of around 900–1800 MHz, corresponding to that emitted by mobile phones (Marchionni *et al.* 2006), contradictory stimulatory effects have in fact been observed at very low frequencies, less than 100 MHz (Marchionni *et al.* 2006, Iorio *et al.* 2011). Indeed, in marked contrast to the negative effects of RF-EMR, extremely low-frequency EMR (50 Hz) has in fact been shown to encourage sperm motility (Iorio *et al.* 2011). This effect is also believed to be a consequence of altered mitochondrial activity; however, in this instance, it appears that the EMR exposure leads to an increase in mitochondrial membrane potential (Iorio *et al.* 2011). Such a discrepancy may be explained, at least in part, by the variable degree of penetration achieved with EMR of different wavelengths (Lin 1976; Fig. 1). In this context, it is well established that the intensity of the RF-EMR decays exponentially as it penetrates the skin, whereas penetration depth varies between different tissues and organs (Fig. 1; De Iuliis *et al.* 2012, Markov & Grigoriev 2015). This radiation exposure generally depends on emitted power, but to some extent, it also depends on other parameters such as the frequency, antenna position relative to the body and the material properties of the absorbing tissue (Balzano 1999). In any case, the biophysics involved in these types of interactions is unresolved and represents a major limitation regarding RF-EMR studies (Lerchl 2013). We have also observed subtle variations in the response to RF-EMR when assessing mitochondrial function in male germ cells at different stages of maturation, with vulnerabilities to RF-EMR appearing to be dependent on the stage of development (B Houston & R J Aitken 2015, unpublished observations). This again highlights the potential difficulties with interpreting and rationalising the effects of RF-EMR on biology, given the diversity of cells that are potentially exposed by mobile phone use.

It is also probable that the variation in mitochondrial membrane potential stimulated by EMR is dependent on SAR, as extremely low-intensity radiation (2.5×10^{-5} W/kg) fails to alter mitochondrial membrane potential in human promyelotic leukaemia cells (Jin *et al.* 2012). Similarly, mitochondrial membrane potential also remains unaffected when exposed to low doses of EMR (150–570 μW/cm^2) in mouse endometrial glandular cells, but it is successfully impaired with higher intensities (1400 μW/cm^2) (Liu *et al.* 2012). In human spermatozoa, mitochondrial ROS generation was evident at SAR values above 2.8 W/kg (De Iuliis *et al.* 2009*a*), although there are no data linking such ROS generation to a change in mitochondrial membrane potential. Nevertheless, an increase in ROS generation has been consistently reported in studies focusing on the impacts of RF-EMR on spermatozoa (Agarwal *et al.* 2009, De Iuliis *et al.* 2009*a*, Kesari *et al.* 2011, Al-Damegh 2012).

It should be noted that within the electron transport chain, small concentrations of superoxide are a normal by-product of this essential redox process. However, the magnitude of ROS leakage varies between the ETC complexes, with Complex I (NADH oxidase) responsible

for a bulk of the superoxide, and with the substrate used for energy production, as observed in isolated mitochondria (Quinlan *et al.* 2013). It is also important to note that superoxide production at Complex I is much more damaging than at Complex III in spermatozoa, due to the mode of emigration of ROS from Complex I to the matrix, allowing for subsequent peroxidative damage (Koppers *et al.* 2008). Meanwhile, ROS generated at Complex III escapes to the intermembrane space, where it encounters the pool of mitochondrial antioxidant protection. The movement of electrons through the electron transport chain is a highly regulated process, partly to limit the production of deleterious amounts of ROS. Perturbation of the electron flow through this chain by RF-EMR, and the subsequent promotion of electron leakage within the mitochondria, would provide a gateway for the formation of ROS such as the superoxide anion (Martino & Castello 2011) as part of a two-step process (Fig. 3). Considering that RF-EMR specifically promotes mitochondrial ROS production (De Iuliis *et al.* 2009*a*, Burlaka *et al.* 2013) associated with increased expression of mitochondrial apoptotic markers (Liu *et al.* 2015) and decreased mitochondrial membrane potential (Lu *et al.* 2012), we propose that this radiation potentiates the leakage of electrons within the electron transport chain. Such electron leakage may be achieved through interference with proton transmission through the transmembrane complexes of the inner mitochondrial membrane. This is caused by the ability of modulated EMR (such as that emitted from mobile phones) to augment the oscillation of ions, interfering with their transport through membrane proteins, thus potentially perturbing the strict membrane potentials (Panagopoulos *et al.* 2000, 2002, 2015) enforced in the specific intermembrane compartments of the mitochondria, which otherwise stabilise proton flow (Fig. 3; Perry *et al.* 2011). A consequence of reduced proton emigration is a reduced proton motive force and a subsequent reduction in ATP production (Perry *et al.* 2011). Under these conditions, when the NADH/NAD$^+$ ratio is high and associated with low or compromised mitochondrial respiration, as previously shown to be induced by EMR (Sanders & Joines 1984), superoxide is formed at Complex I (Kudin *et al.* 2004, Murphy 2009). This scenario is accompanied by the ability of RF-EMR treatment to significantly impair the conformation of proteins and DNA, including key antioxidant proteins (Lu *et al.* 2012), preventing them from participating in the elimination of radicals generated during respiration. Thus, as a first step, the combined effects of RF-EMR results in an imbalance of free radical formation and antioxidant status, driving a state of oxidative stress (Fig. 3). The ROS formed through this process, modified to hydrogen peroxide via mitochondrial superoxide dismutase, would in turn have the ability to drive a lipid peroxidation cascade (Al-Damegh, 2012), resulting in the production of electrophilic aldehydes including malondialdehyde (Mailankot *et al.* 2009, Kesari *et al.* 2011) and 4HNE (Moazamian *et al.* 2015). Once formed, these potent electrophiles activate the second

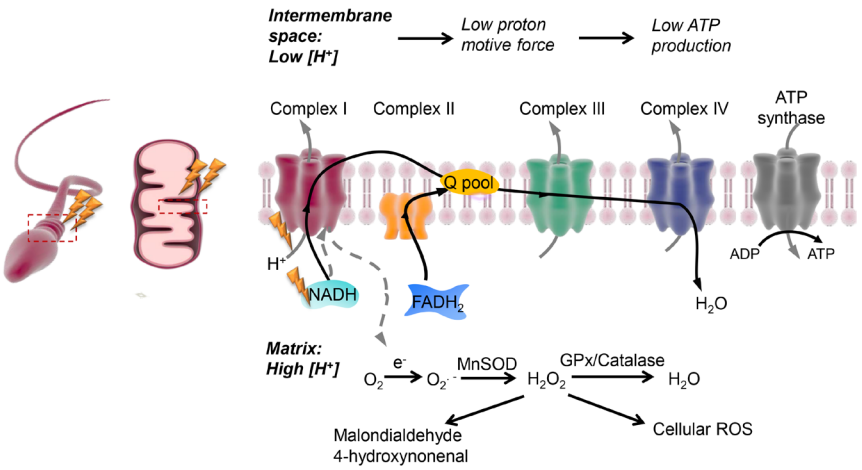


Figure 3 Potential effects of RF-EMR on the mitochondrial electron transport chain. Electron flow within the transport chain usually involves transfer of electrons through Complexes I and II into the Q pool where the electrons then feed into Complex III, interact with cytochrome-C and finally Complex IV where water acts as the terminal electron acceptor. Step 1, the presence of EMR may interfere with proton flow through these complexes, reducing proton motive force and ATP production. Via such mechanisms, EMR would also increase the NADH/NAD+ ratio (Sanders & Joines 1984), which would, in turn, promote the leakage of electrons from NADH to oxygen, forming superoxide anion – a progenitor ROS molecule. Subsequent dismutation of superoxide to H_2O_2 allows for step 2, where an imbalance of ROS results in lipid peroxidation and the formation of electrophilic aldehydes. These nucleophilic compounds impair the electron transport chain further by binding to the complexes of the ETC, promoting additional dislocation of electron flow and generating yet more superoxide, promoting extensive lipid peroxidation, motility loss and oxidative DNA damage. Grey arrows represent proton movement, black arrows represent electron flow, dashed lines represent electron leakage and thunderbolts denote EMR. C, cytochrome-C; F, FADH; N, NADH; Q pool, quinone pool; GPx, glutathione peroxidase.

step of this response, inducing widespread interference within the electron transport chain by directly alkylating key proteins associated with the protein complexes of this pathway. As mentioned previously, Complex II (succinate dehydrogenase) of this chain is preferentially targeted by 4HNE (Aitken *et al.* 2012*b*). Modification or inhibition of Complex II prevents the oxidation of FAD in the succinate dehydrogenase-A subunit, forcing the flow of electrons to oxygen and thus resulting in elevated mitochondrial perturbation with consequential increases in superoxide formation (Zhang *et al.* 1998, Aitken *et al.* 2012*b*). Moreover, as mitochondria are responsible for a majority of ROS production within spermatozoa (Koppers *et al.* 2008), it is conceivable that disrupting the function of these organelles accounts for the elevated ROS production observed with RF-EMR treatment in several studies, as exemplified by De Iuliis and coworkers (2009*b*). An important feature of this putative mechanism is that it would account for the subtle or variable changes that RF-EMR has been recorded to induce in terms of sperm motility, owing to the fact that in species such as humans, mice and rats, the energy demands required to support motility are not exclusively dependent on oxidative phosphorylation (Williams & Ford 2001, Storey 2008). However, it should be taken into account that these cells are susceptible to a state of oxidative stress.

Conclusion

To date, contradictory studies surrounding the impacts of RF-EMR on biological systems maintain controversy over this subject. Nevertheless, research on the biological responses stimulated by RF-EMR is particularly important given our ever-increasing use of mobile phone technology. Although clinical studies are identifying possible detrimental effects of RF-EMR, it is imperative that mechanistic studies are conducted that elucidate the manner in which RF-EMR perturbs biological function, thus supplying a rational cause. A focus on the male reproductive system is justified given the potentially elevated levels of exposure this system may experience as consequences of the personal storage of mobile devices, the unique vulnerability of the highly specialised sperm cell, and the future health burden that may be created if conception proceeds with defective, DNA-damaged spermatozoa. Although this subject remains a topic of active debate, this review has considered the growing body of evidence suggesting a possible role for RF-EMR-induced damage of the male germline. In a majority of studies, this damage has been characterised by loss of sperm motility and viability as well as the induction of ROS generation and DNA damage. We have therefore given consideration to the potential mechanisms through which RF-EMR may elicit these effects on spermatozoa, which we used as a sensitive model system. We propose a mechanistic model in which RF-EMR exposure leads to defective mitochondrial function associated with elevated levels of ROS production and culminates in a state of oxidative stress that would account the varying phenotypes observed in response to RF-EMR exposure. With further complementary data, this model will provide new impetus to the field and stimulate research that will allow us to confidently assess the reproductive hazards of mobile phone usage.

Declaration of interest

The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Australian Research Council Discovery Project scheme (grant number DP110103951) to R J A, and B V K. B H is the recipient of an Australian Postgraduate Award PhD scholarship.

References

Adams JA, Galloway TS, Mondal D, Esteves SC & Mathews F 2014 Effect of mobile telephones on sperm quality: a systematic review and meta-analysis. *Environment International* **70** 106–112. (doi:10.1016/j.envint.2014.04.015)

Agarwal A, Desai NR, Makker K, Varghese A, Mouradi R, Sabanegh E & Sharma R 2009 Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an in vitro pilot study. *Fertility and Sterility* **92** 1318–1325. (doi:10.1016/j.fertnstert.2008.08.022)

Aitken RJ 2013 Human spermatozoa: revelations on the road to conception. *F1000 Prime Reports* **5** 39. (doi:10.12703/p5-39)

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z & Irvine DS 1998 Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biology of Reproduction* **59** 1037–1046. (doi:10.1095/biolreprod59.5.1037)

Aitken RJ, Bennetts LE, Sawyer D, Wiklendt AM & King BV 2005 Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline. *International Journal of Andrology* **28** 171–179. (doi:10.1111/j.1365-2605.2005.00531.x)

Aitken RJ, De Iuliis GN, Gibb Z & Baker MA 2012*a* The Simmet lecture: new horizons on an old landscape – oxidative stress, DNA damage and apoptosis in the male germ line. *Reproduction in Domestic Animals* **47** (Supplement 4) 7–14. (doi:10.1111/j.1439-0531.2012.02049.x)

Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA & Baker MA 2012*b* Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *Journal of Biological Chemistry* **287** 33048–33060. (doi:10.1074/jbc.M112.366690)

Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS & De Iuliis GN 2012*c* Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biology of Reproduction* **87** 110. (doi:10.1095/biolreprod.112.102020)

Aitken RJ, Smith TB, Jobling MS, Baker MA & De Iuliis GN 2014 Oxidative stress and male reproductive health. *Asian Journal of Andrology* **16** 31–38. (doi:10.4103/1008-682X.122203)

Al-Damegh MA 2012 Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E. *Clinics* **67** 785–792. (doi:10.6061/clinics/2012(07)14)

Baker MA, Weinberg A, Hetherington L, Villaverde AI, Velkov T, Baell J & Gordon CP 2015 Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biology of Reproduction* **92** 108. (doi:10.1095/biolreprod.114.126680)

Balode Z 1996 Assessment of radio-frequency electromagnetic radiation by the micronucleus test in bovine peripheral erythrocytes. *Science of the Total Environment* **180** 81–85. (doi:10.1016/0048-9697(95)04923-1)

Balzano Q 1999 Exposure metrics for RF epidemiology: cellular phone handsets. *Radiation Protection Dosimetry* **83** 165–169. (doi:10.1093/oxfordjournals.rpd.a032654)

Bilgici B, Akar A, Avci B & Tuncel OK 2013 Effect of 900 MHz radiofrequency radiation on oxidative stress in rat brain and serum. *Electromagnetic Biology and Medicine* **32** 20–29. (doi:10.3109/15368378.2012.699012)

Bin-Merefij MM & El-Kott AF 2015 The radioprotective effects of *Moringa oleifera* against mobile phone electromagnetic radiation-induced infertility in rats. *International Journal of Clinical and Experimental Medicine* **8** 12487–12497.

Bolte JF & Eikelboom T 2012 Personal radiofrequency electromagnetic field measurements in The Netherlands: exposure level and variability for everyday activities, times of day and types of area. *Environment International* **48** 133–142. (doi:10.1016/j.envint.2012.07.006)

Burlaka A, Tsybulin O, Sidorik E, Lukin S, Polishuk V, Tsehmistrenko S & Yakymenko I 2013 Overproduction of free radical species in embryonal cells exposed to low intensity radiofrequency radiation. *Experimental Oncology* **35** 219–225.

Chen ZN 2007 *Antennas for Portable Devices*. New York, NY, USA: Wiley.

Christman MF, Morgan RW, Jacobson FS & Ames BN 1985 Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. *Cell* **41** 753–762. (doi:10.1016/S0092-8674(85)80056-8)

CTIA 2011 Semi-Annual Wireless Industry Survey (CTIA–The Wireless Association). Washington, DC, USA

Curley SA, Palalon F, Lu X & Koshkina NV 2014 Noninvasive radiofrequency treatment effect on mitochondria in pancreatic cancer cells. *Cancer* **120** 3418–3425. (doi:10.1002/cncr.28895)

d'Ambrosio G, Massa R, Scarfi MR & Zeni O 2002 Cytogenetic damage in human lymphocytes following GMSK phase modulated microwave exposure. *Bioelectromagnetics* **23** 7–13. (doi:10.1002/bem.93)

Dasdag S, Ketani MA, Akdag Z, Ersay AR, Sari I, Demirtas OC & Celik MS 1999 Whole-body microwave exposure emitted by cellular phones and testicular function of rats. *Urological Research* **27** 219–223. (doi:10.1007/s002400050113)

Dasdag S, Zulkuf Akdag M, Aksen F, Yilmaz F, Bashan M, Mutlu Dasdag M & Salih Celik M 2003 Whole body exposure of rats to microwaves emitted from a cell phone does not affect the testes. *Bioelectromagnetics* **24** 182–188. (doi:10.1002/bem.10083)

Dasdag S, Akdag MZ, Ulukaya E, Uzunlar AK & Ocak AR 2009 Effect of mobile phone exposure on apoptotic glial cells and status of oxidative stress in rat brain. *Electromagnetic Biology and Medicine* **28** 342–354. (doi:10.3109/15368370903206556)

Dasdag S, Akdag MZ, Erdal ME, Erdal N, Ay OI, Ay ME, Yilmaz SG, Tasdelen B & Yegin K 2015 Long term and excessive use of 900 MHz radiofrequency radiation alter microRNA expression in brain. *International Journal of Radiation Biology* **91** 306–311. (doi:10.3109/09553002.2015.997896)

De Iuliis GN, Newey RJ, King BV & Aitken RJ 2009*a* Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. *PLoS ONE* **4** e6446. (doi:10.1371/journal.pone.0006446)

De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B & Aitken RJ 2009*b* DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. *Biology of Reproduction* **81** 517–524. (doi:10.1095/biolreprod.109.076836)

De Iuliis GN, King BV & Aitken RJ 2012 Electromagnetic radiation and oxidative stress in the male germ line. In *Studies on Men's Health and Fertility,* pp 119–130. Eds A Agarwal, RJ Aitken & JG Alvarez. New York, NY, USA: Humana Press.

Demirel S, Doganay S, Turkoz Y, Dogan Z, Turan B & Firat PG 2012 Effects of third generation mobile phone-emitted electromagnetic radiation on oxidative stress parameters in eye tissue and blood of rats. *Cutaneous and Ocular Toxicology* **31** 89–94. (doi:10.3109/15569527.2012.657725)

Di Carlo A, White N, Guo F, Garrett P & Litovitz T 2002 Chronic electromagnetic field exposure decreases HSP70 levels and lowers cytoprotection. *Journal of Cellular Biochemistry* **84** 447–454. (doi:10.1002/jcb.10036)

Duan W, Liu C, Zhang L, He M, Xu S, Chen C, Pi H, Gao P, Zhang Y, Zhong M *et al.* 2015 Comparison of the genotoxic effects induced by 50 Hz extremely low-frequency electromagnetic fields and 1800 MHz radiofrequency electromagnetic fields in GC-2 cells. *Radiation Research* **183** 305–314. (doi:10.1667/RR13851.1)

Durney C 1986 *Radiofrequency Radiation Dosimetry Handbook*, 4th edn. The University of Utah, Salt Lake City, UT, USA

Erogul O, Oztas E, Yildirim I, Kir T, Aydur E, Komesli G, Irkilata HC, Irmak MK & Peker AF 2006 Effects of electromagnetic radiation from a cellular phone on human sperm motility: an in vitro study. *Archives of Medical Research* **37** 840–843. (doi:10.1016/j.arcmed.2006.05.003)

Falzone N, Huyser C, Becker P, Leszczynski D & Franken DR 2011 The effect of pulsed 900-MHz GSM mobile phone radiation on the acrosome reaction, head morphometry and zona binding of human spermatozoa. *International Journal of Andrology* **34** 20–26. (doi:10.1111/j.1365-2605.2010.01054.x)

Fejes I, Zavaczki Z, Szollosi J, Koloszar S, Daru J, Kovacs L & Pal A 2005 Is there a relationship between cell phone use and semen quality? *Archives of Andrology* **51** 385–393. (doi:10.1080/014850190924520)

French PW, Penny R, Laurence JA & McKenzie DR 2001 Mobile phones, heat shock proteins and cancer. *Differentiation* **67** 93–97. (doi:10.1046/j.1432-0436.2001.670401.x)

Friedman J, Kraus S, Hauptman Y, Schiff Y & Seger R 2007 Mechanism of short-term ERK activation by electromagnetic fields at mobile phone frequencies. *Biochemical Journal* **405** 559–568. (doi:10.1042/BJ20061653)

Furtado-Filho OV, Borba JB, Dallegrave A, Pizzolato TM, Henriques JA, Moreira JC & Saffi J 2014 Effect of 950 MHz UHF electromagnetic radiation on biomarkers of oxidative damage, metabolism of UFA and antioxidants in the livers of young rats of different ages. *International Journal of Radiation Biology* **90** 159–168. (doi:10.3109/09553002.2013.817697)

Gabriel S, Lau RW & Gabriel C 1996 The dielectric properties of biological tissues: III. Parametric models for the dielectric spectrum of tissues. *Physics in Medicine and Biology* **41** 2271–2293. (doi:10.1088/0031-9155/41/11/003)

Gajda GB, McNamee JP, Thansandote A, Boonpanyarak S, Lemay E & Bellier PV 2002 Cylindrical waveguide applicator for in vitro exposure of cell culture samples to 1.9-GHz radiofrequency fields. *Bioelectromagnetics* **23** 592–598. (doi:10.1002/bem.10055)

Ghanbari M, Mortazavi SB, Khavanin A & Khazaei M 2013 The effects of cell phone waves (900 MHz-GSM Band) on sperm parameters and total antioxidant capacity in rats. *International Journal of Fertility & Sterility* **7** 21–28.

Gharagozloo P & Aitken RJ 2011 The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Human Reproduction* **26** 1628–1640. (doi:10.1093/humrep/der132)

Gorpinchenko I, Nikitin O, Banyra O & Shulyak A 2014 The influence of direct mobile phone radiation on sperm quality. *Central European Journal of Urology* **67** 65–71. (doi:10.5173/ceju.2014.01.art14)

Guney M, Ozguner F, Oral B, Karahan N & Mungan T 2007 900 MHz radiofrequency-induced histopathologic changes and oxidative stress in rat endometrium: protection by vitamins E and C. *Toxicology and Industrial Health* **23** 411–420. (doi:10.1177/0748233707080906)

Hossmann KA & Hermann DM 2003 Effects of electromagnetic radiation of mobile phones on the central nervous system. *Bioelectromagnetics* **24** 49–62. (doi:10.1002/bem.10068)

Hou Q, Wang M, Wu S, Ma X, An G, Liu H & Xie F 2015 Oxidative changes and apoptosis induced by 1800-MHz electromagnetic radiation in NIH/3T3 cells. *Electromagnetic Biology and Medicine* **34** 85–92. (doi:10.3109/15368378.2014.900507)

Imai N, Kawabe M, Hikage T, Nojima T, Takahashi S & Shirai T 2011 Effects on rat testis of 1.95-GHz W-CDMA for IMT-2000 cellular phones. *Systems Biology in Reproductive Medicine* **57** 204–209. (doi:10.3109/19396368.2010.544839)

Iorio R, Delle Monache S, Bennato F, Di Bartolomeo C, Scrimaglio R, Cinque B & Colonna RC 2011 Involvement of mitochondrial activity in mediating ELF-EMF stimulatory effect on human sperm motility. *Bioelectromagnetics* **32** 15–27. (doi:10.1002/bem.20602)

Irmak MK, Fadillioglu E, Gulec M, Erdogan H, Yagmurca M & Akyol O 2002 Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits. *Cell Biochemistry and Function* **20** 279–283. (doi:10.1002/cbf.976)

Jin Z, Zong C, Jiang B, Zhou Z, Tong J & Cao Y 2012 The effect of combined exposure of 900 MHz radiofrequency fields and doxorubicin in HL-60 cells. *PLoS ONE* **7** e46102. (doi:10.1371/journal.pone.0046102)

Jones R, Mann T & Sherins R 1979 Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertility and Sterility* **31** 531–537. (doi:10.1016/S0015-0282(16)43999-3)

Kahya MC, Naziroglu M & Cig B 2014 Selenium reduces mobile phone (900 MHz)-induced oxidative stress, mitochondrial function, and apoptosis in breast cancer cells. *Biological Trace Element Research* **160** 285–293. (doi:10.1007/s12011-014-0032-6)

Kesari KK & Behari J 2012 Evidence for mobile phone radiation exposure effects on reproductive pattern of male rats: role of ROS. *Electromagnetic Biology and Medicine* **31** 213–222. (doi:10.3109/15368378.2012.700292)

Kesari KK, Kumar S & Behari J 2011 Effects of radiofrequency electromagnetic wave exposure from cellular phones on the reproductive pattern in male Wistar rats. *Applied Biochemistry and Biotechnology* **164** 546–559. (doi:10.1007/s12010-010-9156-0)

Khalil AM, Abu Khadra KM, Aljaberi AM, Gagaa MH & Issa HS 2014 Assessment of oxidant/antioxidant status in saliva of cell phone users. *Electromagnetic Biology and Medicine* **33** 92–97. (doi:10.3109/15368378.2013.783855)

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA & Aitken RJ 2008 Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *Journal of Clinical Endocrinology and Metabolism* **93** 3199–3207. (doi:10.1210/jc.2007-2616)

Koppers AJ, Garg ML & Aitken RJ 2010 Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radical Biology and Medicine* **48** 112–119. (doi:10.1016/j.freeradbiomed.2009.10.033)

Kudin AP, Bimpong-Buta NY, Vielhaber S, Elger CE & Kunz WS 2004 Characterization of superoxide-producing sites in isolated brain mitochondria. *Journal of Biological Chemistry* **279** 4127–4135. (doi:10.1074/jbc.M310341200)

La Vignera S, Condorelli RA, Vicari E, D'Agata R & Calogero AE 2012 Effects of the exposure to mobile phones on male reproduction: a review of the literature. *Journal of Andrology* **33** 350–356. (doi:10.2164/jandrol.111.014373)

Lenzi A, Gandini L, Maresca V, Rago R, Sgro P, Dondero F & Picardo M 2000 Fatty acid composition of spermatozoa and immature germ cells. *Molecular Human Reproduction* **6** 226–231. (doi:10.1093/molehr/6.3.226)

Lerchl A 2013 Electromagnetic pollution: another risk factor for infertility, or a red herring? *Asian Journal of Andrology* **15** 201–203. (doi:10.1038/aja.2012.104)

Li HW, Yao K, Jin HY, Sun LX, Lu DQ & Yu YB 2007 Proteomic analysis of human lens epithelial cells exposed to microwaves. *Japanese Journal of Ophthalmology* **51** 412–416. (doi:10.1007/s10384-007-0483-9)

Lin JC 1976 Interaction of two cross-polarized electromagnetic waves with mammalian cranial structures. *IEEE Transactions on Biomedical Engineering* **23** 371–375. (doi:10.1109/TBME.1976.324646)

Liu W, Zheng X, Qu Z, Zhang M, Zhou C, Ma L & Zhang Y 2012 Effect of 935-MHz phone-simulating electromagnetic radiation on endometrial glandular cells during mouse embryo implantation. *Journal of Huazhong University of Science and Technology* **32** 755–759. (doi:10.1007/s11596-012-1030-6)

Liu C, Duan W, Xu S, Chen C, He M, Zhang L, Yu Z & Zhou Z 2013*a* Exposure to 1800 MHz radiofrequency electromagnetic radiation induces oxidative DNA base damage in a mouse spermatocyte-derived cell line. *Toxicology Letters* **218** 2–9. (doi:10.1016/j.toxlet.2013.01.003)

Liu C, Gao P, Xu SC, Wang Y, Chen CH, He MD, Yu ZP, Zhang L & Zhou Z 2013*b* Mobile phone radiation induces mode-dependent DNA damage in a mouse spermatocyte-derived cell line: a protective role of melatonin. *International Journal of Radiation Biology* **89** 993–1001. (doi:10.3109/09553002.2013.811309)

Liu K, Li Y, Zhang G, Liu J, Cao J, Ao L & Zhang S 2014 Association between mobile phone use and semen quality: a systemic review and meta-analysis. *Andrology* **2** 491–501. (doi:10.1111/j.2047-2927.2014.00205.x)

Liu Q, Si T, Xu X, Liang F, Wang L & Pan S 2015 Electromagnetic radiation at 900 MHz induces sperm apoptosis through bcl-2, bax and caspase-3 signaling pathways in rats. *Reproductive Health* **12** 65. (doi:10.1186/s12978-015-0062-3)

Lord T & Aitken RJ 2015 Fertilization stimulates 8-hydroxy-2′-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Developmental Biology* **406** 1–13. (doi:10.1016/j.ydbio.2015.07.024)

Lu YS, Huang BT & Huang YX 2012 Reactive oxygen species formation and apoptosis in human peripheral blood mononuclear cell induced by 900 MHz mobile phone radiation. *Oxidative Medicine and Cellular Longevity* **2012** 740280. (doi:10.1155/2012/740280)

Mailankot M, Kunnath AP, Jayalekshmi H, Koduru B & Valsalan R 2009 Radio frequency electromagnetic radiation (RF-EMR) from GSM (0.9/1.8GHz) mobile phones induces oxidative stress and reduces sperm motility in rats. *Clinics* **64** 561–565. (doi:10.1590/S1807-59322009000600011)

Manta AK, Stravopodis DJ, Papassideri IS & Margaritis LH 2014 Reactive oxygen species elevation and recovery in Drosophila bodies and ovaries following short-term and long-term exposure to DECT base EMF. *Electromagnetic Biology and Medicine* **33** 118–131. (doi:10.3109/15368378.2013.791991)

Marchionni I, Paffi A, Pellegrino M, Liberti M, Apollonio F, Abeti R, Fontana F, D'Inzeo G & Mazzanti M 2006 Comparison between low-level 50 Hz and 900 MHz electromagnetic stimulation on single channel ionic currents and on firing frequency in dorsal root ganglion isolated neurons. *Biochimica et Biophysica Acta* **1758** 597–605. (doi:10.1016/j.bbamem.2006.03.014)

Markov M & Grigoriev Y 2015 Protect children from EMF. *Electromagnetic Biology and Medicine* **34** 251–256. (doi:10.3109/15368378.2015.1077339)

Martino CF & Castello PR 2011 Modulation of hydrogen peroxide production in cellular systems by low level magnetic fields. *PLoS ONE* **6** e22753. (doi:10.1371/journal.pone.0022753)

Masuda H, Sanchez S, Dulou PE, Haro E, Anane R, Billaudel B, Leveque P & Veyret B 2006 Effect of GSM-900 and -1800 signals on the skin of hairless rats. I: 2-hour acute exposures. *International Journal of Radiation Biology* **82** 669–674. (doi:10.1080/09553000600930079)

Meral I, Mert H, Mert N, Deger Y, Yoruk I, Yetkin A & Keskin S 2007 Effects of 900-MHz electromagnetic field emitted from cellular phone on brain oxidative stress and some vitamin levels of guinea pigs. *Brain Research* **1169** 120–124. (doi:10.1016/j.brainres.2007.07.015)

Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P & Aitken RJ 2015 Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Molecular Human Reproduction* **21** 502–515. (doi:10.1093/molehr/gav014)

Murphy MP 2009 How mitochondria produce reactive oxygen species. *Biochemical Journal* **417** 1–13. (doi:10.1042/BJ20081386)

Nahomi RB, DiMauro MA, Wang B & Nagaraj RH 2015 Identification of peptides in human Hsp20 and Hsp27 that possess molecular chaperone and anti-apoptotic activities. *Biochemical Journal* **465** 115–125. (doi:10.1042/BJ20140837)

Ozguner F, Altinbas A, Ozaydin M, Dogan A, Vural H, Kisioglu AN, Cesur G & Yildirim NG 2005 Mobile phone-induced myocardial oxidative stress: protection by a novel antioxidant agent caffeic acid phenethyl ester. *Toxicology and Industrial Health* **21** 223–230. (doi:10.1191/0748233705th228oa)

Ozlem Nisbet H, Nisbet C, Akar A, Cevik M & Karayigit MO 2012 Effects of exposure to electromagnetic field (1.8/0.9 GHz) on testicular function and structure in growing rats. *Research in Veterinary Science* **93** 1001–1005. (doi:10.1016/j.rvsc.2011.10.023)

Ozorak A, Naziroglu M, Celik O, Yuksel M, Ozcelik D, Ozkaya MO, Cetin H, Kahya MC & Kose SA 2013 Wi-Fi (2.45 GHz)- and mobile

phone (900 and 1800 MHz)-induced risks on oxidative stress and elements in kidney and testis of rats during pregnancy and the development of offspring. *Biological Trace Element Research* **156** 221–229. (doi:10.1007/s12011-013-9836-z)

Panagopoulos DJ, Messini N, Karabarbounis A, Philippetis AL & Margaritis LH 2000 A mechanism for action of oscillating electric fields on cells. *Biochemical and Biophysical Research Communications* **272** 634–640. (doi:10.1006/bbrc.2000.2746)

Panagopoulos DJ, Karabarbounis A & Margaritis LH 2002 Mechanism for action of electromagnetic fields on cells. *Biochemical and Biophysical Research Communications* **298** 95–102. (doi:10.1016/S0006-291X(02)02393-8)

Panagopoulos DJ, Chavdoula ED & Margaritis LH 2010 Bioeffects of mobile telephony radiation in relation to its intensity or distance from the antenna. *International Journal of Radiation Biology* **86** 345–357. (doi:10.3109/09553000903567961)

Panagopoulos DJ, Johansson O & Carlo GL 2015 Polarization: a key difference between man-made and natural electromagnetic fields, in regard to biological activity. *Scientific Reports* **5** 14914. (doi:10.1038/srep14914)

Perry SW, Norman JP, Barbieri J, Brown EB & Gelbard HA 2011 Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques* **50** 98–115. (doi:10.2144/000113610)

Polla BS, Kantengwa S, Francois D, Salvioli S, Franceschi C, Marsac C & Cossarizza A 1996 Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *PNAS* **93** 6458–6463. (doi:10.1073/pnas.93.13.6458)

Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Orr AL & Brand MD 2013 Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox Biology* **1** 304–312. (doi:10.1016/j.redox.2013.04.005)

Redmayne M, Smith E & Abramson MJ 2011 Adolescent in-school cellphone habits: a census of rules, survey of their effectiveness, and fertility implications. *Reproductive Toxicology* **32** 354–359. (doi:10.1016/j.reprotox.2011.08.006)

Roberts JA, Yaya LH & Manolis C 2014 The invisible addiction: cell-phone activities and addiction among male and female college students. *Journal of Behavioral Addictions* **3** 254–265. (doi:10.1556/JBA.3.2014.015)

Sanders AP & Joines WT 1984 The effects of hyperthermia and hyperthermia plus microwaves on rat brain energy metabolism. *Bioelectromagnetics* **5** 63–70. (doi:10.1002/bem.2250050107)

Sommer AM, Grote K, Reinhardt T, Streckert J, Hansen V & Lerchl A 2009 Effects of radiofrequency electromagnetic fields (UMTS) on reproduction and development of mice: a multi-generation study. *Radiation Research* **171** 89–95. (doi:10.1667/RR1460.1)

Storey BT 2008 Mammalian sperm metabolism: oxygen and sugar, friend and foe. *International Journal of Developmental Biology* **52** 427–437. (doi:10.1387/ijdb.072522bs)

Singer TM, Lambert IB, Williams A, Douglas GR & Yauk CL 2006 Detection of induced male germline mutation: correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. *Mutation Research* **598** 164–193. (doi:10.1016/j.mrfmmm.2006.01.017)

Tas M, Dasdag S, Akdag MZ, Cirit U, Yegin K, Seker U, Ozmen MF & Eren LB 2014 Long-term effects of 900 MHz radiofrequency radiation emitted from mobile phone on testicular tissue and epididymal semen quality. *Electromagnetic Biology and Medicine* **33** 216–222. (doi:10.3109/15368378.2013.801850)

Tattersall JE, Scott IR, Wood SJ, Nettell JJ, Bevir MK, Wang Z, Somasiri NP & Chen X 2001 Effects of low intensity radiofrequency electromagnetic fields on electrical activity in rat hippocampal slices. *Brain Research* **904** 43–53. (doi:10.1016/S0006-8993(01)02434-9)

Tosic J & Walton A 1950 Metabolism of spermatozoa. The formation and elimination of hydrogen peroxide by spermatozoa and effects on motility and survival. *Biochemical Journal* **47** 199–212. (doi:10.1042/bj0470199)

Trosic I, Matausic-Pisl M, Pavicic I & Marjanovic AM 2013 Histological and cytological examination of rat reproductive tissue after short-time intermittent radiofrequency exposure. *Arhiv za Higijenu Rada i Toksikologiju* **64** 513–519.

Tumkaya L, Kalkan Y, Bas O & Yilmaz A 2013 Mobile phone radiation during pubertal development has no effect on testicular histology in rats. *Toxicology and Industrial Health* **32** 328–336. (doi:10.1177/0748233713500820)

Valbonesi P, Franzellitti S, Bersani F, Contin A & Fabbri E 2014 Effects of the exposure to intermittent 1.8 GHz radio frequency electromagnetic fields on HSP70 expression and MAPK signaling pathways in PC12 cells. *International Journal of Radiation Biology* **90** 382–391. (doi:10.3109/09553002.2014.892225)

Wdowiak A, Wdowiak L & Wiktor H 2007 Evaluation of the effect of using mobile phones on male fertility. *Annals of Agricultural and Environmental Medicine* **14** 169–172.

Williams AC & Ford WC 2001 The role of glucose in supporting motility and capacitation in human spermatozoa. *Journal of Andrology* **22** 680–695.

Yan JG, Agresti M, Bruce T, Yan YH, Granlund A & Matloub HS 2007 Effects of cellular phone emissions on sperm motility in rats. *Fertility and Sterility* **88** 957–964. (doi:10.1016/j.fertnstert.2006.12.022)

Yao K, Wu W, Wang K, Ni S, Ye P, Yu Y, Ye J & Sun L 2008 Electromagnetic noise inhibits radiofrequency radiation-induced DNA damage and reactive oxygen species increase in human lens epithelial cells. *Molecular Vision* **14** 964–969.

Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y & Mostafa T 2015 In vitro effect of cell phone radiation on motility, DNA fragmentation and clusterin gene expression in human sperm. *International Journal of Fertility & Sterility* **9** 129–136.

Zhang L, Yu L & Yu CA 1998 Generation of superoxide anion by succinate-cytochrome c reductase from bovine heart mitochondria. *Journal of Biological Chemistry* **273** 33972–33976. (doi:10.1074/jbc.273.51.33972)

Zhao L, Peng RY, Wang SM, Wang LF, Gao YB, Dong J, Li X & Su ZT 2012 Relationship between cognition function and hippocampus structure after long-term microwave exposure. *Biomedical and Environmental Sciences* **25** 182–188.

Received 9 March 2016
First decision 8 April 2016
Revised manuscript received 2 August 2016
Accepted 2 September 2016

1.2: The effects of heating on the male reproductive system

As another environmental factor with potential to perturb the male germ line, heating induces effects to this system like that of RF-EMR. By extension, heat is generated by high power RF-EMR, above SAR values of approximately 4 W/kg, meaning it has potential to confound studies utilising these exposure conditions. On the other hand, in many well-designed and low power exposure experiments, RF-EMR has been shown to induce a state of oxidative stress, as commonly identified in studies concerning heat stressed male reproductive organs. Furthermore, the effect of heating on fertility is timely as the reproductive consequences of global warming are not currently understood.

The reproductive system of mammalian species is unique in its arrangement, where the testis and epididymis are adapted to allow for cooler temperature of operation; multiple degrees below that of core body temperature (Hansen, 2009; Waites, 1991). This specialization is likely to allow for efficient spermatogenesis, to reduce gamete mutation rates, and/or supports sperm maturation and storage in the epididymis (Gallup, 2009). On the other hand, this also means that this system is vulnerable to heat stress supplied by the application of inguinal clothing or elevated environmental temperatures. Many studies have been undergone to mimic these effects, by direct application of heat to the testis via scrotal heat stress in water baths (Perez-Crespo *et al*., 2008) and heating apparatuses (Zhang *et al*., 2015), or by placing model animal species in heating chambers (Zhu *et al*. 2004). To further understand the mechanisms involved in the decline in semen quality under these conditions, it has been proposed that specific populations of germ cells are susceptible to heat stress, including the pachytene spermatocyte (PS) and round spermatid (RS) stages (Perez-Crespo *et al*., 2008; Wettemann and Desjardins, 1979; Zhu *et al*. 2015). Heat stress has been postulated to impair spermatogenesis and drive an overall reduction in sperm count, motility and normal morphology (Hansen *et al*., 2009; Perez-Crespo *et al*., 2008; Zhang *et al*., 2015). In contrast to the negative consequences of heat stress in more developed germ cell

stages, type A spermatogonia appear resilient to heat stress (McLean *et al*., 2002). As such, sperm quality is regenerated post heat stress when germ cell populations are re-established from these progenitor cells (Zhu *et al*., 2004).

It has been shown that IVF performed with spermatozoa treated with heat stress *in vivo* results in embryo losses following exposure to an elevated temperature of 35ºC for as little as 24 h (Zhu *et al*., 2004). In order to further understand the cause of embryo losses under this stress and the ontogeny of germ cell susceptibility to heat stress, we must first investigate a complete profile of response in the spermatozoa produced under these conditions. PS and RS germ cell populations respond to acute heat stress with elevated levels of DNA damage (Perez-Crespo *et al*., 2008), which may be harboured throughout their development to spermatozoa. This presents as our leading hypothesis for the decline in embryonic success, as DNA damage in spermatozoa has been correlated with a variety of negative outcomes, such as impaired embryonic development and elevated levels of miscarriage (Aitken and Koppers, 2011). Furthermore, oxidative stress has been suggested as a mediator of cellular defects resulting from heat stress, including a hallmark generation of ROS (Hansen, 2009). Therefore, the resulting DNA damage is likely to be of an oxidative means and detectable by oxidative base lesions in these spermatozoa.

Many published studies have been designed to investigate the effects of direct heating testicular heating, in order to mimic conditions experienced in response to inguinal clothing. Comparatively, the impact(s) of ambient temperature heating models, where the whole body is subjected to thermal stress, have received less emphasis. However, these whole body studies also provide a more precise characterisation of the impacts of environmental heating on the reproductive system. Environmental factors have been suggested as a driver of reduction in semen quality (Virtanen *et al*., 2017), which is now widespread in our species (Huang *et al*., 2017). Men who are occupationally exposed to extreme heat conditions commonly experience spermatogenic arrest,

characterised by the onset of azoospermia, oligozoospermia or teratozoospermia (Dada *et al*., 2003) and men exhibit reduced fertility and sperm counts in summer months across the world (Gyllenborg *et al*.,1999; Jorgensen *et al*., 2001; Levine *et al*., 1988; 1990). This theme is consistent in the agriculturally bovine model, where sperm quality is severely impaired following heat stress. Therefore, it is important to understand the mechanisms involved in heat stress impairment to fertility, which may affect the reproductive capacity of our species and also the livestock we utilize as a food source.

1.3 References:

Aitken, R. J. and Koppers, A. J. (2011). Apoptosis and DNA damage in human spermatozoa. *Asian J Androl* **13**, 36-42.

Dada, R., Gupta, N. P. and Kucheria, K. (2003). Spermatogenic arrest in men with testicular hyperthermia. *Teratog Carcinog Mutagen* **Suppl 1**, 235-43.

Gallup GG J, Finn MM & Sammis B. (2009). On the Origin of Descended Scrotal Testicles: The Activation Hypothesis. *Evolutionary Psychology* **7** 517-526.

Gyllenborg, J., Skakkebaek, N. E., Nielsen, N. C., Keiding, N. and Giwercman, A. (1999). Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977-1995. *Int J Androl* **22**, 28-36.

Hansen, P. J. (2009). Effects of heat stress on mammalian reproduction. *Philos Trans R Soc Lond B Biol Sci* **364**, 3341-50.

Huang, C., Li, B., Xu, K., Liu, D., Hu, J., Yang, Y., Nie, H., Fan, L. and Zhu, W. (2017). Decline in semen quality among 30,636 young Chinese men from 2001 to 2015. *Fertil Steril* **107**, 83-88 e2.

Jorgensen, N., Andersen, A. G., Eustache, F., Irvine, D. S., Suominen, J., Petersen, J. H., Andersen, A. N., Auger, J., Cawood, E. H., Horte, A. et al. (2001). Regional differences in semen quality in Europe. *Hum Reprod* **16**, 1012-9.

Levine, R. J., Bordson, B. L., Mathew, R. M., Brown, M. H., Stanley, J. M. and Star, T. B. (1988). Deterioration of semen quality during summer in New Orleans. *Fertil Steril* **49**, 900-7.

Levine, R. J., Mathew, R. M., Chenault, C. B., Brown, M. H., Hurtt, M. E., Bentley, K. S., Mohr, K. L. and Working, P. K. (1990). Differences in the quality of semen in outdoor workers during summer and winter. *N Engl J Med* **323**, 12-6.

McLean, D. J., Russell, L. D. and Griswold, M. D. (2002). Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. *Biol Reprod* **66**, 1374-9.

Perez-Crespo, M., Pintado, B. and Gutierrez-Adan, A. (2008). Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev* **75**, 40-7.

Virtanen, H. E., Jorgensen, N. and Toppari, J. (2017). Semen quality in the 21st century. *Nat Rev Urol* **14**, 120-130.

Waites, G. M. (1991). Thermoregulation of the scrotum and testis: studies in animals and significance for man. *Adv Exp Med Biol* **286**, 9-17.

Wettemann, R. P. and Desjardins, C. (1979). Testicular function in boars exposed to elevated ambient temperature. *Biol Reprod* **20**, 235-41.

Zhang, M. H., Shi, Z. D., Yu, J. C., Zhang, Y. P., Wang, L. G. and Qiu, Y. (2015). Scrotal heat stress causes sperm chromatin damage and cysteinyl aspartate-spicific proteinases 3 changes in fertile men. *J Assist Reprod Genet* **32**, 747-55.

Zhu, B., Walker, S. K., Oakey, H., Setchell, B. P. and Maddocks, S. (2004). Effect of paternal heat stress on the development in vitro of preimplantation embryos in the mouse. *Andrologia* **36**, 384-94.

CHAPTER 2

1800 MHz radiofrequency electromagnetic radiation induces DNA fragmentation in mouse spermatozoa

Submitted: Frontiers in Public Health

Authors: Brendan J Houston[1], Brett Nixon[1], Bruce V King[2], R John Aitken[1] and Geoffry N De Iullis[1]

[1] Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

[2] School of Mathematical and Physical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

Chapter 2: Overview

The overarching goal of this manuscript was to expand on the current literature in order to further understand how RF-EMR is capable of eliciting cellular stress. Here, we exposed both cultured murine male germ cells and spermatozoa to RF-EMR to define the vulnerability of the male germ line to RF-EMR and characterise their response profiles to this factor.

For the purpose of these studies, cultured spermatogonial and spermatocyte male germ cells, and spermatozoa were treated with RF-EMR via a waveguide device, thereby enabling finely tuned exposure conditions. A major consequence of exposure to RF-EMR was elevated mitochondrial reactive oxygen species (ROS) content in both male germ cell lines. Through the use of inhibitors of key components of the electron transport chain in the mitochondria in combination with RF-EMR exposure, we further pinpointed the vulnerable junction of this chain to likely be Complex III. While a similar increase in mitochondrial ROS levels was not detected in the spermatozoa, we identified increased DNA fragmentation in these cells arising from exposure to RF-EMR. This impairment of sperm DNA integrity was detected in association with a loss of sperm motility and the onset of oxidative DNA lesions.

As introduced in the previous chapter, the data in this manuscript further supports the potential for RF-EMR to induce oxidative stress in the male germ line. Furthermore, this data also adds light to the mechanisms controlling this stress, where we identified Complex III of the mitochondrial electron transport chain as a likely candidate of inhibition by RF-EMR. Such findings are crucial in directing this field of research forward, for a deeper understanding of the molecular mechanisms at play, and to address the controversy surrounding the effects of RF-EMR on biological systems.

TITLE: 1800 MHz radiofrequency electromagnetic radiation induces DNA fragmentation in mouse spermatozoa

AUTHORS: Brendan J. HOUSTON[1], Brett NIXON[1], Bruce V. KING[2], R. John AITKEN[1] and Geoffry N. DE IULIIS[1]

AUTHOR AFFLIATIONS

[1] Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, Discipline of Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

[2] School of Mathematical and Physical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

CORRESPONDENCE: Brendan HOUSTON, School of Environmental and Life Sciences, Discipline of Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

Tel: +61 24921 2043

Fax: +61 24921 6308

Email: brendan.houston@uon.edu.au

KEY WORDS:

RF-EMR, spermatozoa, germ cells, DNA damage, mobile phone radiation

RUNNING TITLE:

RF-EMR impairs the DNA integrity of spermatozoa.

ABSTRACT

As the use of mobile phone devices continues to escalate, many studies have sought to evaluate the effects of the radiofrequency-electromagnetic radiation (RF-EMR) on both human health and biology. While several such studies have shown RF-EMR is capable of inducing cellular stress, the physicobiological origin of this stress remains largely unresolved. To explore the effect of RF-EMR on the male reproductive system, we exposed cultured mouse spermatogonial GC1 and spermatocyte GC2 cell lines, and cauda epididymal spermatozoa to a waveguide generating RF-EMR (1.8 GHz, 0.15 W/kg). This study demonstrated that a 4 h RF-EMR exposure is capable of inducing the generation of mitochondrial reactive oxygen species (ROS) in GC1 (7 vs 18 %; $p < 0.001$) and GC2 cells (11.5 vs 16 %; $p < 0.01$), potentially from Complex III of the electron transport chain (ETC). Assessing the generation of ROS in the presence of the aldehyde scavenger, penicillamine, and lipid peroxidation via levels of 4-hydroxynonenal adducted protein, indicated that the increased ROS generation observed under our exposure conditions did not necessarily induce overt cellular oxidative stress. However, exposure to RF-EMR induced significant DNA fragmentation in the form of single strand breaks assessed by the alkaline comet assay (tail intensity) in spermatozoa ($p < 0.05$), following 3 h of exposure. Furthermore, this fragmentation was accompanied by an induction of oxidative DNA damage in the form of 8-hydroxy-2'-deoxyguanosine after spermatozoa were exposed to RF-EMR for 4 h. Meanwhile, this exposure also led to a decline in sperm motility ($p < 0.05$). This study contributes new evidence toward elucidating a mechanism to account for the effects of RF-EMR on biological systems, proposing Complex III of the mitochondrial ETC as the key target of this radiation.

INTRODUCTION

Mobile phone usage is becoming increasingly popular worldwide and, consequently, our exposure to the radiofrequency-electromagnetic radiation (RF-EMR) emitted by these devices is now unprecedented (Agarwal *et al*., 2009; French *et al*., 2001; Meral *et al*., 2007). Currently, the biological effects of this radiation remain the subject of active debate (Consales *et al*., 2012; Merhi, 2012; Lerchl, 2013; *et al*., 2015) and no robust clinical impacts have been established (Lagorio and Roosli, 2014). In addition, the mechanism(s) by which RF-EMR could affect biological systems is unknown. Despite this, an emerging body of evidence supports the potential of RF-EMR to elicit a suite of detrimental effects in a variety of cell and tissues types. Notable among these effects are the degeneration of the blood brain barrier (Salford, 1993), altered brain nerve branching (Narayan *et al*., 2015), oxidative stress (Hou *et al*., 2014; Kahya *et al*., 2014; Ozguner *et al*., 2005; De Iuliis *et al*., 2009a; Yao *et al.,* 2009), genotoxic assault highlighted by micronuclei formation (d'Ambrosio *et al*., 2002; Balode, 1996) and DNA fragmentation (De Iuliis et al., 2009a; Liu *et al*., 2013a; Zalata *et al*., 2015). However, the absence of a widely accepted mechanism-of-action to account for these effects, complicates our ability to determine safe RF-EMR exposure limits.

Interest in the vulnerability of the male reproductive system to RF-EMR exposure has fueled an increasing number of recent studies. While such studies have yet to reach a firm consensus, they have revealed that sperm motility (Erogul *et al.,* 2006; Gorpinchenko *et al*., 2014; Zalata *et al*., 2015) and vitality (Agarwal *et al*., 2009; De Iuliis *et al.,* 2009a; Yan *et al*., 2007) represent two key functional parameters that exhibit susceptibility to RF-EMR and can be significantly impaired following certain exposure regimes (Adams *et al*., 2014). Similarly, RF-EMR is capable of eliciting elevated reactive oxygen species (ROS) generation (De Iuliis *et al*., 2009a; Al-Damegh, 2012; Kesari *et al*., 2011; Liu *et al.,* 2015), and also significant DNA fragmentation in spermatozoa (Aitken *et al.,* 2005; Gorpinchenko *et al*., 2014; Liu *et al*., 2013a; Zalata *et al*., 2015). In work conducted by our group (De Iuliis *et al*., 2009a), it was established that RF-EMR is capable of inducing oxidative stress in purified human spermatozoa.

Hallmarks of this process included elevated generation of mitochondrial ROS that, in turn, resulted in impaired sperm motility and vitality culminating in DNA fragmentation and oxidative DNA base adduct formation. Such results may be viewed as clinically important given that the RF-EMR intensity eliciting these responses (1 - 2.8 W/kg) falls comfortably within the non-damaging exposure levels currently prescribed for this form of radiation (4 W/kg). Nevertheless, it is important to note that recent studies have revealed a level of variability in the responses documented following RF-EMR exposure. This variability may arise by virtue of the diverse exposure conditions employed in individual studies involving differing microwave intensity (SAR) and frequency (MHz/GHz), as well as variable exposure time (Adams *et al*., 2014), mode (continuous/intermittent) and method (waveguide/mobile phone device) of exposure (Agarwal *et al*., 2009; 2011). Notwithstanding these variations, a consistent theme may be emerging from studies reporting biological effects.

Oxidative stress is a major cause of defective sperm function, contributing to male infertility and DNA damage in the male germ line (Aitken and Gharagozloo, 2011; Aitken *et al*., 2012b, 2014; Tremellen, 2008). Such a state of oxidative stress arises in spermatozoa predominantly as a result of increased ROS production from the mitochondria. The deleterious effects of excess ROS extend to the peroxidation of membrane lipids, generating cytotoxic aldehydes such as 4-hydroxynonenal (4HNE) (Jones et al., 1979; Aitken et al., 2012a) and lead to the oxidation of DNA, generating adducts such as 8-hydroxy-2′-deoxyguanosine (8OHdG) (De Iuliis *et al*., 2009b).

While many studies are now focusing on the biological effects of RF-EMR on reproductive systems, only four of these (Duan *et al*., 2015; Liu *et al*., 2013a, b, 2015) have investigated germ cell specific stages. In the current study, we have sought to extend the findings of our previous research (De Iuliis *et al*., 2009a) by focusing on whether key stages of germ cell development differ in their overall susceptibility to RF-EMR, seeking to uncover mechanism(s) that could account for any variability in response between the different cell types. Male germ cells present a key developmental model to utilize for studying the effects of

RF-EMR during spermatogenesis. For this purpose, we employed cultured immortalized mouse germ cell lines (GC1, spermatogonial; GC2, spermatocyte) and caudal epididymal spermatozoa to determine the impact of RF-EMR exposure on immature germ cells and their mature counterparts. Using a similar experimental design to that reported in our previous study (De Iuliis *et al*., 2009a), these cells were exposed to RF-EMR in a waveguide for up to 6 h while being maintained at 23ºC to mitigate any bulk thermal effects of this treatment. This temperature was crucial to confidently dismiss the effects of heat in our study, which has been a criticism of past studies concerning RF-EMR exposure. Following exposure, cells were assessed using a suite of functional assays to probe the potential impact of RF-EMR on oxidative stress in male germ cells, with a focus on the mitochondria as a potential source of RF-EMR-induced ROS generation.

MATERIALS AND METHODS

Chemical reagents

The chemicals and reagents used in this study were purchased from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA) unless stated otherwise, and were of research grade. The fluorescent probes were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Mouse germ cell lines were purchased from the American Tissue Culture Collection (ATCC; Rockville, MD, USA). These cell lines included type B spermatogonia-like GC1 (ATCC CRL-2053) and primary spermatocyte-like GC2 (ATCC CRL-2196) strains. Human embryonic kidney (HEK) 293 (ATCC CRL-1573), McCoy mouse fibroblast (ATCC CRL-1696) and COV434 human granulosa (Sigma Aldrich) cell lines were also used for comparison.

Cell culture

All cell lines were grown at 37ºC in 5% CO_2, 95% air in Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher Scientific, Taren Point, NSW, Australia) supplemented with 100 mg/ml sodium pyruvate, 4.5 g/l glucose, 0.5 mM L-glutamine, 100 U/ml penicillin-streptomycin and 10% fetal bovine serum (ThermoFisher Scientific). For each experiment, cells were seeded to glass coverslips overnight at a concentration of ~1 × 10^5 cells in 1 ml media in Greiner

CELLSTAR multiwell culture plates (Sigma Aldrich). The passage number used was on average between 5 and 20, but this was maintained less than passage 25, where we observed no changes to proliferation or confluency. Cells were then subjected to EMR exposure for 0-6 h in DMEM media as described below.

Primary cell and spermatozoa isolation

Primary spermatogonial germ cells were isolated from neonatal Swiss mice, as previously described (Baleato *et al*., 2005). Testes were dissected, followed by removal of the tunica albuginea, washing in DMEM at 600 × g and 4ºC for 5 min. Seminiferous tubules were then digested in 0.5 mg/ml collagenase for 15 min, resuspended in 0.5% v/v trypsin-EDTA and rotated for 15 min at 21ºC. This sample was subsequently resuspended in DMEM and strained through a 70 µm filter to remove cell aggregates. The resulting suspension was layered above a continuous 2-4% BSA/DMEM gradient and allowed to sediment under gravity for 3 h to enrich for spermatogonia. The bottom 30 ml layer of this gradient was discarded and the second 40 ml layer, containing an enriched population of spermatogonia, was collected.

Studies undertaken with mouse spermatozoa followed experimental protocols approved by the University of Newcastle Animal Care and Ethics Committee (Ethics Number 2014-423). To isolate spermatozoa, epididymides were dissected from adult Swiss mice (>8 weeks) killed via CO_2 asphyxiation. Mature spermatozoa were collected from the cauda epididymis by retrograde perfusion via the vas deferens (Aitken *et al.,* 2005; Smith *et al*., 2013). These cells were resuspended at a concentration of 1×10^6/ml in 1 ml of modified Biggers, Whitten, and Whittingham medium (BWW; Biggers *et al*., 1971) in 35 mm petri dishes and, due to their short lifespan, were exposed to RF-EMR for up to a maximum of 4 h. Objective sperm motility was assessed by computer assisted sperm analysis (CASA; IVOS, Hamilton Thorne, Danvers, MA, USA). For this purpose, a minimum of 100 spermatozoa in five fields were assessed using 2X-CEL slides (Hamilton Thorne) suspended on a pre-warmed stage (37ºC) (Smith *et al*., 2013). The following settings were utilised: negative phase-contrast

optics, 60 frames/sec recording rate, minimum cell size of 9 pixels, minimum contrast of 80, low size gate of 0.3, high size gate of 1.95, low intensity gate of 0.5, high intensity gate of 1.3, nonmotile head size of 45 pixels, nonmotile head intensity of 75, progressive average path velocity (VAP) threshold of 10 µm/sec, slow (static) cells VAP threshold of 5 µm/sec, slow (static) cells straight-line velocity (VSL) threshold of 0 µm/sec, and threshold straightness (STR) of 75%. Cells exhibiting a VAP of >10 µm/sec and a STR >0 were considered progressive. Cells with a VAP greater than that of the mean VAP of progressive cells were considered rapid. Sperm vitality was assessed via the eosin exclusion method (World Health Organization, 2010).

EMR waveguide exposure system

Cells were exposed to EMR in a waveguide apparatus emitting radiofrequency waves produced by a SMC100A signal generator (Rohde and Schwarz, Macquarie Park, NSW, Australia). The signal intensity was adjusted to appropriate levels with a signal amplifier as used by De Iuliis *et al.* (2009) and output was split through a network antenna to direct the RF-EMR to the aluminum exposure cage, and with minimal wave discharge to a spectrum analyzer to assess the radiation levels (Advantest, Tokyo, Japan). RF-EMR reflection within the cage was minimized by addition of carbon-impregnated foam (RFI Industries, Bayswater, VIC, Australia) around the exposure setting. Microwaves were generated at a frequency of 1.8 GHz and intensity of 0.15 W/kg or 1.5 W/kg specific absorption rate (SAR) as previously calculated by De Iuliis *et al*. (2009). For exposure, germ cells seeded to coverslips or spermatozoa were situated in a small petri dish inside the apparatus. Untreated controls were placed outside of the Faraday cage of the unit and were maintained under identical environmental conditions, in the dark at 23°C. The use of 23ºC was important in our study to discern between the effects of RF-EMR exposure and potential associated heating. The temperature of these media was measured over the course of the experiments, with no significant fluctuations observed in both exposed and control samples, with a stable reading of 23°C (+/- 0.2°C; Supp. Figure 1D) observed.

Alkaline Comet assay

The Comet assay was performed as detailed by Katen *et al*., (2016a, b). Germ cells and spermatozoa were pelleted and stored at -80ºC before being resuspended in phosphate buffered saline (PBS) at a concentration of 4 × 10^4 cells/µl. A 10 µl sample of this cell suspension was mixed with 70 µl agarose (Trevigen, Gaithersburg, MA, USA) and allowed to set on Dakin G376 slides pre-coated with 1% low melting point agarose (ProSciTech, Kirwan, QLD, Australia) sealed with a coverslip overnight at 4ºC. After removing the coverslip, slides were treated with lysis solution 1 (pH 7.5; 0.8 M Tris–HCl, 0.8 M dithiothreitol [DTT], 1% SDS; Ribas-Maynou *et al*., 2014) and sealed with a coverslip for 30 min, followed by lysis solution 2 (pH 7.5, 0.4 M Tris –HCl, 50 mM EDTA, 2 M NaCl, 0.4 M DTT) under the same conditions. Again, coverslips were removed and slides were washed in tris-boric acid-EDTA (TBE) solution (0.445 M Tris–HCl, 0.445 M boric acid, 10 mM EDTA) for 10 min. In preparation for electrophoresis, slides were treated with alkaline solution (pH 11.5; 0.03 M NaOH, 1 M NaCl) for 15 min at 4 ºC, followed by electrophoresis in alkaline buffer (pH 12; 0.03 M NaOH) for 4 min at 1 V/cm. To neutralize the assay, slides were washed in neutralization solution (pH 7.5; 0.4 M Tris–HCl) for 5 min. SYBR green nucleic acid stain (ThermoFisher Scientific, Taren Point, NSW, Australia) (diluted to 1 × from a 10,000 × stock solution in 10 mM Tris/PBS) was applied to the slides immediately before viewing on the microscope, and a coverslip was added. Slides were imaged with a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging Inc., Kirchdorf, Germany), and the integrity of the cells was analyzed using Comet Assay IV software (Perceptive Instruments, Suffolk, UK).

Oxidative DNA damage assay (8-OH-dG)

In order to determine the level of 8-OH-dG DNA base adduction following RF-EMR exposure, DNA was extracted from GC1, GC2 and sperm cells by the phenol/chloroform method. A sample of 5 × 10^6 cells were suspended in 1 ml STE buffer (500 mM NaCl, 100 mM Tris-HCl [pH 8], 10 mM EDTA) and were supplemented with 50 µl 20% SDS, 10 µl 2-mercaptoethanol

and 100 µl 20 mg/mL proteinase K (Roche, Castle Hill, NSW, Australia). After overnight incubation at 55ºC, an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to each tube and vortexed for 30 sec. Each tube was then centrifuged at 14,000 × g for 15 min. The top layer of each sample was collected and transferred to a new 1.5 ml Eppendorf tube. Sodium acetate (3 M) was then added at a volume of 1/9 in addition to two volumes of ice-cold 100% ethanol. The tubes were mixed by inversion and placed at -20ºC overnight. Following this, the DNA was pelleted by centrifugation at for 15 min at room temperature. Next, the supernatant was decanted and the pellet was washed with 100 µl 70% ethanol to remove salts. Finally, the DNA was pelleted by centrifugation at 20,000 × *g* for 15 min, air dried and resuspended in water. DNA concentration was revealed via spectrophotometry at 260 nm and quantification of 8-OH-dG formation was then performed with an 8-OH-dG ELISA kit (Abcam, Cambridge, UK). The ELISA plate was developed in the dark on an orbital shaker for 60 min before being read on a Fluostar Optima plate reader (BMG Labtech, Mornington, Victoria, Australia) at a wavelength of 405-10 nm.

Sperm chromatin dispersion (Halo) assay

The halo assay is a qualitative method to assess DNA integrity of spermatozoa, whereby cells treated with DTT and SDS will have their DNA splay out, if intact. The DNA is then stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize a halo-like pattern (Fernandez *et al*., 2003). Cells snap frozen and stored at -80ºC were mixed with 1% low melting agarose at 37ºC to achieve a concentration of 0.7% agarose. A 70 µl aliquot of this solution was then transferred to a Superfrost microscope slide precoated with 0.65% agarose, sealed with a coverslip and placed at 4ºC for 5 min to solidify. Following this, the coverslip was gently removed and the slides were treated with 0.08 N HCl for 7 min in the dark. The slides were then treated with halo solution 1 (pH 7.5; 0.4 M tris, 1% SDS, 50 mM EDTA, 0.8 M DTT) for 10 min, followed by halo solution 2 (pH 7.5; 0.4 M tris, 1% SDS, 2 M NaCl) for 5 min at room temperature to lyse the cells, relax and neutralize the DNA. Next, the slides were treated with tris-boric acid-EDTA buffer (pH 7.5; 0.1 M tris, 0.09 M boric acid, 0.002 M EDTA) for 2 min,

followed by washes in increasing concentrations of 70%, 90% and 100% ethanol for 2 min each, to dehydrate the slides. The slides were allowed to air dry before staining with DAPI for 10 min at room temperature. Finally, the slides were rinsed in PBS and mounted for viewing with a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss). A minimum of 100 cells were assessed for the number of intact halos.

Assessment of germ cell mitochondria following RF-EMR exposure

Coverslips containing seeded, irradiated cell lines were incubated (15 min at 37°C) in the dark in 50 µl droplets of DMEM containing 1 µM MitoSOX Red (MSR) to detect mitochondrial superoxide generation and 5 nM SYTOX green stain for assessment of cell vitality. Following incubation, coverslips were washed in DMEM and mounted in 5 µl DMEM on microscope slides. A minimum of 100 cells were then assessed using a Zeiss Axioplan 2 fluorescence microscope. For experiments in which mitochondrial electron transfer was inhibited, seeded coverslips were treated with a final concentration of 10 µM of either antimycin A or rotenone for 0-6 h at 21ºC and again assayed with the MSR probe, as detailed above. Finally, for experiments involving succinate as the electron source, germ cells were seeded overnight in DMEM described above, and refreshed with DMEM or DMEM devoid of glucose, containing 5 mM succinate for the duration of the experiment (4 h).

Determination of sperm oxidative stress following RF-EMR exposure

Spermatozoa were used for determination of mitochondrial ROS generation (MSR), mitochondrial membrane potential (JC1), lipid peroxidation (BODIPY C11) and protein tyrosine phosphorylation level (α-PT66) using a FACS-Canto flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser and 633 nm helium-neon laser. Gating was used to prevent incorporation of non-sperm cells and the evaluations were based on at least 5,000 gated cells. Regarding the MSR and JC1 assays, sperm cells were centrifuged at 450 × g for 5 min and resuspended in a final concentration of 500 nM MSR or JC1 coupled with a 5 nM SYTOX green vitality stain. Incubation of this probe was for 15 min

at 37°C in the dark, followed by resuspension in BWW media in flow cytometry tubes for analysis on the flow cytometer. The BOPIDY probe was preloaded at 5 µM for 1 h at 37°C. Cells were then washed and treated, with arachidonic acid (50 µM) employed as a positive control. Analysis of these data was undertaken using CellQuest software (BD Biosciences, San Jose, CA, USA).

Chemiluminescence was used to investigate hydrogen peroxide generation in treated populations of germ cells and spermatozoa as previously described (Houston *et al*., 2015). Briefly, 2×10^6 cells were resuspended in BWW supplemented with 4 µl of 250 µM luminol and 8 µl of 2 mg/ml horseradish peroxidase (HRP, type VI from horseradish) in a total of 400 µl BWW. These samples were assessed for chemiluminescence in Rohren tubes (Sarstedt, Numbrecht, Germany) for 2 h at 37°C in a Berthold 9505C luminometer (Berthold, Wilbad, Germany). Control Version 1.04B was used for the system software.

Statistical analysis

JMP version 11 (SAS Institute Inc., Cary, NC) was used to analyze the data in each experiment, which were performed with at least 3 independent replicates. EMR treatment was analyzed using a one-way ANOVA at each time point, paired with Tukey's honest significant difference test (significance $p < 0.05$). Error bars are presented as standard error values around the mean.

RESULTS

Mouse male germ cells are vulnerable to RF-EMR

Cell lines representing both spermatogonial (GC1) and spermatocyte (GC2) phases of development, exposed to RF-EMR at a dose of 0.15 W/kg exhibited significant increases in the formation of mitochondrial ROS generation following 2 h ($p < 0.001$) and 4 h ($p < 0.05$) of exposure, respectively (Fig. 1A and B). This phenomenon persisted up to the 6 h time point for both cell types ($p < 0.01$). Furthermore, as shown in Figure 1C, this result was recapitulated in populations of primary spermatogonial cells isolated from neonatal mice. Here, we again observed significantly elevated mitochondrial ROS generation, after 2, 4 and 6 hours of exposure ($p < 0.05$) compared to unexposed control populations. In these primary cultures we again observed no effect of RF-EMR exposure on vitality, or in any cell type exposed in this study (Supplementary Figure 2). While we documented a modest decrease in vitality after 6 h from the initial assessment 93% ±0.7, RF-EMR exposure did not significantly decrease this measure (88% ±1.1) with respect to the untreated control spermatogonia (83% ±2.9) at this time. An identical RF-EMR treatment regime failed to elicit any overt changes in mitochondrial ROS generation (MitoSOX labelling) of the three somatic cell lines examined (Fig. 1D, E and F; HEK293, COV434 and McCoy, respectively) beyond that of the untreated control samples. In both GC1 and GC2 cell lines, ROS generation was not notably increased following exposure with an elevated dose of 1.5 W/kg EMR (Supplementary Fig. 1A, B) compared to the dose of 0.15 W/kg. Importantly, the effects of exposure were generated independent of any significant reduction in cell viability, in all cell types and treatment regimes employed in this study.

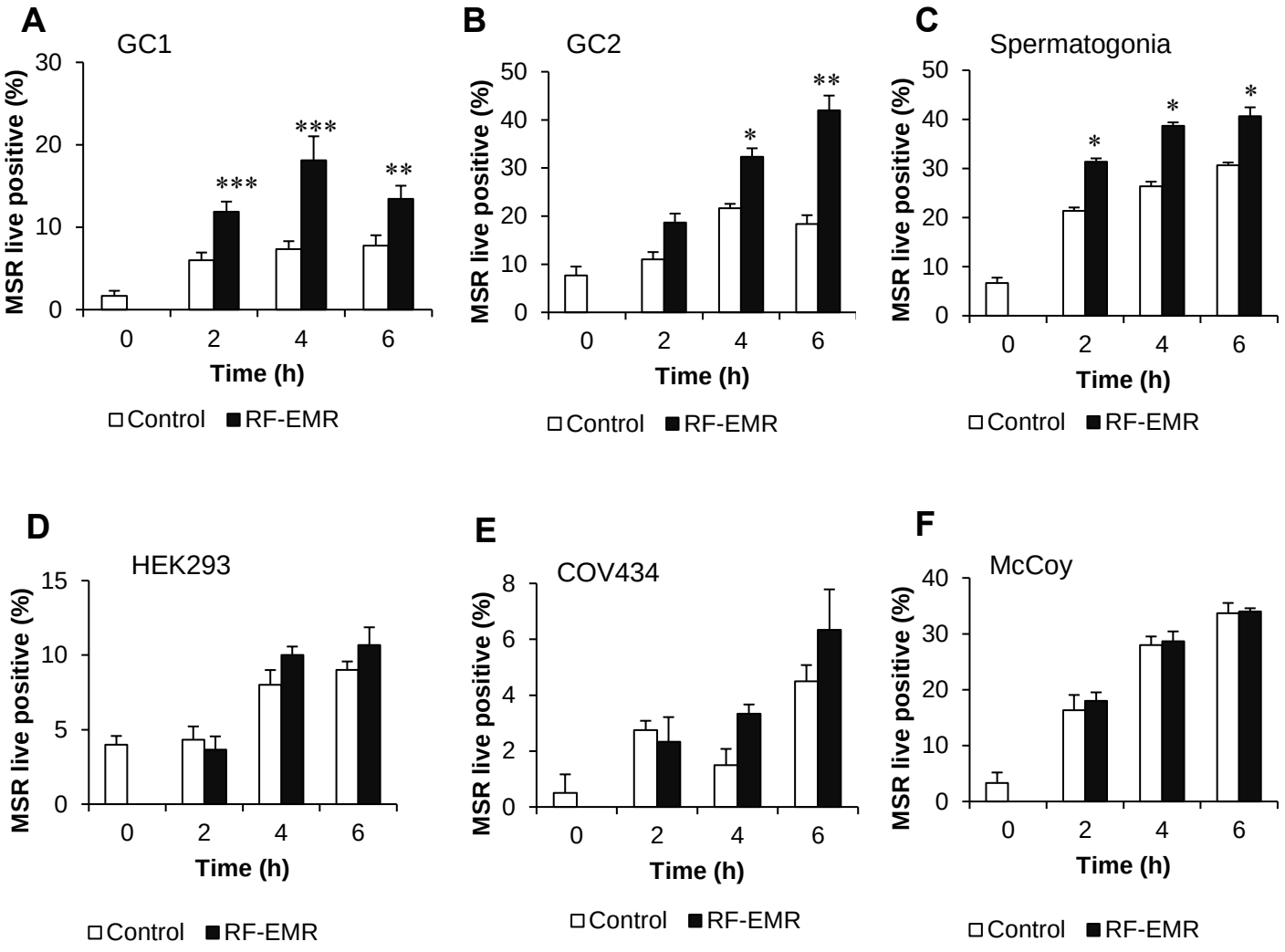


Figure 1. RF-EMR exposure (1.8 GHz, 0.15 W/kg) induces mitochondrial superoxide generation in male germ cells. (A) Spermatogonia-like (GC1) cell line, (B) spermatocyte-like (GC2) cell line, and (C) spermatogonia isolated from neonatal mice were seeded to glass coverslips overnight and exposed to RF-EMR (1.8 GHz, 0.15 W/kg) for periods of up to 6 h. Somatic cell lines comprising (D) human embryonic kidney cells (HEK293), (E) granulosa cells (COV434) and (F) mouse fibroblasts (McCoy), were treated under an identical exposure regime (1.8 GHz, 0.15 W/kg) as negative controls. At regular intervals during exposure, a portion of the cells were assessed for mitochondrial ROS production using the MitoSOX red (MSR) probe. This analysis was restricted to the live cell population as determined by co-labeling with SYTOX green vitality stain. These analyses were performed at least three times and data are presented as mean ± SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to unexposed controls. Panel A n=9; B n =5, C-F n=3

The origin of EMR mediated ROS generation in male germ cells

Following the demonstration that GC1 cells responded to EMR exposure in a similar manner to primary spermatocytes, we focused our characterization of downstream effects of EMR on the GC1 and GC2 cell lines. Given that the mitochondria account for a majority of ROS production within the mature spermatozoon (Aitken *et al*., 2012a,b) and the observed increase in mitochondrial ROS generation following germ cell exposure to RF-EMR (Fig. 1A and B), we next investigated the effects of treating GC1/GC2 germ cell lines with a combination of RF-EMR and inhibitors that selectively target either Complex I or III of the electron transport chain (Fig. 2). This study demonstrated that inhibition of Complex I with rotenone had a marked impact on both CG1 and CG2 cell types, dramatically increasing ROS production via mechanisms that were independent of RF-EMR exposure (Fig. 2A, B). In contrast, while inhibition of Complex III with antimycin A also induced a significant increase in mitochondrial ROS generation in the GC1 cell line (Fig. 2C; $p < 0.01$), this inhibitor significantly potentiated the impact of RF-EMR exposure in both cell lines (Fig. 2C, D; $p < 0.01$). Accordingly, after 2 h, antimycin A treated GC1 cells were characterized by ROS levels that were significantly elevated above that of non-exposed cells (Fig. 2C; $p < 0.01$). A similar, although delayed, response was also recorded in GC2 cells, with significance differences in mitochondrial ROS generation only being achieved after a period of 4 h (Fig. 2D $p < 0.05$). To aid in pinpointing the components of the mitochondrial ETC vulnerable to RF-EMR, succinate was employed as a metabolic substrate; driving electrons to enter this pathway via Complex II. In GC1 cells (Fig. 2E) the introduction of this metabolic substrate elevated basal ROS levels substantially in both control ($p < 0.01$) and RF-EMR ($p < 0.01$) treated cells. However, RF-EMR exposure did not induce a significant increase in mitochondrial ROS generation in comparison to control cells when utilizing succinate as substrate, even though a positive change was observed in the presence of glucose (Fig. 2E). A similar profile was observed with GC2 cells (Fig. 2F), which did not exhibit an increase in ROS generation following exposure to RF-EMR in the presence of succinate, although a significant response again was generated when using glucose as the energy substrate.

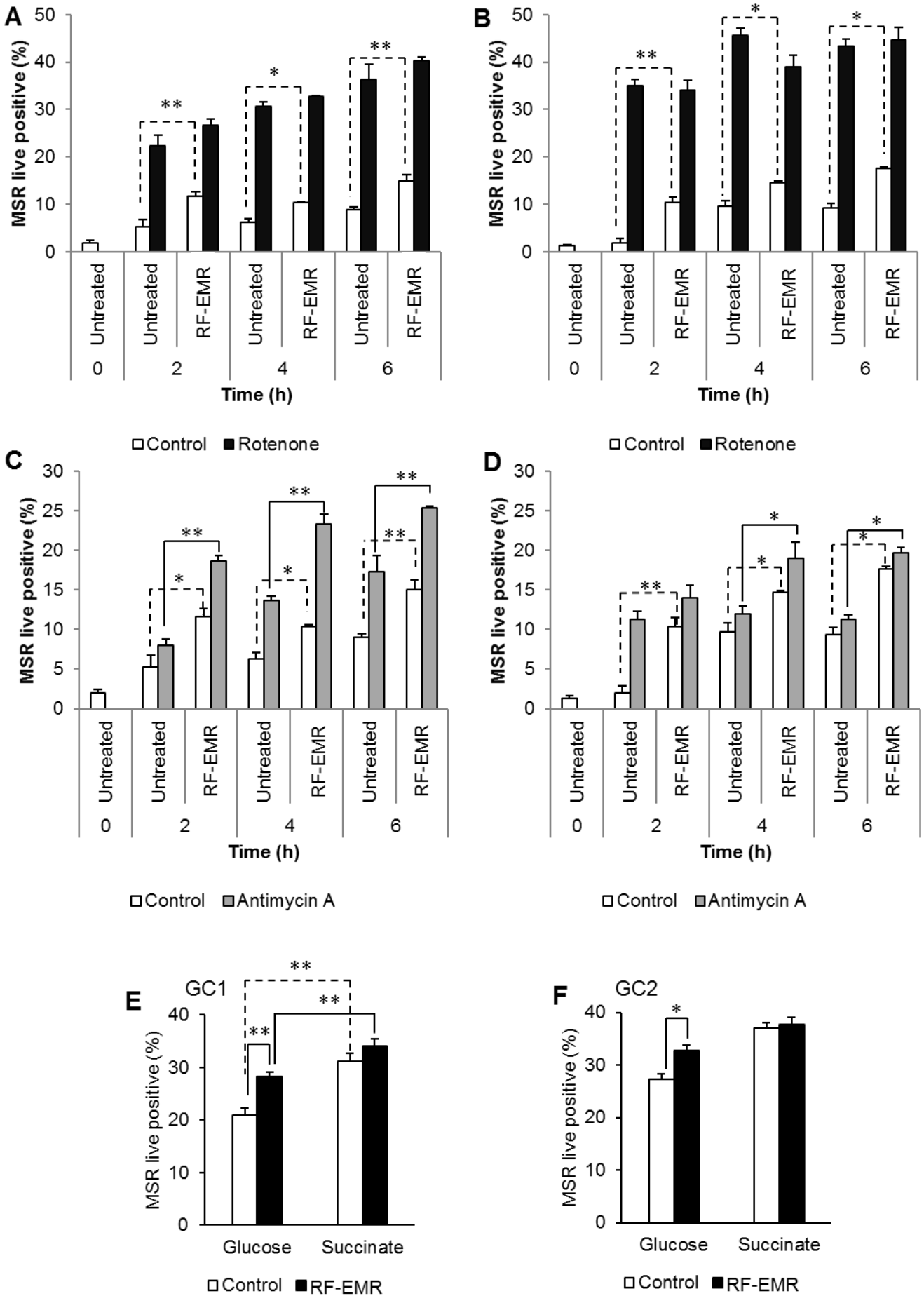

A
B
C
D
E
F
MSR live positive (%)
Time (h)
Untreated
RF-EMR
Control
Rotenone
Antimycin A
GC1
GC2
Glucose
Succinate

Figure 2. Inhibition of mitochondrial respiration in the presence of RF-EMR is associated with increased ROS production. (A) Spermatogonia-like (GC1) and (B) spermatocyte-like (GC2) cell lines were seeded to glass coverslips overnight and treated with mitochondrial electron transport chain inhibitor rotenone, in the presence or absence of RF-EMR exposure (1.8 GHz, 0.15 W/kg), for periods of up to 6 h. Alongside these experiments, another electron transport inhibitor, antimycin A, was also utilized for GC1 (C) and GC2 cells (D). Mitochondrial ROS production was assessed using the MSR probe. This analysis was again restricted to the live population, determined by co-labeling with SYTOX green vitality stain. Glucose and succinate substrates were utilized for comparison of mitochondrial ROS generation in GC1 (E) and GC2 (F) cells in the presence of RF-EMR. Cells were seeded to coverslips overnight in DMEM media as detailed above, and refreshed with this DMEM media or 5 mM succinate media DMEM (devoid of glucose) for the course of the experiment. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to unexposed controls, n=3

In order to determine if the increase in mitochondrial ROS in the irradiated germ cells could advance cellular oxidative stress as shown in previous studies (Agarwal *et al*., 2009; De Iuliis *et al*., 2009a; Mailankot *et al.*, 2009), we next investigated markers of cellular ROS production and lipid peroxidation following application of RF-EMR. Examination of extracellular ROS production using a luminol-peroxidase chemiluminescence assay revealed no significant increases in the release of ROS from these cells following RF-EMR exposure (0.15 W/kg) for any of the mouse germ cell types examined (Fig. 3A). Meanwhile, incorporation of the potent nucleophile scavenger penicillamine, which inhibits the propagation of oxidative stress by blocking damaging alkylation events mediated by lipid peroxidation by-products (Aitken *et al*., 2012a), provided no significant reduction in the elevated levels of ROS production observed in RF-EMR treated GC1 cells (Fig. 3B). While a similar MSR response to RF-EMR was observed in GC2 cells (Fig. 3C), penicillamine treatment did achieve a significant decrease in MSR positive cells following 4 h of exposure ($p < 0.05$). However, this observation did not persist to the 6 h time point.

RF-EMR does not induce significant DNA damage in male germ cell lines

To confirm the potential of RF-EMR to induce genotoxic effects in male germ cells (as documented in previous studies [Liu *et al*., 2013a, b]) we next investigated the incidence of DNA strand breakage, utilizing the alkaline comet assay. Here, it was revealed that RF-EMR induced non-significant DNA fragmentation in GC1 cells ($p = 0.07$) following 6 h exposure (Fig. 4A), which was completely absent in GC2 cells at any time point examined (Fig. 4B). Furthermore, to confirm the apparent disconnect between DNA damage and ROS production, we investigated the formation of the oxidative DNA base adduct, 8-OH-dG, in GC1 and GC2 cells (Fig. 4C). In keeping with the inability of RF-EMR to induce lipid peroxidation (Fig. 4), this exposure induced no significant increases in the generation of 8-OH-dG in either germ cell population ($p > 0.1$).

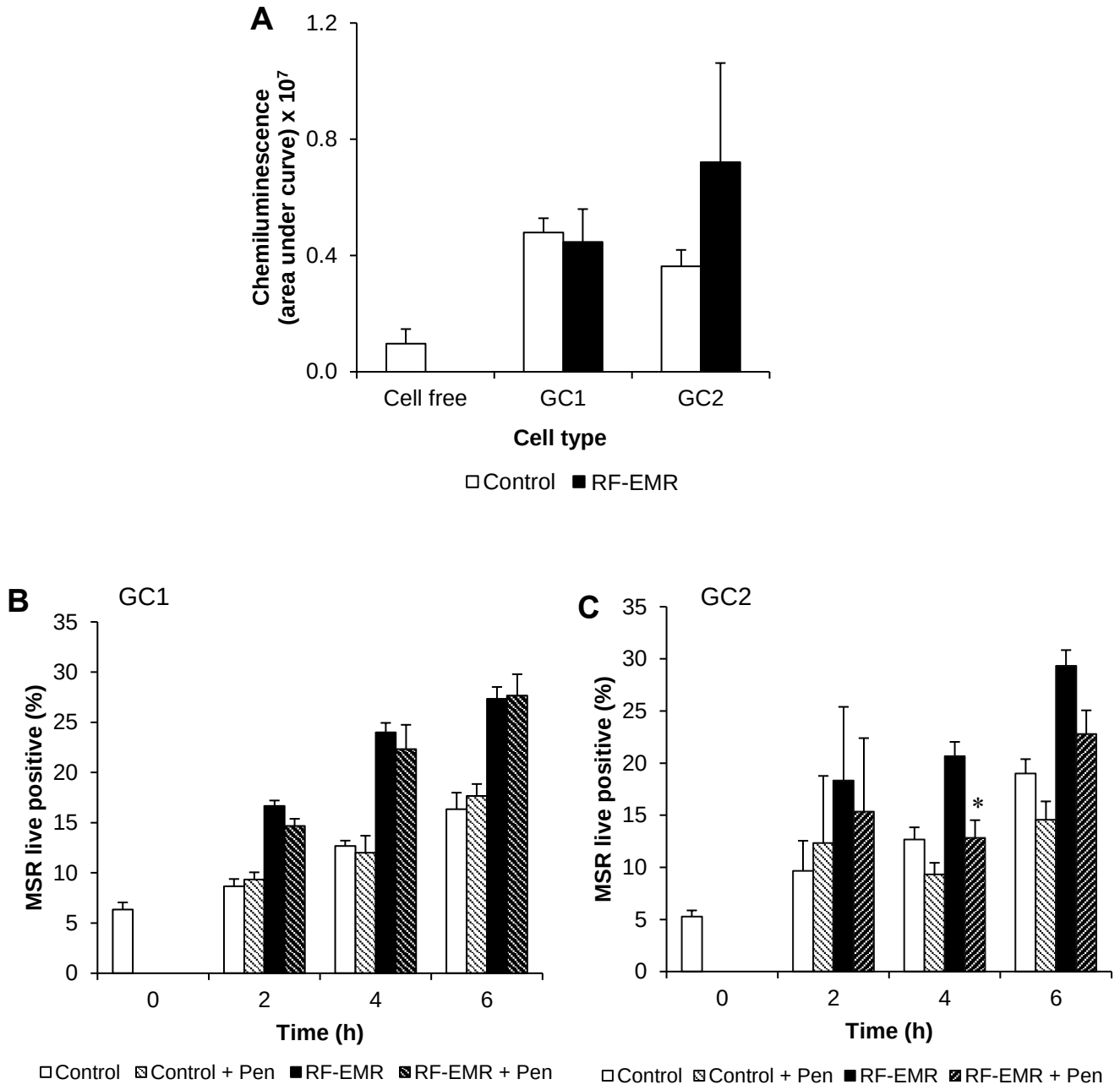


Figure 3. The effects of RF-EMR on production of reactive oxygen species and lipid peroxidation in male germ cells. GC1 and GC2 cells were seeded to coverslips overnight then exposed to RF-EMR of an intensity of 0.15 W/kg and frequency of 1.8 GHz. (A) Luminol-peroxidase chemiluminescence assessment of ROS production was conducted on populations of GC1 and GC2 cells following 6 h of exposure. The ability of penicillamine (100 µM), a potent aldehyde scavenger, to prevent RF-EMR induced ROS production was also assessed over the course of exposure in both (B) GC1 and (C) GC2 cells. * $p < 0.05$ compared to RF-EMR treatment. Panel A n=6, B n=3, C n=6.

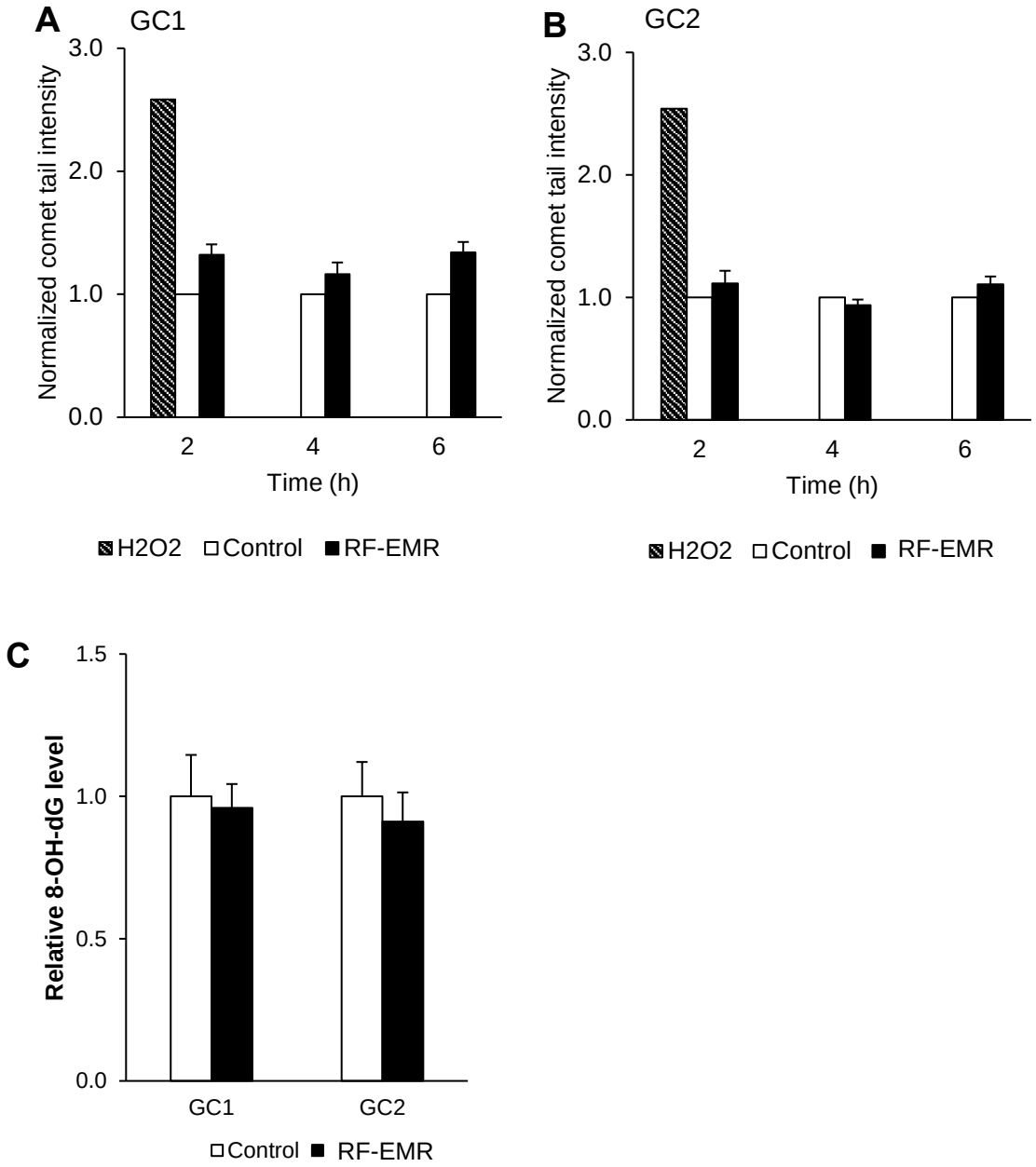


Figure 4. The effect of RF-EMR on DNA fragmentation and DNA oxidation within mouse germ cells. GC1 and GC2 cell lines seeded to glass coverslips overnight were exposed to RF-EMR (1.8 GHz, 0.15 W/kg) to determine its ability to impair DNA integrity. These cells were subsequently assessed for DNA fragmentation using an alkaline comet assay; (A) GC1, (B) GC2. DNA was also extracted from these cells via the use of phenol-chloroform methodology in order to assess oxidative DNA damage in the form of 8-hydroxy, 2-deoxyguanosine (8-OH-dG) base adducts, as evaluated by an 8-OH-dG ELISA (C), n=3.

The effects of RF-EMR on mature mouse spermatozoa

In marked contrast to the response elicited by RF-EMR in germ cell lines and purified spermatogonia, mature populations of mouse spermatozoa sampled from the cauda epididymis proved refractory to this exposure. In this regard, we failed to detect any substantive increase in either mitochondrial ROS (Fig. 5A) or cell death (Fig. 5B) following exposure of spermatozoa to a dose of 0.15 W/kg RF-EMR. Rather, these terminally differentiated cells exhibited a highly significant ($p < 0.001$) spontaneous, time-dependent increase in ROS generation in association with sperm capacitation that was not influenced by RF-EMR exposure (Fig. 5A). These changes were accompanied by significant, time-dependent reductions in sperm vitality (Fig. 5B; $p < 0.001$) and mitochondrial membrane potential (MMP) (Fig 5C; $p < 0.001$) that were again uninfluenced by exposure to RF-EMR. Increasing the intensity of this radiation to 1.5 W/kg (Supplementary Fig. 1C) resulted in a significant reduction in ROS generation after 1 h ($p < 0.05$) of exposure. This trend of reduced ROS production was held over the ensuing 3 h incubation, however, did not maintain significance ($p = 0.11$).

Next, we further investigate levels of ROS generation via chemiluminescence, which demonstrated that global cellular ROS generation in mature mouse spermatozoa was unaffected following exposure to RF-EMR (Fig. 6A). Accordingly, the levels of lipid peroxidation associated with spermatozoa, as assessed using the BODIPY C11 probe (and arachidonic acid positive control), were also not significantly elevated following RF-EMR exposure (Fig. 6B). Furthermore, both the qualitative profile and relative levels of 4-hydroxynonenal-alkylated sperm proteins remained unchanged with exposure to RF-EMR (Fig. 6C and D).

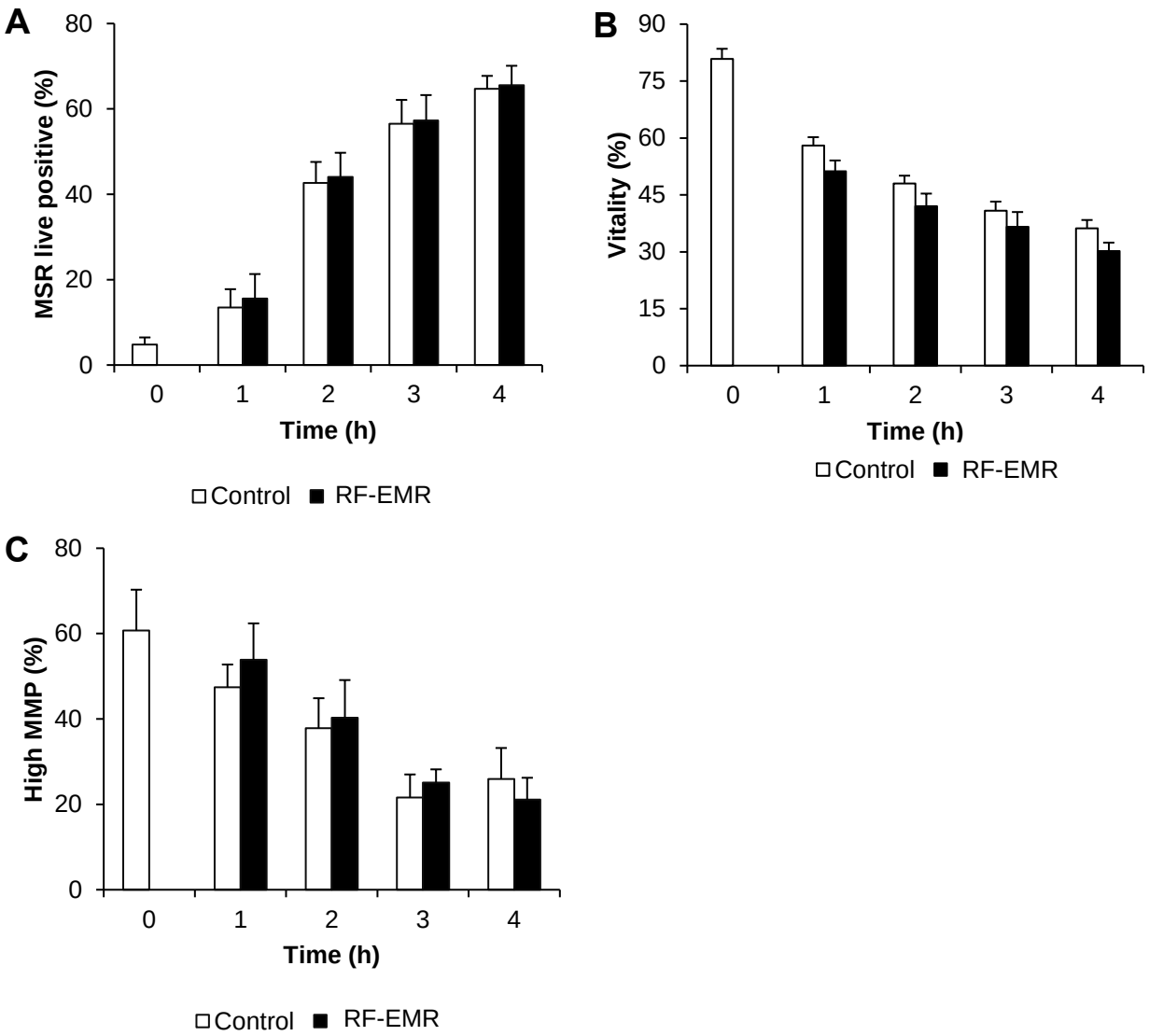


Figure 5. Susceptibility of mouse spermatozoa to RF-EMR (1.8 GHz, 0.15 W/kg). Mature mouse spermatozoa isolated from the cauda epididymis were exposed to RF-EMR of an intensity of 0.15 W/kg. At regular intervals during exposure, a portion of the live cell population was assessed for (A) mitochondrial ROS generation using the MSR probe via flow cytometry. (B) Similarly, total vitality was evaluated with an eosin stain. (C) Alternatively, perturbation of mitochondrial membrane potential was determined through incubation with the JC1 probe. In this instance, the percentage of cells displaying green fluorescence indicative of high mitochondrial membrane potential was determined, again via flow cytometry. These analyses were performed 3 times and data are presented as mean ± SEM. Panels A n=6, B n=5, C n=6.

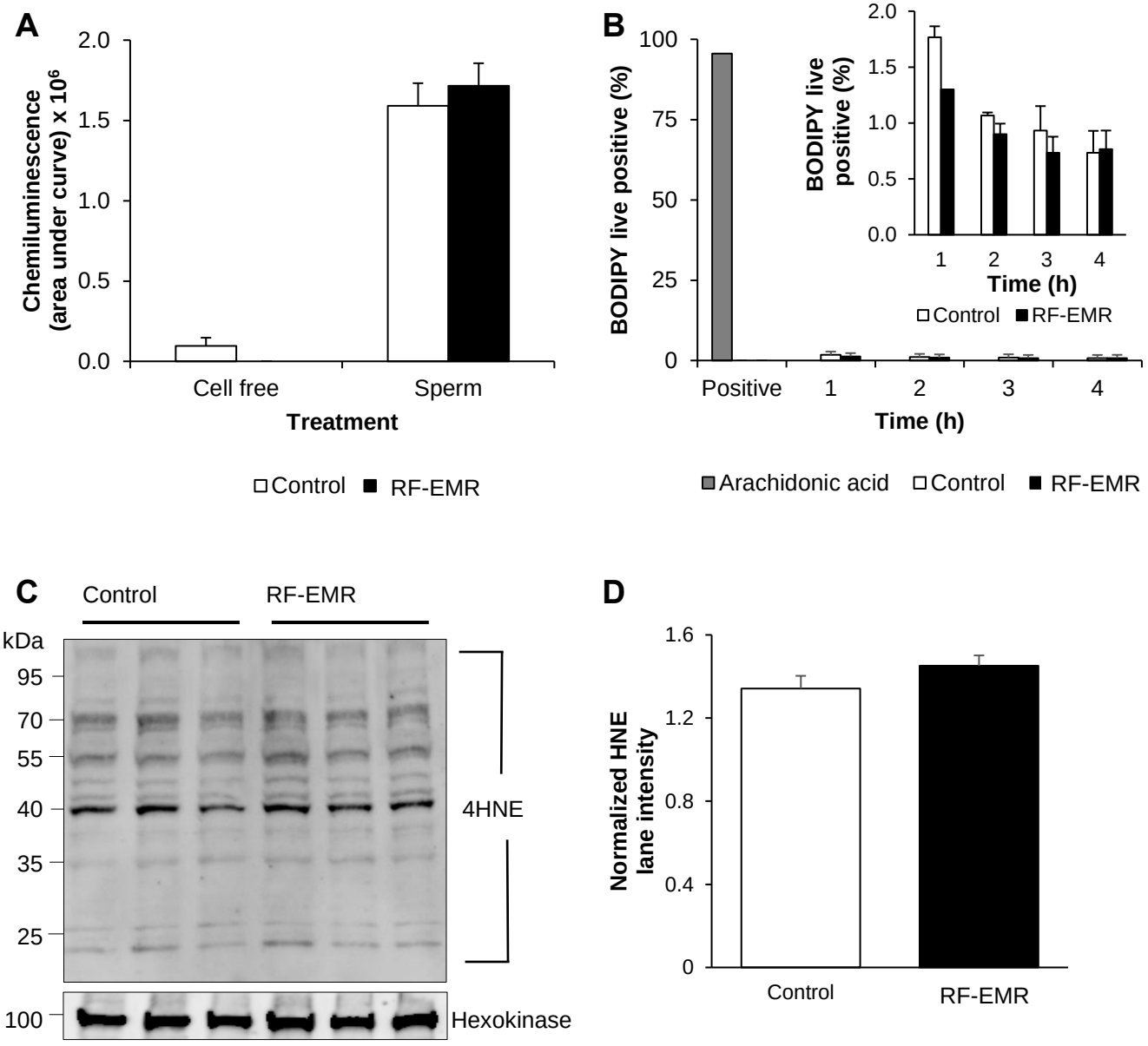


Figure 6. The effects of RF-EMR on production of reactive oxygen species and lipid peroxidation in mature spermatozoa. Spermatozoa were isolated from the cauda epididymis and exposed to RF-EMR of an intensity of 0.15 W/kg. (A) Luminol-peroxidase chemiluminescence was used to assess ROS production following 4 h of exposure. (B) The lipid peroxidation status of these spermatozoa was evaluated with the BODIPY C11 probe via flow cytometry. (C) The profile of 4HNE alkylated-proteins in RF-EMR exposed spermatozoa was assessed via immunoblotting, with hexokinase expression featuring as a loading control. Three replicates were performed for both control and RF-EMR treated sperm protein extracts. (D) The corresponding intensity of all 4HNE labeled protein bands extracted from untreated control, and RF-EMR exposed spermatozoa, was determined by densitometric analysis of pixel intensity. Densitometry was performed on the principal bands from 70-40 kDa, relative to the 100 kDa hexokinase band presented below the 4HNE immunoblot. Panels A n=4, C-D n=3.

RF-EMR induces DNA damage in mature mouse spermatozoa

In order to determine if spermatozoa were also sensitive to DNA damage following RF-EMR exposure we repeated our alkaline comet assay in these mature cells (Fig. 7A). This assay demonstrated a significant, 20% increase in DNA fragmentation in RF-EMR treated spermatozoa following 3 h of exposure ($p < 0.05$). We next utilized the halo assay to explore the impact of such RF-EMR mediated fragmentation on overall sperm DNA integrity (Fig. 7B). However, this assay failed to detect a concomitant reduction in DNA integrity in response to RF-EMR exposure. Nevertheless, our subsequent investigation of the burden of oxidative DNA lesions present within RF-EMR treated spermatozoa, unveiled a significant increase in the oxidative stress biomarker, 8-OH-dG, following 4 h of radiation exposure in these cells (Fig. 7C).

Functional consequences of RF-EMR exposure in mouse spermatozoa

Considering RF-EMR did not induce marked alterations to mitochondrial function in spermatozoa, but was capable of inducing significant DNA fragmentation and oxidative DNA damage in this cell type, we next explored its effect on basic sperm physiology. RF-EMR exposure was able to induce DNA fragmentation within 3 h of exposure (Fig. 7A). At an equivalent exposure time, spermatozoa retained motility profiles that were indistinguishable from that of untreated control samples (Fig. 8A - D). In fact, significant impacts on motility induced by RF-EMR exposure did not appear until 4 h of exposure, where a significant decrease in total sperm motility (46% vs 35%; $p < 0.05$) was observed. This overall decrease was accompanied by significant reductions in progressive motility ($p < 0.05$; Fig. 8B), rapid motility ($p < 0.05$; Fig. 8C), and the straight line velocity ($p < 0.05$; Fig. 8D) of the treated spermatozoa. These effects of RF-EMR exposure were not associated with any change in the capacitation status of the spermatozoa as reflected in their patterns of protein phosphotyrosine expression, which remained uniformly high (Fig. 8E).

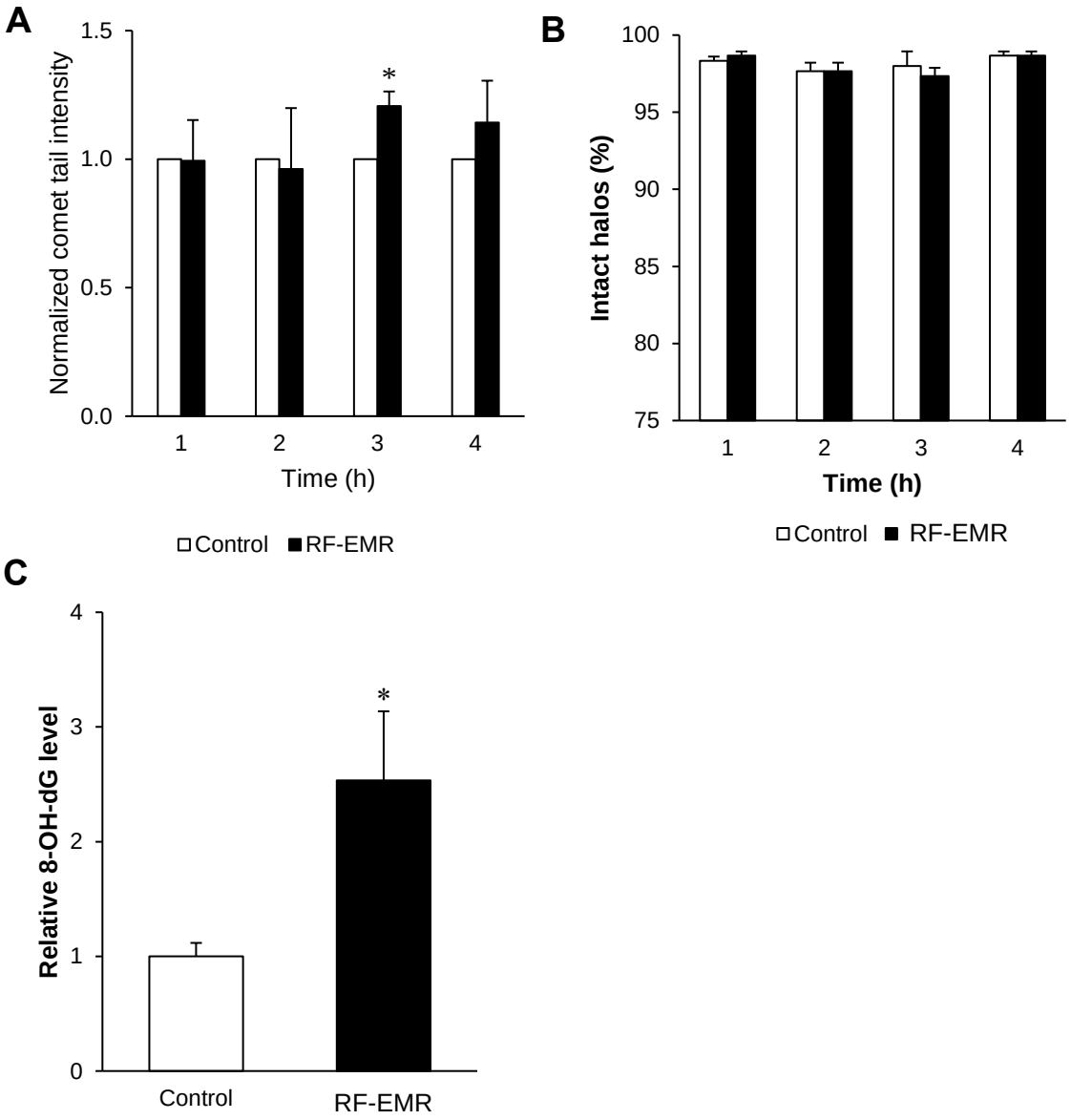


Figure 7. The effect of RF-EMR on DNA oxidation and fragmentation within mature sperm cells. Mature caudal mouse spermatozoa were exposed to RF-EMR (0.15 W/kg) to determine its ability to impair DNA integrity. These spermatozoa were subsequently assessed for DNA damage using the (A) alkaline comet assay (B) and halo assay. (C) DNA was extracted from these cells via the use of phenol-chloroform and analyzed for the presence of oxidative DNA damage utilizing an 8-OH-dG ELISA. * $p < 0.05$ compared to unexposed controls, n=3

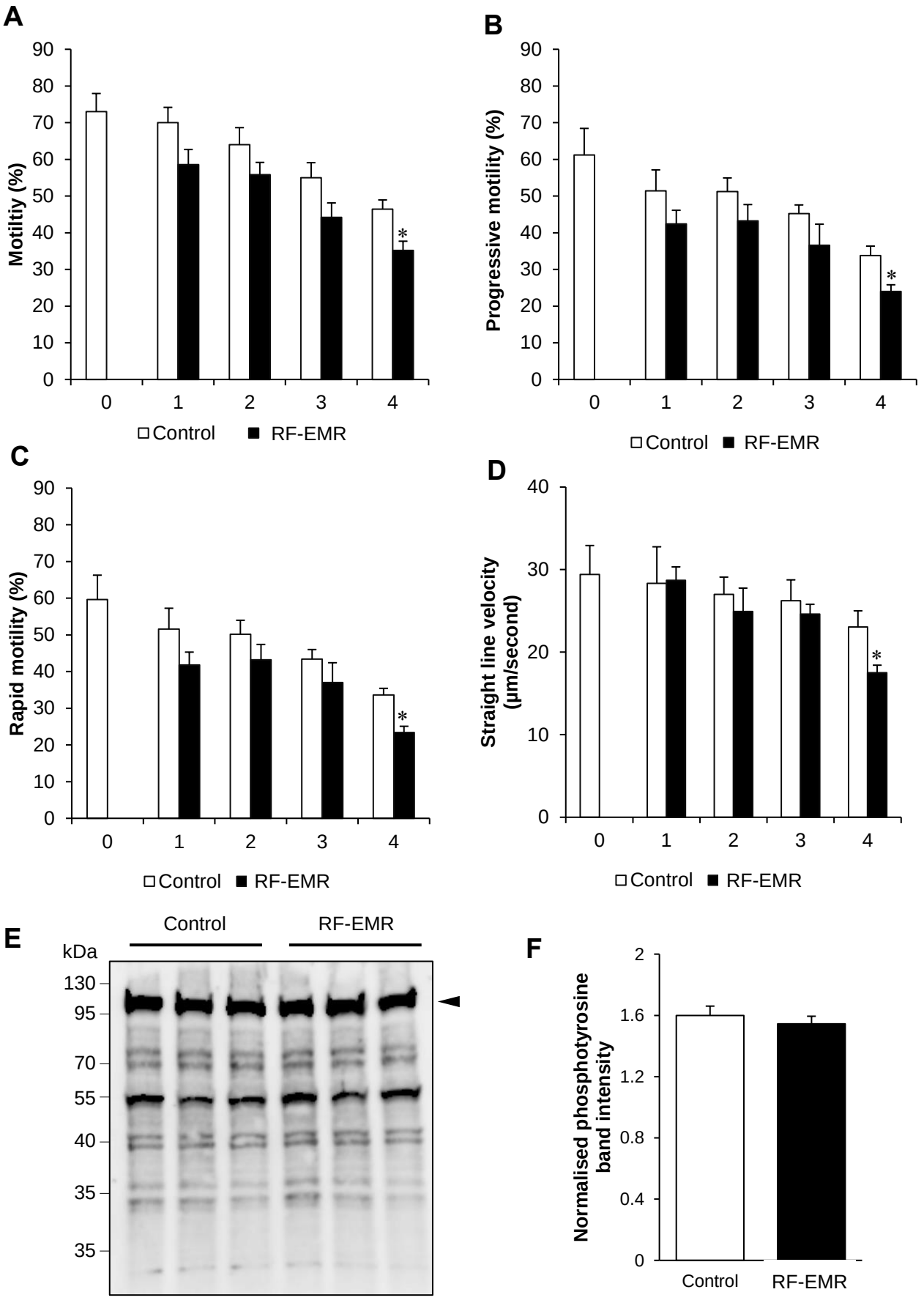

A
Motiltiy (%)
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4
Control RF-EMR
*
B
Progressive motility (%)
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4
Control RF-EMR
*
C
Rapid motility (%)
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4
Control RF-EMR
*
D
Straight line velocity (µm/second)
40
30
20
10
0
0 1 2 3 4
Control RF-EMR
*
E
Control
RF-EMR
kDa
130
95
70
55
40
35
35
F
Normalised phosphotyrosine band intensity
2
1.6
1.2
0.8
0.4
0
Control
RF-EMR

Figure 8. RF-EMR alters sperm motility but not tail tyrosine phosphorylation levels. Mature mouse spermatozoa isolated from the cauda epididymis were exposed to RF-EMR of an intensity of 0.15 W/kg for periods of up to 4 h. At regular intervals during exposure, computer assisted sperm analysis was performed for parameters of sperm motility. (A) Sperm total, (B) progressive, (C) rapid motility and (D) straight-line velocity. (E) Spontaneous sperm tyrosine phosphorylation levels were assessed via immunoblotting. Three replicates were performed for both control and RF-EMR treated sperm protein extracts. The intensity of each lane was then quantified via pixel intensity (F). The entire lane was quantified relative to hexokinase, as the loading control (arrow). * $p < 0.05$ compared to unexposed controls. Panels A-D n=5, E-F n=3.

DISCUSSION

In this study we have explored the effects of RF-EMR on both cultured male germ cell lines and spermatozoa isolated from the mouse. Our results align with previous studies in this field which demonstrate the capacity of RF-EMR to induce DNA strand breakage, mitochondrial free radical generation and motility loss. These results were attained in the absence of an overt state of oxidative stress in our germ cell models, and are thus unlikely to be attributed to this pathological mechanism in these models. Meanwhile, the response of cultured spermatozoa to this insult, suggests that oxidative stress is induced in these mature cells. The body of evidence revealing the genotoxic impacts of RF-EMR in spermatozoa is steadily growing (d'Ambrosio *et al*., 2002; Aitken *et al*., 2005; Balode, 1996; Duan *et al*., 2015; Liu *et al*., 2013a). However, the need to understand the physicobiological details of how non-ionizing radiation results in cellular damage remains unmet. Elucidating such a mechanism has been confounded by the considerable amount of conflicting data published to date (Houston *et al*., 2016). The difficulties establishing a recognized mechanism, is a major constraint when examining the potential clinical impacts of research in this field. This now not only warrants the investigation into potentially new safe exposure levels but also highlights the importance of probing the mechanisms of action.

Origin of RF-EMR induced mitochondrial ROS production in immortalised male germ cells

To the best of our knowledge, the present study provides the first evidence that RF-EMR is capable of inducing mitochondrial ROS generation in isolated precursor mouse germ cells (Fig. 1A-C). The fact that similar responses were not elicited in any of the somatic cell lines examined (Fig. 1D-F), suggests the male reproductive system may possess a unique vulnerability to RF-EMR and therefore supports the male germline as a potentially sensitive model system. Within the immortalized germ cell types examined, the spermatogonial-like GC1 cell line appeared more susceptible to RF-EMR than that of the later stage spermatocyte-like GC2 cells, as indicated by the earlier onset of elevated levels of mitochondrial ROS (2 vs

4 h, respectively). One explanation for this may involve the morphological change of the germ cell mitochondria following progression to the spermatocyte stage, whereupon these organelles experience increased vacuolarization (Meinhardt *et al*., 2000). Irrespective of this, the contrasting outcomes highlight that the effect of RF-EMR may vary greatly, depending on the stage of germ cell development experiencing RF-EMR exposure. On one hand, it has been well established that isolated spermatozoa are susceptible to elevated ROS production (Aitken *et al.,* 1989; Houston *et al*., 2015; Moazamian *et al*., 2015) because of their lack of intrinsic antioxidant defense, but, *in vivo,* germ cells may be protected by the reproductive system through the provision of antioxidant protection, including superoxide dismutase and glutathione peroxidase (Kaur *et al*., 2006).

It has previously been reported that spermatozoa exhibit a particular susceptibility to RF-EMR revolving around mitochondrial dysfunction (De Iuliis *et al*., 2009a). However, unlike human spermatozoa exposed to RF-EMR, mouse spermatozoa experienced no substantial increase in mitochondrial ROS generation following RF-EMR exposure in this study (Fig. 5A). In line with the high variation in overall sperm cell quality in the human compared to the mouse, the lack of mitochondrial dysfunction in comparison in the mouse may reflect the relatively poor capacity of human sperm mitochondria to control the flow of electrons through the ETC (Koppers et al., 2008). This in turn, may reflect a heavier reliance of human spermatozoa on glycolysis for ATP production in comparison to murine spermatozoa (Storey, 2008). As a consequence of this pattern of metabolism, human sperm mitochondria may not only leak electrons more readily than their mouse counterparts but also be less competent at dealing with mitochondrial dysfunction induced by RF-EMR. Furthermore, it should be noted that, for human spermatozoa, De Iuliis (*et al*., 2009) utilized extended exposure periods in comparison to this study (16 h versus 4 h) and higher intensities of exposure (up to 27.5 W/kg). This difference in exposure is likely to be the major reason for the discrete set of results generated between these two studies. However, this disparity is unable to be addressed as mouse

spermatozoa do not survive long enough *in vitro* for such extended exposure times to be assessed.

Within the mitochondria, electron flow is generally strictly regulated. Any interruption of electron transport and associated electron leakage would be expected to elevate the production of ROS. To probe the origin of ROS under RF-EMR exposure, we utilized inhibitors of Complex I, rotenone (Fig. 2A, B), and Complex III, antimycin A (Fig. 2C, D) in both cell lines. These compounds work to impede the flow of electrons through the electron transport chain, by inhibiting the oxidation of the electron carrier ubiquinone in key intermediate sites of these complexes (Quinlan *et al*., 2013). As expected, we documented an induction of ROS in unexposed cells treated with rotenone, and to a lesser, yet still significant, extent with antimycin A. In this study, the ability of RF-EMR to induce addtional ROS production in the presence of antimycin A, within both GC1 and GC2 cell lines (Fig. 2C, D), suggests that this ETC complex may be a key target for RF-EMR induced mitochondrial dysfunction. Furthermore, we document a different profile of ROS production in GC1 cells than in GC2 when treated with this combination of antimycin A and RF-EMR, which, again, is likely to do with their differences in mitochondrial architecture as these cells mature from spermatogonia to spermatocytes (Meinhardt *et al*., 2000). While Complex I is responsible for a majority of the overt ROS leakage involved during normal mammalian cellular respiration, perturbation of Complex III fails to induce global oxidative stress in spermatozoa (Koppers *et al*., 2008; Murphy, 2009). Furthermore, complete inhibition of Complex III alone does not induce downstream peroxidative damage to the sperm lipid membranes but does encourage a positive MSR response (Koppers *et al*., 2008). This aligns with the data of the present study and may be accounted for by the leakage of electrons via this complex to the intermembrane space of the mitochondria, where they encounter the defenses of the mitochondrial antioxidant pool (Koppers *et al*., 2008). Therefore, the subtle yet significant increases in ROS levels observed following irradiation, in the presence of antimycin A, uncovers Complex III as a potential target for RF-EMR.

In order to add strength to this observation, we examined the impact of using succinate as an energy substrate for germ cell metabolism (Fig. 2E, F). While both GC1 and GC2 cells displayed no significant increases in mitochondrial ROS generation when exposed to RF-EMR in the presence of succinate, a significant response was observed when such exposures were conducted in the presence of glucose. With succinate as the energy substrate, the majority of ROS generation has previously been attributed to Complex I (~90%), with a modest portion liberated at Complex III (~10%) (Quinlan *et al*., 2013). In the presence of glucose, where NADH is produced as the major electron source, the elevated mitochondrial ROS generation resulting from the exposure to RF-EMR, is therefore likely to have been driven by Complex III, possibly involving the reduction of ubiquinone to ubiquinol (Koppers *et al*., 2008; Quinlan *et al*., 2013). This can be further rationalized by the absence of a mitochondrial ROS production in both GC1 and GC2 cells, following RF-EMR exposure, when succinate is utilized as an energy source. Succinate metabolism drives electrons through Complex II and ROS production via this pathway has been characterized via the flow of electrons to Complex I via a mechanism of reverse electron flow (Lambert and Brand, 2004; Quinlan *et al*., 2013). In light of our observations, Complex I does not appear to be sensitive to RF-EMR exposure and provides further evidence that processes involving Complex III are responsible for the ROS associate with exposure to RF-EMR.

The relationship between RF-EMR and DNA damage

While the RF-EMR levels used in this study were capable of inducing elevated mitochondrial ROS in the vulnerable spermatogonia and spermatocyte germ cell stages, this was not apparent in the mature spermatozoa (Fig. 5A) and did not, in any cell type, translate to downstream effects on lipid peroxidation (Figs. 3B-C; 6B-D). This lack of oxidative damage in isolated germ cells is consistent with Complex III being the source of electron leakage following the exposure of male germ cells to RF-EMR (Koppers et al., 2008). Furthermore, we did not detect an increase in the presence of 8-OH-dG in either germ cell line exposed to RF-EMR (Fig. 4C), which may suggest these cells were capable of attenuating ROS propagation

with their antioxidant defenses. While no comet sensitive DNA insults were detected for GC2 spermatocytes in our study (Fig. 4B), Liu *et al*. (2013a, b) have previously implicated RF-EMR in the formation of DNA fragmentation and the formation of oxidative DNA lesions in equivalent GC2 cell lines. Although our exposure conditions encompassed the same radiation intensity as in the Liu study, our exposure duration was four-fold shorter (6 vs 24 h). Such timing may account for differences between our observations, but also demonstrates the importance for precise experimental design in this field. In marked contrast, the response profile elicited within mature spermatozoa bears clear hallmarks of oxidative stress, with enhanced detection of the oxidative DNA lesion 8OHdG (Fig. 7C). This damage was accompanied by significant losses to sperm motility (Fig. 8A-E) and increased DNA fragmentation (Fig. 7A). Due to the sequence of damage onset in the oxidative stress pathway and the sensitivity of the ELISA utilized, the sperm motility and DNA fragmentation fold changes were not as obvious as the highly significant change of the oxidative damage readout. Meanwhile, previous studies have concluded that RF-EMR induced oxidative stress is also a driver of DNA damage in spermatozoa (Agarwal *et al*., 2009; De Iuliis *et al*., 2009a). While our MitoSOX red assay was not capable of differentiating the potential subtle increases in sperm ROS generation (Fig. 5A), previous studies by De Iuliis *et al*. (2009a) and Zalata *et al.* (2015) support our findings, identifying that human spermatozoa exposed to RF-EMR suffer DNA fragmentation and oxidation. In the former study, however, DNA damage occurred in association with increased mitochondrial ROS production, supporting oxidative stress as a causal factor in this setting (De Iuliis *et al*., 2009a).

RF-EMR compromises sperm function

Within human spermatozoa, inhibition of Complex I in the electron transport chain results in a pronounced elevation of ROS and concomitant reduction to sperm motility, effects that are not readily apparent upon comparable inhibition of Complex III (Koppers *et al.,* 2008). Indeed, numerous studies have reinforced a causal link between oxidative stress and motility loss (Agarwal *et* al., 2009; De Iuliis *et al*., 2009a; Yan *et al*., 2007; Zalata *et al*., 2015). As we state

above, while we did not observe elevated ROS production or lipid peroxidation in spermatozoa exposed to RF-EMR, we did detect increased 8OHdG, suggesting that oxidative stress may be involved in the loss of sperm motility. There are two main mechanisms implicated in the regulation of sperm motility, where motility is elevated during capacitation with the onset of tyrosine phosphorylation signaling (Mitchell *et al*., 2008), or inhibited during membrane peroxidation in the event of oxidative stress. Meanwhile, impeding tyrosine phosphorylation events in spermatozoa also has a negative impact on sperm motility (Mitchell *et al*., 2008). We demonstrated that exposure to RF-EMR did not impact spontaneous protein tyrosine phosphorylation levels in exposed spermatozoa (Fig. 8E-F), further implicating oxidative stress as a contributing factor in motility loss.

With regard to the vulnerability of mouse spermatozoa to RF-EMR, direct comparison to published literature is challenging as former studies have largely focused on either rat or human models (Agarwal *et al*., 2009; De Iuliis *et al*., 2009a; Kesari *et al*., 2011; Zalata *et al., 2015*). In a study utilizing *in vivo* exposed Swiss mice, RF-EMR did not influence sperm motility or vitality, but these cells did present with extensive DNA degradation within the mitochondrial genome (Aitken *et al*., 2005). Meanwhile, rat and human spermatozoa appear to exhibit a greater vulnerability to RF-EMR; which diminishes sperm motility, viability and exacerbates ROS production in these cells (Agarwal *et al*., 2009; Bin-Meferij and El-kott, 2015; De Iuliis *et al.,* 2009; Ghanbari *et al*., 2013; Yan *et al*., 2007). Here, we add to the small pool of data reporting the effects of RF-EMR on mouse spermatozoa. Our data proposes that Complex III of the ETC is a potential biological target of RF-EMR and provides impetus for the continuation of studies to further contribute toward our understanding of this mechanism. The fact that our study again revealed RF-EMR is capable of inducing genotoxicity complements the body of evidence detailing the range of impacts elicited by this insult. Importantly, such responses can occur at a range of intensities, thus encouraging further exploration of the impact of this form of radiation on biological systems.

CONFLICT OF INTEREST

The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

FUNDING

This work was supported by the Australian Research Council Discovery Project scheme (grant number DP110103951) to R.J.A. and B.K. B.H. is the recipient of an Australian Postgraduate Award PhD scholarship.

ACKNOWLEDGEMENTS

We would like to thank Aimee Katen for technical assistance with the comet assay.

REFERENCES

Adams JA, Galloway TS, Mondal D, Esteves SC & Mathews F. (2014) Effect of mobile telephones on sperm quality: a systematic review and meta-analysis. *Environ Int* 70, 106-112.

Agarwal A, Desai NR, Makker K, Varghese A, Mouradi R, Sabanegh E & Sharma R. (2009) Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an in vitro pilot study. *Fertil Steril* 92, 1318-1325.

Aitken RJ, Bennetts LE, Sawyer D, Wiklendt AM & King BV. (2005) Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline. *Int J Androl* 28, 171-179.

Aitken RJ, Clarkson JS & Fishel S. (1989) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 41, 183-197.

Aitken RJ, De Iuliis GN, Gibb Z & Baker MA. (2012b) The Simmet lecture: new horizons on an old landscape--oxidative stress, DNA damage and apoptosis in the male germ line. *Reprod Domest Anim* 47 Suppl 4, 7-14.

Aitken RJ, Smith TB, Jobling MS, Baker MA & De Iuliis GN. (2014) Oxidative stress and male reproductive health. *Asian J Androl* 16, 31-38.

Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA & Baker MA. (2012a) Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J Biol Chem* 287, 33048-33060.

Al-Damegh MA. (2012) Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E. *Clinics (Sao Paulo)* 67, 785-792.

Baleato RM, Aitken RJ & Roman SD. (2005) Vitamin A regulation of BMP4 expression in the male germ line. *Dev Biol* 286, 78-90.

Balode Z. (1996) Assessment of radio-frequency electromagnetic radiation by the micronucleus test in bovine peripheral erythrocytes. *Sci Total Environ* 180, 81-85.

Biggers, J. D., Whitten, W. K., and Whittingham, D. G. (1971) The culture of mouse embryos in vitro. In 'Methods in Mammalian Embryology'. (Eds J. C. Jnr Daniel.) 86–116. (Freeman: San Francisco.)

Bin-Meferij MM & El-Kott AF. (2015) The radioprotective effects of Moringa oleifera against mobile phone electromagnetic radiation-induced infertility in rats. *Int J Clin Exp Med* 8, 12487-12497.

Consales C, Merla C, Marino C & Benassi B. (2012) Electromagnetic fields, oxidative stress, and neurodegeneration. *Int J Cell Biol* 2012, 683897.

d'Ambrosio G, Massa R, Scarfi MR & Zeni O. (2002) Cytogenetic damage in human lymphocytes following GMSK phase modulated microwave exposure. *Bioelectromagnetics* 23, 7-13.

De Iuliis GN, Newey RJ, King BV & Aitken RJ. (2009a) Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. *PLoS One* 4, e6446.

De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B & Aitken RJ. (2009b) DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* 81, 517-524.

Duan W, Liu C, Zhang L, He M, Xu S, Chen C, Pi H, Gao P, Zhang Y, Zhong M, Yu Z & Zhou Z. (2015) Comparison of the genotoxic effects induced by 50 Hz extremely low-frequency electromagnetic fields and 1800 MHz radiofrequency electromagnetic fields in GC-2 cells. *Radiat Res* 183, 305-314.

Erogul O, Oztas E, Yildirim I, Kir T, Aydur E, Komesli G, Irkilata HC, Irmak MK & Peker AF. (2006) Effects of electromagnetic radiation from a cellular phone on human sperm motility: an in vitro study. *Arch Med Res* 37, 840-843.

Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R & Alvarez JG. (2003) The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J Androl* 24, 59-66.

French PW, Penny R, Laurence JA & McKenzie DR. (2001) Mobile phones, heat shock proteins and cancer. *Differentiation* 67, 93-97.

Ghanbari M, Mortazavi SB, Khavanin A & Khazaei M. (2013) The Effects of Cell Phone Waves (900 MHz-GSM Band) on Sperm Parameters and Total Antioxidant Capacity in Rats. *Int J Fertil Steril* 7, 21-28.

Gharagozloo P & Aitken RJ. (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* 26, 1628-1640.

Gorpinchenko I, Nikitin O, Banyra O & Shulyak A. (2014) The influence of direct mobile phone radiation on sperm quality. *Cent European J Urol* 67, 65-71.

Hou Q, Wang M, Wu S, Ma X, An G, Liu H & Xie F. (2015) Oxidative changes and apoptosis induced by 1800-MHz electromagnetic radiation in NIH/3T3 cells. *Electromagn Biol Med* 34, 85-92.

Houston B, Curry B & Aitken RJ. (2015) Human spermatozoa possess an IL4I1 l-amino acid oxidase with a potential role in sperm function. *Reproduction* 149, 587-596.

Houston BJ, Nixon B, King BV, De Iuliis GN & Aitken RJ. (2016) The effects of radiofrequency electromagnetic radiation on sperm function. *Reproduction* 152, R263-R276.

Jones R, Mann T & Sherins R. (1979) Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* 31, 531-537.

Kahya MC, Naziroglu M & Cig B. (2014) Selenium reduces mobile phone (900 MHz)-induced oxidative stress, mitochondrial function, and apoptosis in breast cancer cells. *Biol Trace Elem Res* 160, 285-293.

Katen AL, Chambers CG, Nixon B & Roman SD. (2016a) Chronic Acrylamide Exposure in Male Mice Results in Elevated DNA Damage in the Germline and Heritable Induction of CYP2E1 in the Testes. *Biol Reprod*.

Katen AL, Stanger SJ, Anderson AL, Nixon B & Roman SD. (2016b) Chronic acrylamide exposure in male mice induces DNA damage to spermatozoa; Potential for amelioration by resveratrol. *Reprod Toxicol* 63, 1-12.

Kaur P, Kaur G & Bansal MP. (2006) Tertiary-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice: role of transcription factor NF-kappaB and testicular antioxidant enzymes. *Reprod Toxicol* 22, 479-484.

Kesari KK, Kumar S & Behari J. (2011) Effects of radiofrequency electromagnetic wave exposure from cellular phones on the reproductive pattern in male Wistar rats. *Appl Biochem Biotechnol* 164, 546-559.

Kim J, Kwon WS, Rahman MS, Lee JS, Yoon SJ, Park YJ, You YA & Pang MG. (2015) Effect of sodium fluoride on male mouse fertility. *Andrology* 3, 544-551.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA & Aitken RJ. (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 93, 3199-3207.

Lagorio S & Roosli M. (2014) Mobile phone use and risk of intracranial tumors: a consistency analysis. *Bioelectromagnetics* 35, 79-90.

Lambert AJ & Brand MD. (2004) Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem J* 382, 511-517.

Lerchl A. (2013) Electromagnetic pollution: another risk factor for infertility, or a red herring? *Asian J Androl* 15, 201-203.

Lerchl A, Klose M, Grote K, Wilhelm AF, Spathmann O, Fiedler T, Streckert J, Hansen V & Clemens M. (2015) Tumor promotion by exposure to radiofrequency electromagnetic fields below exposure limits for humans. *Biochem Biophys Res Commun* 459, 585-590.

Liu C, Duan W, Xu S, Chen C, He M, Zhang L, Yu Z & Zhou Z. (2013b) Exposure to 1800 MHz radiofrequency electromagnetic radiation induces oxidative DNA base damage in a mouse spermatocyte-derived cell line. *Toxicol Lett* 218, 2-9.

Liu C, Gao P, Xu SC, Wang Y, Chen CH, He MD, Yu ZP, Zhang L & Zhou Z. (2013a) Mobile phone radiation induces mode-dependent DNA damage in a mouse spermatocyte-derived cell line: a protective role of melatonin. *Int J Radiat Biol* 89, 993-1001.

Liu Q, Si T, Xu X, Liang F, Wang L & Pan S. (2015) Electromagnetic radiation at 900 MHz induces sperm apoptosis through bcl-2, bax and caspase-3 signaling pathways in rats. *Reprod Health* 12, 65.

Meinhardt A, McFarlane JR, Seitz J & de Kretser DM. (2000) Activin maintains the condensed type of mitochondria in germ cells. *Mol Cell Endocrinol* 168, 111-117.

Meral I, Mert H, Mert N, Deger Y, Yoruk I, Yetkin A & Keskin S. (2007) Effects of 900-MHz electromagnetic field emitted from cellular phone on brain oxidative stress and some vitamin levels of guinea pigs. *Brain Res* 1169, 120-124.

Merhi ZO. (2012) Challenging cell phone impact on reproduction: a review. *J Assist Reprod Genet* 29, 293-297.

Mitchell LA, Nixon B, Baker MA & Aitken RJ. (2008) Investigation of the role of SRC in capacitation-associated tyrosine phosphorylation of human spermatozoa. *Mol Hum Reprod* 14, 235-243.

Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P & Aitken RJ. (2015) Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol Hum Reprod* 21, 502-515.

Murphy MP. (2009) How mitochondria produce reactive oxygen species. *Biochem J* 417, 1-13.

Narayanan SN, Kumar RS, Karun KM, Nayak SB & Bhat PG. (2015) Possible cause for altered spatial cognition of prepubescent rats exposed to chronic radiofrequency electromagnetic radiation. *Metab Brain Dis*.

Ozguner F, Altinbas A, Ozaydin M, Dogan A, Vural H, Kisioglu AN, Cesur G & Yildirim NG. (2005) Mobile phone-induced myocardial oxidative stress: protection by a novel antioxidant agent caffeic acid phenethyl ester. *Toxicol Ind Health* 21, 223-230.

Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Orr AL & Brand MD. (2013) Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox Biol* 1, 304-312.

Salford LG, Brun A, Sturesson K, Eberhardt JL & Persson BR. (1994) Permeability of the blood-brain barrier induced by 915 MHz electromagnetic radiation, continuous wave and modulated at 8, 16, 50, and 200 Hz. *Microsc Res Tech* 27, 535-542.

Smith TB, Baker MA, Connaughton HS, Habenicht U & Aitken RJ. (2013) Functional deletion of Txndc2 and Txndc3 increases the susceptibility of spermatozoa to age-related oxidative stress. *Free Radic Biol Med* 65, 872-881.

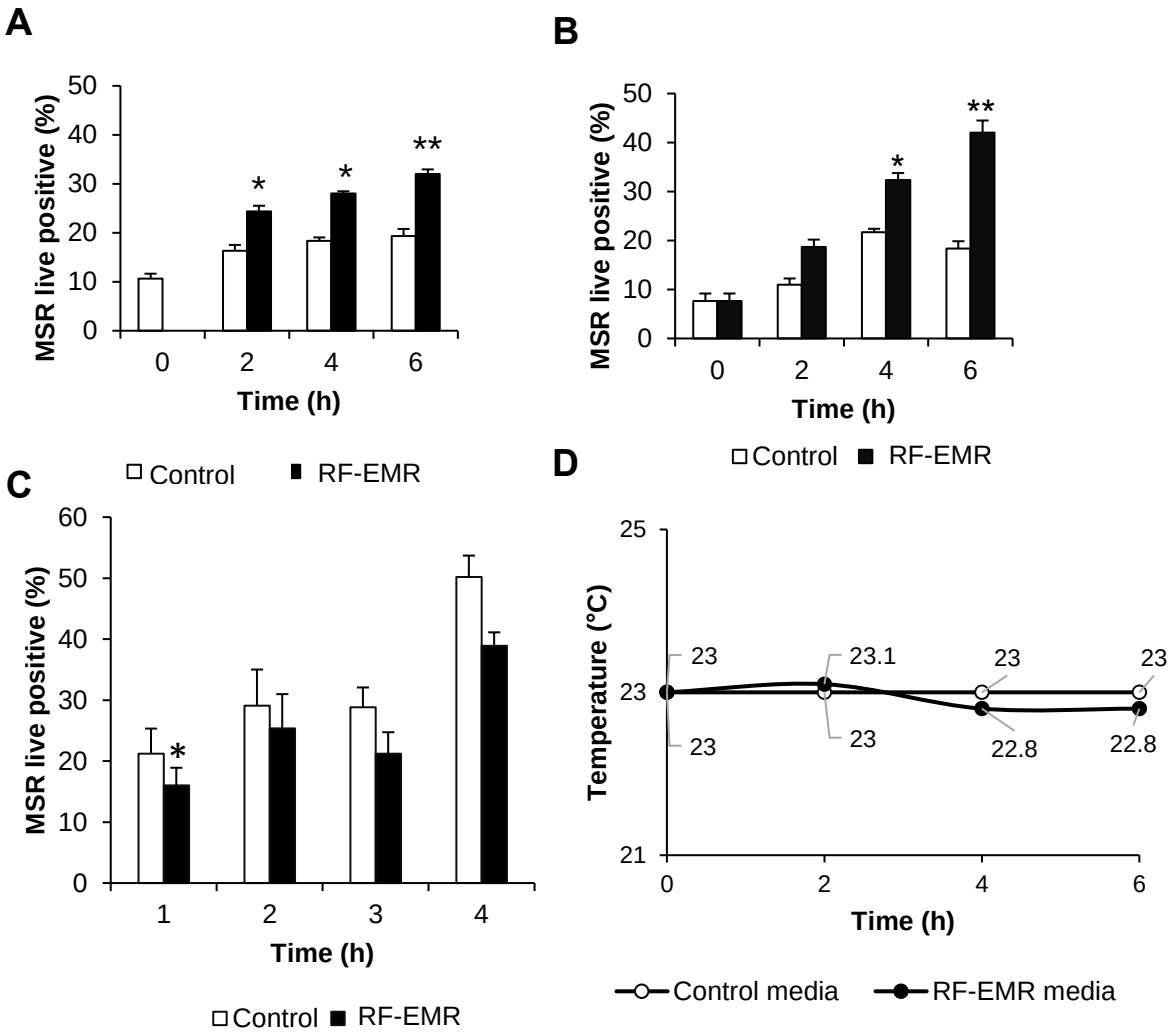
Storey BT. (2008) Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int J Dev Biol* 52, 427-437.

Tremellen K. (2008) Oxidative stress and male infertility--a clinical perspective. *Hum Reprod Update* 14, 243-258.

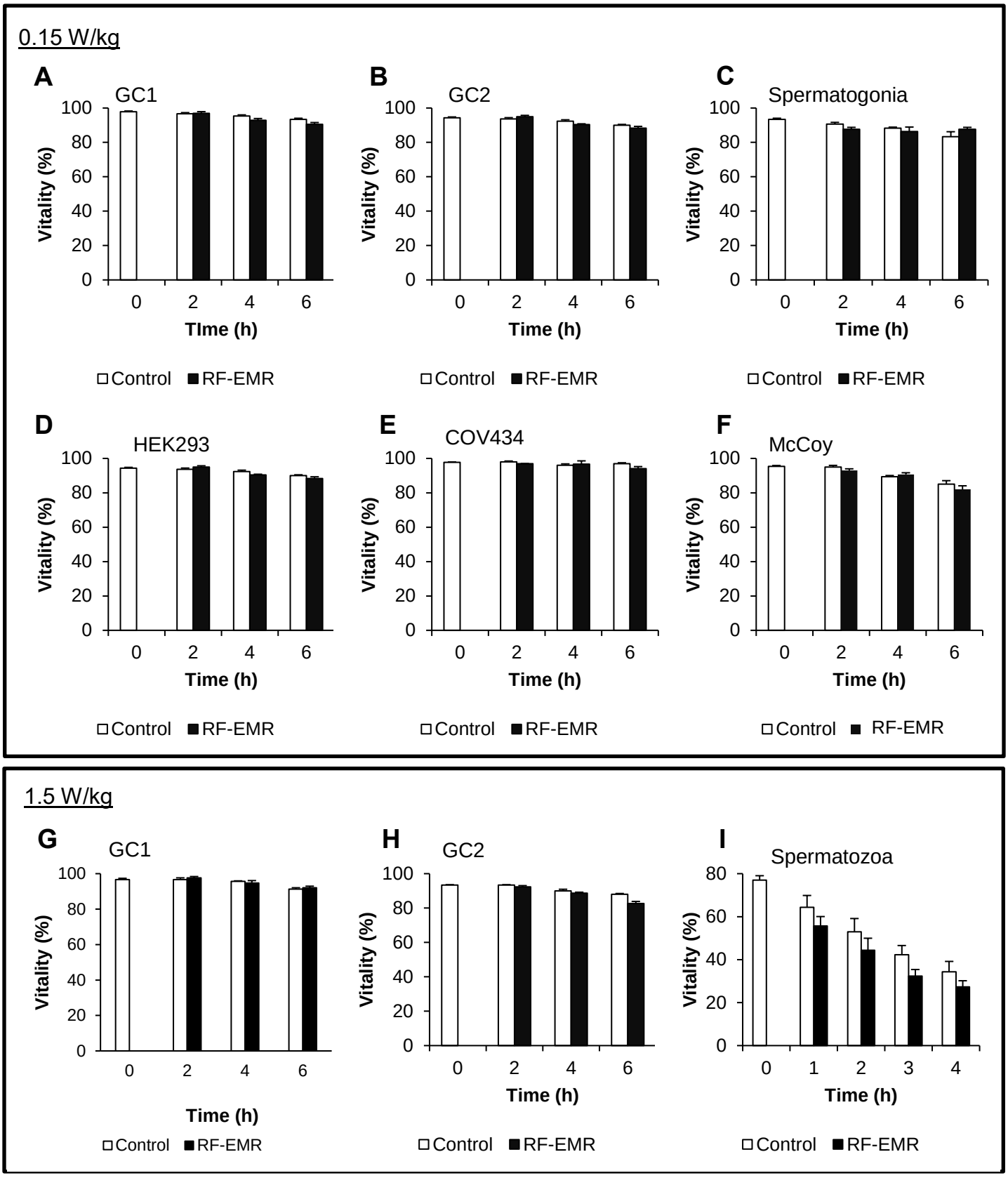
Yan JG, Agresti M, Bruce T, Yan YH, Granlund A & Matloub HS. (2007) Effects of cellular phone emissions on sperm motility in rats. *Fertil Steril* 88, 957-964.

Yao K, Wu W, Wang K, Ni S, Ye P, Yu Y, Ye J & Sun L. (2008) Electromagnetic noise inhibits radiofrequency radiation-induced DNA damage and reactive oxygen species increase in human lens epithelial cells. *Mol Vis* 14, 964-969.

Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y & Mostafa T. (2015) In vitro effect of cell phone radiation on motility, DNA fragmentation and clusterin gene expression in human sperm. *Int J Fertil Steril* 9, 129-136.



Supplementary Figure 1. Examination of the effect of exposing male germ cells and spermatozoa to an elevated dose of RF-EMR. The studies reported in Figure 1 of this manuscript were replicated on (A) GC1 (B) GC2 cell lines and (C) spermatozoa exposed to an elevated intensity of RF-EMR (1.5 W/kg). Mitochondrial ROS generation in both germ cell populations and spermatozoa was subsequently assessed with the MSR probe. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to unexposed controls. (D) Media temperature for the RF-EMR exposed treatments in the waveguide, and control counterparts over the exposure time course, n=3.



Supplementary Figure 2. Cell viability under RF-EMR exposure. Associated viability counts were performed for all cell types exposed to RF-EMR treatment. (A) GC1, (B) GC2, (C) spermatogonia, (D) HEK293, (E) COV434 and (F) McCoy cells exposed to 0.15 W/kg RF-EMR (top box). (G) GC1, (H) GC2 and (I) spermatozoa exposed to 1.5 W/kg RF-EMR (bottom box), n=3.

CHAPTER 3

Whole body exposure to radiofrequency-electromagnetic radiation induces DNA fragmentation in mouse spermatozoa

Authors: Brendan J Houston[1], Brett Nixon[1], Jacinta H Martin[1], Kristen E McEwan[1], Bruce V King[2], R John Aitken[1] and Geoffry N De Iullis[1]

[1] Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

[2] School of Mathematical and Physical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

Chapter 3: Overview

In this chapter, we extend on our previous study investigating the effects of radiofrequency-electromagnetic radiation (RF-EMR) on the male reproductive system, with an *in vivo* study. Here, we irradiated C57BL/6 male mice with RF-EMR to further characterise the effects on sperm development and quality.

The data generated in this chapter demonstrates that RF-EMR exposure is capable of inducing a state of oxidative stress in spermatozoa in the absence of overt disruption to spermatogenesis in the testis. Treatment with RF-EMR was documented to elevate the generation of mitochondrial ROS, that led to reductions in sperm motility and significant induction to multiple forms of DNA damage. While these cells were harbouring such DNA damage, they were not impaired in their ability to recognise and bind the oocyte. Furthermore, the integral role of the oocyte in detecting and repairing DNA damage was supported in this study; embryos sired by RF-EMR exposed spermatozoa were capable of progressing in development to the blastocyst stage with similar success rates to unexposed spermatozoa.

In characterising this response profile to RF-EMR, we add weight to the literature documenting oxidative stress a major pathway activated by this factor. We again demonstrated a mitochondrial origin of ROS generated by RF-EMR, which supports our hypothesis detailed Chapter 1, as well as our findings in Chapter 2 and previous research from our group. While this subtle insult was insufficient at completely impeding sperm function, it is important to remember that we are constantly exposed to RF-EMR, and this exposure is only increasing with time. This, alongside the data provided in this thesis, warrants further research around this topic, to further understand potential genetic changes in embryos generated via RF-EMR treated spermatozoa.

TITLE: Whole body exposure to radiofrequency-electromagnetic radiation induces DNA fragmentation in mouse spermatozoa.

AUTHORS: Brendan Houston[1*], Brett Nixon[1], Kristen McEwan[1], Jacinta Martin[1], Bruce King[2], Geoffry De Iuliis[1] and R. John Aitken[1]

AUTHOR AFFILIATIONS

[1] Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, Discipline of Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

[2] School of Mathematical and Physical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

CORRESPONDENCE: Brendan HOUSTON, School of Environmental and Life Sciences, Discipline of Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

Tel: +61 24921 2043

Fax: +61 24921 6308

Email: brendan.houston@uon.edu.au

KEY WORDS:

RF-EMR, spermatozoa, germ cells, DNA damage, mobile phone radiation

RUNNING TITLE:

In vivo RF-EMR exposure induces DNA damage in spermatozoa

ABSTRACT

We live in a world where we are now constantly exposed to the radiofrequency-electromagnetic radiation (RF-EMR) emitted by mobile phone and other communication devices. While it is imperative to establish public safety guidelines around the use of these devices, it is difficult to meet these demands due to a current lack of understanding concerning how this form of energy interacts with biology. Several studies have identified that RF-EMR is capable of eliciting cellular stress but the mechanistic basis of stress induction remains largely unresolved. In this study, we extend on the small collection of studies detailing the effects of RF-EMR on the male reproductive system. We exposed C57BL/6 mice to RF-EMR generated via a dedicated waveguide (905 MHz, 2.2 W/kg) for 12 h per day, for 1, 3 and 5 weeks. The testes collected from RF-EMR treated mice exhibited no signs of histological change or hallmarks of elevated stress (γH2A.x; 4-hydroxynonenal). However, the spermatozoa collected from the cauda epididymis experienced significant declines in sperm vitality ($p < 0.05$), as well as several objective parameters of sperm motility (including overall, progressive and rapid motility; $p < 0.05$), following a 5 week exposure regime to RF-EMR. These modifications were associated with increased mitochondrial generation of reactive oxygen species ($p < 0.05$) after 1 and 3 weeks of exposure. Furthermore, this exposure regimen elicited sperm DNA fragmentation ($p < 0.01$) and oxidation ($p < 0.05$) at all exposure periods. Notwithstanding these damaging effects, RF-EMR exposure did not impair the ability of spermatozoa to engage in zona pellucida binding or to support fertilization and embryonic development through to the blastocyst stage. This study further supports oxidative stress as a key mediator in RF-EMR associated biological stress.

INTRODUCTION

With rapid advances in technology and increasing demands for electronic communication, mobile phone usage has become essential in the developed world. Nearly every person in

these countries owns one or more mobile phone units (Houston *et al*, 2016), and more importantly, the use of these devices by adolescents and even children is steadily rising in popularity (Sadetzki *et al*., 2014; Terras and Ramsay, 2016). Mobile phone devices receive and emit radiofrequency-electromagnetic radiation (RF-EMR) to transfer information, and accordingly, our exposure to this form of energy is now unprecedented. While to date, no overwhelming clinical effects have been associated with exposure to RF-EMR (Marchionni *et al*., 2006, Masuda *et al*., 2006, Dasdag *et al.,* 2009, Demirel *et al*., 2012; Khalil *et al*., 2014), multiple emerging studies suggest that there are subtler detrimental effects of this radiation on biological systems (De Iuliis *et al*., 2009; Narayanan *et al*., 2015; Ozguner *et al*., 2005; Salford, 1994). The International Agency for Research on Cancer have not yet confidently dismissed the risks of RF-EMR, classifying this form of radiation as a potential carcinogen, which only strengthens the need for a greater understanding in this field. While, the effects of RF-EMR on biology is a topic of active debate, a substantial body of evidence now proposes oxidative stress as a prominent outcome of RF-EMR exposure (Hou *et al*., 2015; Ozguner *et al*., 2005; De Iuliis *et al*., 2009; Yao *et al.,* 2008). It is therefore our interest to further explore the effects of this form of radiation on an *in vivo* model, in order to strengthen a potential mechanism by which RF-EMR may be affecting biological systems. One leading hypothesis is that RF-EMR targets the mitochondria, which in turn leads to perturbation of the electron transport chain and a subsequent generation of reactive oxygen species (Houston *et al*., 2016).

Due to storage of mobile phone devices in the pant pocket, the male reproductive system of many individuals receives low-intensity, but sustained RF-EMR exposure. This exposure is amplified in the case of handsfree phone calls and during use in rural areas, where people are generally situated further from receiving towers, and extra power is required to reach these structures (Kelsh, 2011). Bringing the relevance of the male reproductive system into focus is the notion that sperm cells in the male reproductive tract are susceptible to RF-EMR and oxidative stress (De Iuliis *et al*., 2009). Indeed, it has been shown that spermatozoa provide a

sensitive model to study the specific physical and chemical responses to EMR (Agarwal *et al*., 2009). The unique architecture and metabolism of spermatozoa renders these cells sensitive to damage by free radicals, their motility provides a readily assessable means of monitoring adverse biological effects, and they are clinically important, since DNA damage in spermatozoa has the potential to influence the health and wellbeing of the offspring (Aitken, 2013; Aitken *et al*., 2014).

While a handful of studies have been undertaken to assess the effects of RF-EMR on the male germ line, these often have included isolated spermatozoa or male germ cells (Agarwal *et al*., 2009; Erogul *et al*., 2006; Liu *et al*., 2013a; Zalata *et al*., 2015). While this approach is conducive to intricate examination of the biochemistry and cell responses following exposures, the utilization of alternate *in vivo* rodent models is likely to present a closer clinical representation of exposure. Such models afford the added advantage that they enable observation of the effects of RF-EMR on all stages of male germ cell development (Ghanbari *et al*., 2013), encompassing the differentiation of germ into sperm cells and their subsequent functional maturation as they transit the epididymis. With a sustained interest in developing a biophysical mechanism of action for RF-EMR on biology, we have utilized a mouse model with optimised exposure conditions, designed to further probe RF-EMR associated reproductive stress. To achieve our findings, we constructed a waveguide machine; similar to that developed by Puranen (*et al*., 2009). Mice were exposed at 905 MHz at 2.2 W/kg (SAR) for 12 h per day, over a period of 1 – 5 weeks (7, 21 and 35 days, respectively; Figure 1). Sham exposed mice were placed inside the exposure unit under identical conditions and treatment lengths, but did not receive radiation exposure. Subsequently, the testes and epididymides were collected to investigate the effects of RF-EMR on spermatogenesis and sperm function.

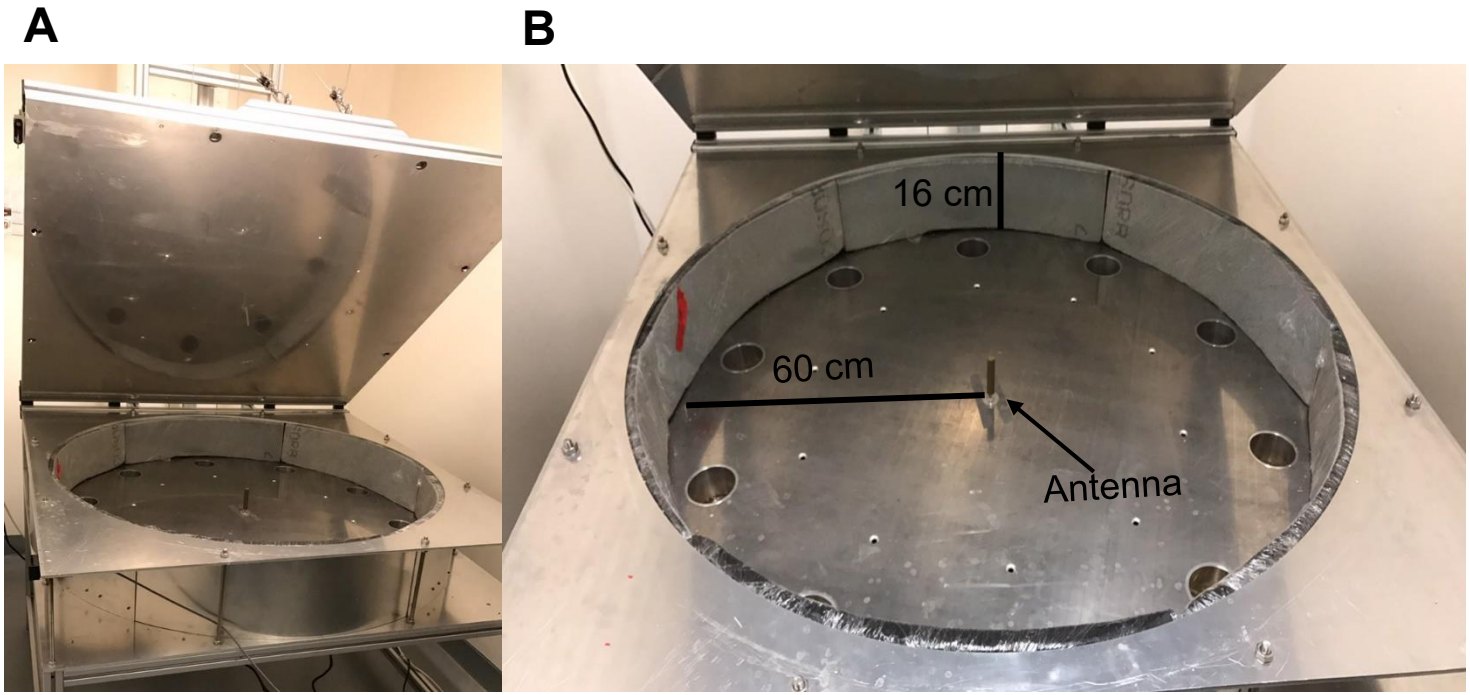


Figure 1. Waveguide photographs and setup while the lid is open. (A) Whole waveguide structure and (B) Chamber close-up view.

MATERIALS AND METHODS

Chemical reagents

The chemicals and reagents used in this study were purchased from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA) unless stated otherwise, and were of research grade. The fluorescent probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA), unless otherwise stated. All fluorescent imaging was performed using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Waveguide exposure

All experimental protocols were approved by the University of Newcastle Animal Care and Ethics Committee (Ethics Number 2014-447). Mature adult male C57BL/6 mice were irradiated with 2.2 W/kg and 905 MHz RF-EMR in a waveguide (Fig. 1) for 12 h daily, during a night (7pm – 7am) cycle, while the waveguide lid was shut. This waveguide was constructed by the Physics Department at the University of Newcastle, NSW, Australia, and comprises a large cylindrical metal chamber, with a radius of 60 cm and depth of 16 cm, containing a mechanically operated lid. The sides of this chamber were insulated with carbon impregnated foam (RFI Industries, Bayswater, VIC, Australia) to prevent RF-EMR reflection. Small fans were implemented to allow external air circulation into the chamber through the base, during lid closure. RF-EMR was generated by a Rohde and Schwarz SMC100A signal generator (Rohde and Schwarz, Macquarie Park, NSW, Australia), which was connected to a signal amplifier. Chamber lid operation was controlled by a motor connected to a timer in order to raise or lower the lid every 12 h. The lid also contained an ‘enable switch’ to shut off the power to the signal amplifier while the lid was open. Mice were housed in plastic cages with Perspex lids and plastic water bottles to ensure there was no metal, which interferes with the distribution of the RF-EMR. The cages were arranged radially around the central antenna, which emitted the RF-EMR. The end of the cage containing the water bottle was oriented such that it was placed furthest from the radiation source to minimise the liquid interference, and

when mice were removed they were replaced with saline 'phantoms'. These phantoms were composed of a 50 ml Falcon tube filled with saline (142 mM NaCl in deionised water) to mimic human blood. Sham exposed males were placed in the waveguide under identical conditions, with the exception that the signal generator was turned off, thus receiving no exposure to RF-EMR. All treatment groups were sacrificed at three time points; 1, 3 and 5 weeks of exposure and compared to a control population of mice that were not placed inside the chamber.

The SAR delivered to the mice was calibrated using a NARDA NBM 520 electric field meter with an EF1891 probe to measure electric fields in the empty irradiation system. Radial electric field measurements were made as a function of distance from the vertical aerial mounted in the centre of the system after the antenna length was adjusted to maximize the power supplied to the system at a frequency of 905 MHz. For 1 W input to the aerial a maximum electric field of 94 V/m was measured 16 cm from the centre, whereas in his slightly larger setup, Puranen *et al*. (2009) measured a maximum electric field of 80V/m at 15cm from the centre. The variation of E field with radial distance and the maximum electric fields in the two setups were found to be similar for the same power input.

The SAR (W kg^{-1}) is related to the electric field, E, in a sample of conductivity σ (S m^{-1}), and density ρ (kg m^{-3}) by

$$SAR = \sigma |E| 2 / \rho \quad (Wkg^{-1}) \qquad (1)$$

where E is the root-mean-square local electric field strength in V m^{-1}. Puranen *et al*. (2009) measured a SAR of 0.11 Wm^{-1} for the above 1 W input to the aerial. During our irradiations the input RF power was 20 W, corresponding to an average SAR of 2.2 Wkg^{-1} since the geometry of our irradiation system is very similar to that of Puranen *et al*. (2009).

Isolation of spermatozoa

Epididymides were dissected from adult C57BL/6 mice culled via CO_2 asphyxiation and mature spermatozoa were collected from the cauda epididymis by retrograde perfusion via the vas

deferens (Nixon *et al*., 2015). These cells were resuspended in 1 ml of modified Biggers, Whiting, Whittingham media (BWW; Biggers *et al*., 1971). Objective sperm motility was assessed by computer assisted sperm analysis (CASA; IVOS, Hamilton Thorne, Danvers, MA, USA). For this purpose, a minimum of 100 spermatozoa in five fields were assessed using 2X-CEL slides (Hamilton Thorne) suspended on a pre-warmed stage (37ºC) (Smith *et al*., 2013a). The following settings were utilised: negative phase-contrast optics, 60 frames/sec recording rate, minimum cell size of 9 pixels, minimum contrast of 80, low size gate of 0.3, high size gate of 1.95, low intensity gate of 0.5, high intensity gate of 1.3, nonmotile head size of 45 pixels, nonmotile head intensity of 75, progressive average path velocity (VAP) threshold of 10 µm/sec, slow (static) cells VAP threshold of 5 µm/sec, slow (static) cells straight-line velocity (VSL) threshold of 0 µm/sec, and threshold straightness (STR) of 75%. Cells exhibiting a VAP of 10 µm/sec and a STR >0 were considered progressive. Cells with a VAP greater than that of the mean VAP of progressive cells were considered rapid. Sperm vitality was assessed via the eosin exclusion method (World Health Organization, 2010).

Fixing and sectioning of testes

Upon dissection, testes were immediately placed in Bouin's fixative (9% formaldehyde, 5% acetic acid, 0.9% picric acid) and fixed for 6 h at 4ºC in a rotator. These organs were then placed in 70% ethanol overnight at 4ºC in a rotator. Finally, residual Bouin's fixative was removed by transferal to 70% ethanol and the testes were stored at 4ºC prior to sectioning by the University of Melbourne Biomedical Sciences Histology Facility (Parkville, VIC, Australia). One section from each testis was stained with hematoxylin and eosin to investigate testis morphology.

Immunohistochemistry

Slides were first dewaxed in 3 xylene baths for 5 min each and then rehydrated in ethanol baths, decreasing in concentration; 100% twice for 5 min each, followed by one suspension in 90%, 70% and 50% for 1 minute each. Antigen retrieval was then performed by microwaving

slides in a solution of 50 mM Tris (pH 10.5) for 9 min. After allowing the solution to cool, individual tissues sections were divided with a pap-pen. Tissue sections were then treated with blocking solution (3% bovine serum albumin (BSA)-PBST, 10% goat serum) for 1 h at room temperature and washed in PBS for 5 min. Following this, primary antibody incubation was performed for phospho-γH2A.x (2 µg/ml), 4-hydroxynonenal (1/300) in 1% BSA-PBST overnight at 4ºC. Slides were then washed 3 times in PBS for 5 min. Secondary antibody incubation was again conducted in 1% BSA-PBST using AlexaFluor-594 (Thermo Fisher Scientific) α-rabbit or α-goat dye (10 µg/ml) for 1 h at 37ºC. Slides were washed 3 times in PBS for 5 min and treated with DAPI (0.5 µg/ml) for 5 min at room temperature. Finally, slides were washed twice in PBS for 5 min and mounted in Mowiol 4-88 (Millipore, Darmstadt, Germany) with antifade for viewing under a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging GmbH). Mean pixel intensity analysis was conducted on images of these sections using ImageJ version 1.48 V (NIH, USA). Pixel intensity determination was performed only on the seminiferous tubules, with surrounding tissue isolated from this analysis. For the case of γH2A.x the meiotic germ cells were excluded from the analysis, due to the presence of naturally occurring high levels of double strand breaks in these cell types.

Determination of ROS production in spermatozoa

Flow cytometry

Spermatozoa were assessed for ROS generation using flow cytometry with the mitochondrial superoxide probe MitoSOX red (MSR) or cytosolic superoxide probe dihydroethidium (DHE) in conjunction with Sytox Green (SYG) vitality stain. Cells were resuspended in 2 µM MSR or DHE, and 20 nM SYG in BWW for 15 min in the dark at 37ºC then centrifuged at 450 × g for 5 min and then resuspended in 400 µL BWW. Each sample was transferred to a flow cytometry tube for analysis with a FACS-Canto flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser and 633 nm helium-neon laser. Analysis of these data was undertaken using CellQuest software (BD Biosciences, San Jose, CA, USA).

Sperm chromatin dispersion (Halo) assay

Spermatozoa were collected and snap frozen in liquid nitrogen, then stored at -80ºC prior to analysis via the Halo assay. Briefly, sperm cells were defrosted and mixed with 1% low melting point agarose at 37ºC to achieve a concentration of 0.7% agarose, which was applied to a Superfrost microscope slide (Thermo Fisher Scientific) that was precoated with 0.65% agarose. This was sealed with a coverslip and placed at 4ºC to solidify for 5 min. After carefully removing the coverslips, the slides were treated with 0.08 N HCl for 7 min in foil, followed by halo solution 1 (pH 7.5; 0.4 M tris, 1% SDS, 50 mM EDTA, 0.8 M DTT) for 10 min and halo solution 2 (pH 7.5; 0.4 M tris, 1% SDS, 2 M NaCl) for 5 min at room temperature to lyse the cells, relax and neutralize the DNA. Next, the slides were exposed to tris-boric acid-EDTA buffer (pH 7.5; 0.1 M tris, 0.09 M boric acid, 0.002 M EDTA) for 2 min, then washed in increasing concentrations of ethanol (70%, 90% and 100%) for 2 min each to dehydrate the slides. After allowing the slides to air dry, DAPI (0.5 µg/ml) staining was applied for 10 min at room temperature. Finally, the slides were rinsed in PBS and mounted for microscope analysis.

Alkaline comet assay

The comet assay was performed as described previously (see Katen *et al*., 2016). Spermatozoa, from samples pelleted and stored at -80ºC, were resuspended in PBS to achieve a concentration of 4×10^4 cells/µl. A 10 µl aliquot of this cell suspension was mixed with 70 µl agarose (Trevigen, Gaithersburg, MA, USA) and allowed to set on Dakin G376 slides pre-coated with 1% low melting point agarose (ProSciTech, Kirwan, QLD, Australia) sealed with a coverslip for 5 min at 4ºC. Briefly, after removing the coverslip, slides were treated with lysis solution 1 (0.8 M Tris-HCl, 0.8 M DTT, 1% SDS; pH 7.5) then lysis solution 2 (0.4 M Tris-HCl, 50 mM EDTA, 2 M NaCl, 0.4 M DTT; pH 7.5), while sealed with a coverslip for 30 min each. Coverslips were removed and slides were washed in tris-boric acid buffer (0.445 M Tris-HCl, 10 mM EDTA, 0.445 M boric acid) for 10 min. In preparation for electrophoresis, slides were treated with alkaline solution (0.03 M NaOH, 1 M NaCl) for 15 min

at 4ºC, followed by electrophoresis in alkaline buffer (0.03 M NaOH) for 4 min at 1 V/cm. Slides were then washed in neutralization solution (0.4 M Tris-HCl; pH 7.5) for 5 min. SYBR green nucleic acid stain (Thermo Fisher Scientific) was then applied to the slides immediately before viewing on the microscope, and a coverslip was added. The level of DNA damage was analyzed using Comet Assay IV software (Perceptive Instruments, Suffolk, UK). Hydrogen peroxide treatment (500 µM) was utilized as a positive control, in which spermatozoa were resuspended for 5 min at room temperature, followed by a wash and resuspension both in PBS.

Oxidative DNA damage assay

Spermatozoa were collected and snap frozen in liquid nitrogen, then stored at -80ºC until assayed for oxidative DNA damage. For this purpose, 2 x 10^6 spermatozoa were suspended in Oxidative DNA/RNA damage antibody (Thermo Fisher Scientific) (1/40) in PBST overnight at 4ºC. Cells were then centrifuged for 5 min at 450 × g and washed in 1 × PBS before incubation in AlexaFluor-488 goat α rabbit secondary (Abcam, Massachusetts, US) (1/400) in 1 × PBST for 1 h at 37ºC. Finally, cells were again washed in 1 × PBS and resuspended in PBS for counting and imaging via fluorescence microscopy.

Oocyte binding and fertilization assays

In vitro fertilization

Mature female C57BL/6 mice (3-4 weeks of age) were administered an intraperitoneal injection regime of 7.5 IU equine chorionic gonadotropin and human chorionic gonadotropin to induce superovulation (Intervet, Sydney NSW, Australia). Mice were left for 13-15 h following the final injection and oocytes were retrieved from the ampulla by stereoscope dissection (Lord *et al*., 2015). Oocyte-cumulus complexes were then washed in human tubal fluid (HTF) in three times prior to being transferred into a droplet of HTF supplemented with 1 mM reduced glutathione (GSH) as previously described (Martin *et al*., 2016). Spermatozoa were simultaneously recovered as described above and capacitated by incubation in BWW

medium supplemented with 1 mg/ml polyvinyl alcohol and 1 mg/ml methyl-beta cyclodextrin, for 1 h at 37˚C under an atmosphere of 5% O_2, 6% CO_2 in N_2. Ultimately, oocytes and spermatozoa (2×10^5) were co-incubated for 4 h at 37˚C under the same atmospheric conditions, after which signs of successful fertilization were analyzed (extrusion of the second polar body and/or pronucleus formation). Zygotes were then cultured in unsupplemented HTF media overnight and transferred into G1 PLUS culture medium (Vitrolife, Stockholm, Sweden) on the morning of day 2 followed by an additional media change into G2 PLUS medium (Vitrolife) on Day 4. Embryos were monitored daily and developmental rates were recorded. The percentage of fertilized oocytes and percentage of embryos that had reached the blastocyst stage by the morning of day 5 was calculated.

Zona pellucida binding assay

Following isolation of spermatozoa from the cauda epididymis, these cells were resuspended in capacitation media (BWW supplemented with 1 mM pentoxifylline, 1 mM dibutyryl cyclic-AMP) for 1 h at 37ºC, 5% CO_2. Next, a sample of 2×10^5 spermatozoa was introduced to a collection of 8-10 salt stored oocytes for assessment of zona pellucida binding efficacy. These oocytes had been previously isolated and stored in high salt storage media (1.5 M $MgCl_2$, 0.1% dextran, 0.01 mM HEPES buffer and 0.1% PVA) for at least 2 h at 4ºC. These gametes were incubated for 15 min at 37ºC, then the oocytes were washed in BWW three times, mounted to slides with Vaseline and assessed for levels of sperm binding.

For the purpose of these studies, a ‘non-capacitated’ negative control was used. This involved spermatozoa that were resuspended in BWW, in the absence of the pro-capacitation factors; bicarbonate, pentoxifylline and dibutyryl cyclic-AMP.

Statistical analysis

JMP version 11 (SAS Institute Inc., Cary, NC) was used to analyze the data in each experiment, which were performed with at least 5 independent replicates (unless stated otherwise), i.e. five animals assessed per group. Normality of datasets was assessed with the

Shapiro-Wilks test (α = 0.05). Following this, a one-way ANOVA was used to compare normally distributed treatments, with a post-hoc Tukey's honest significant difference test (α = 0.05). For data not normally distributed, a Wilcoxon test was used (α = 0.05), with post-hoc Dunn's test. Error bars represent standard error values around the mean.

RESULTS

We first investigated the effects of RF-EMR exposure on the testes of irradiated mice (Fig. 2), where we found that the average mouse growth rate (Fig. 2A), and testis weight (Fig. 2B) remained unchanged following both sham and EMR exposure. Testis morphology of sham and EMR exposed mice also remained similar to that of control mice (Fig. 2C), exhibiting healthy tubule growth and extensive germ cell proliferation, comparable at all lengths of exposure.

As multiple studies have revealed that RF-EMR exposure can induce a state of oxidative stress leading to DNA damage, we next explored the levels of DNA fragmentation and lipid peroxidation markers of oxidative stress invoked by EMR exposure within the testis. Testis sections were probed with the yH2A.x antibody, a marker of DNA double strand breaks (Fig. 3). This analysis revealed modest levels of DNA damage, restricted to meiotic germ cells within the seminiferous tubules. Furthermore, this result was consistent across all treatment types, with no overt changes to labelling patterns or pixel intensity in germ cells other than this meiotic population following exposure to sham or EMR treatments (p = 0.07). With regard to lipid peroxidation (Fig. 4), we documented a similar response, with no substantive increases in the lipid peroxidation product, 4-hydroxynonenal (4-HNE) formation within the testis sections across all treatments (p = 0.22).

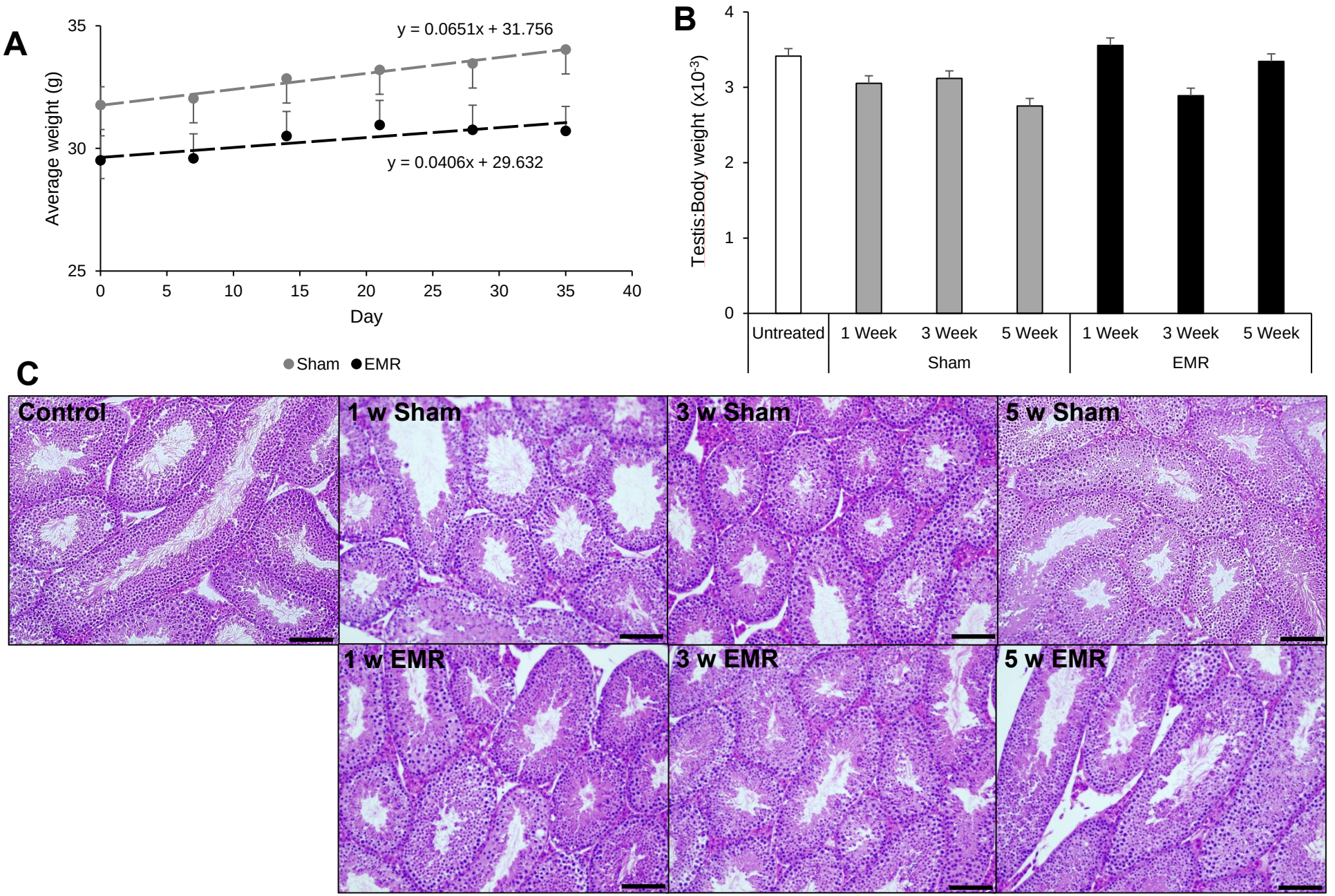


Figure 2. The effect of RF-EMR on the growth, testis weight and morphology of C57BL/6 mice. (A) Mice were weighed weekly to investigate the effects of RF-EMR on body mass against sham exposed males. (B) Testis weight was then recorded with respect to body mass for untreated, sham and RF-EMR exposed populations, across the 1, 3 and 5 week time points. (C) Haematoxylin and eosin staining was then performed on these testes, for comparison of general cell morphology. Scale bar represents 200 µM. Error bars represent standard error values around the mean, n = 5 for panels A-B, and 3 for panel C.

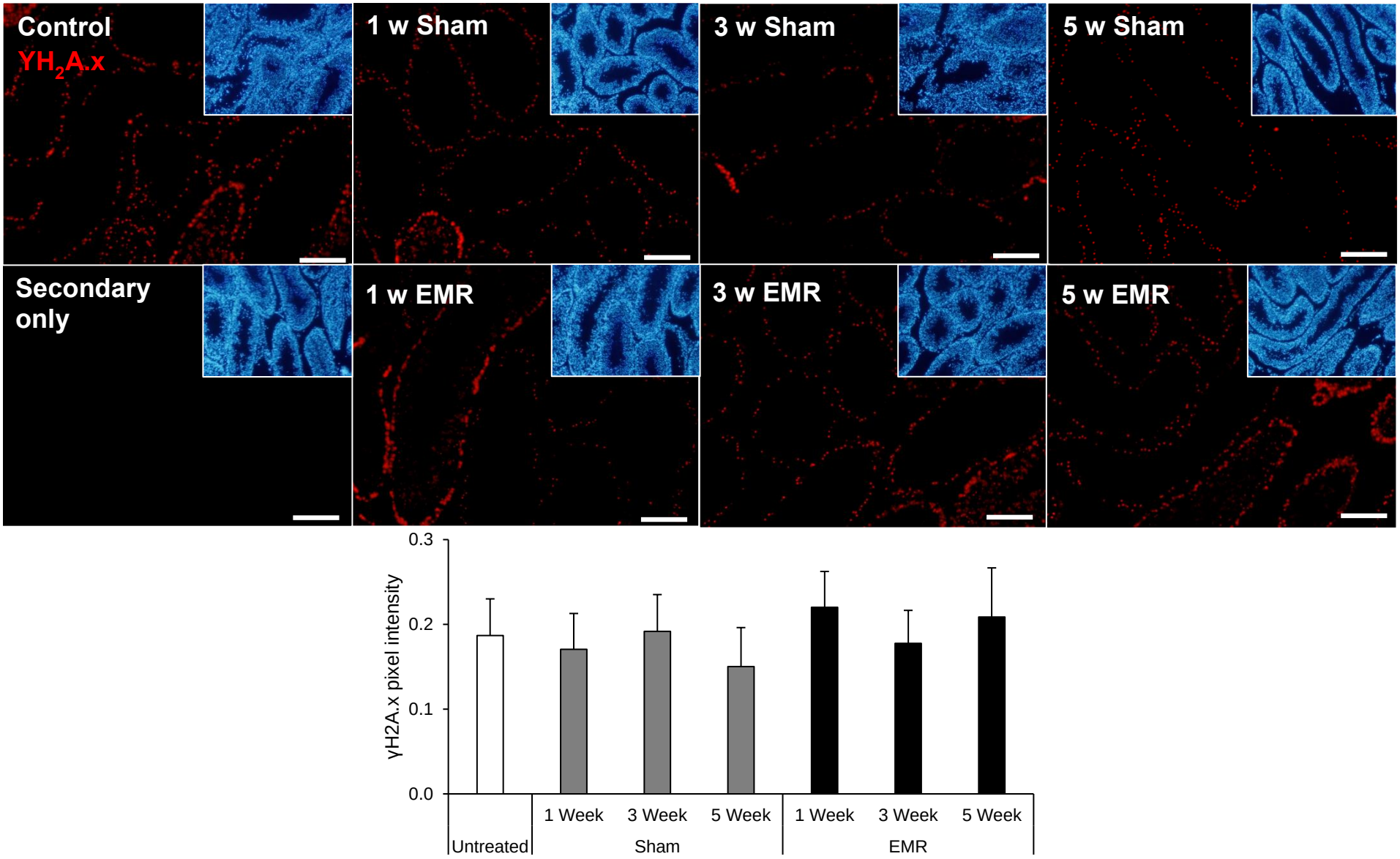


Figure 3. RF-EMR exposure does not induce γH2Ax expression in the testis. Testis sections were probed for gamma-H2Ax, for the untreated control, as well as sham and RF-EMR exposures across all time points. Scale bar represents 400 µM. Alongside this, pixel intensity analysis was performed on the germ cell population inside the seminiferous tubules to quantify expression levels across treatments. Error bars represent standard error values around the mean, n = 3.

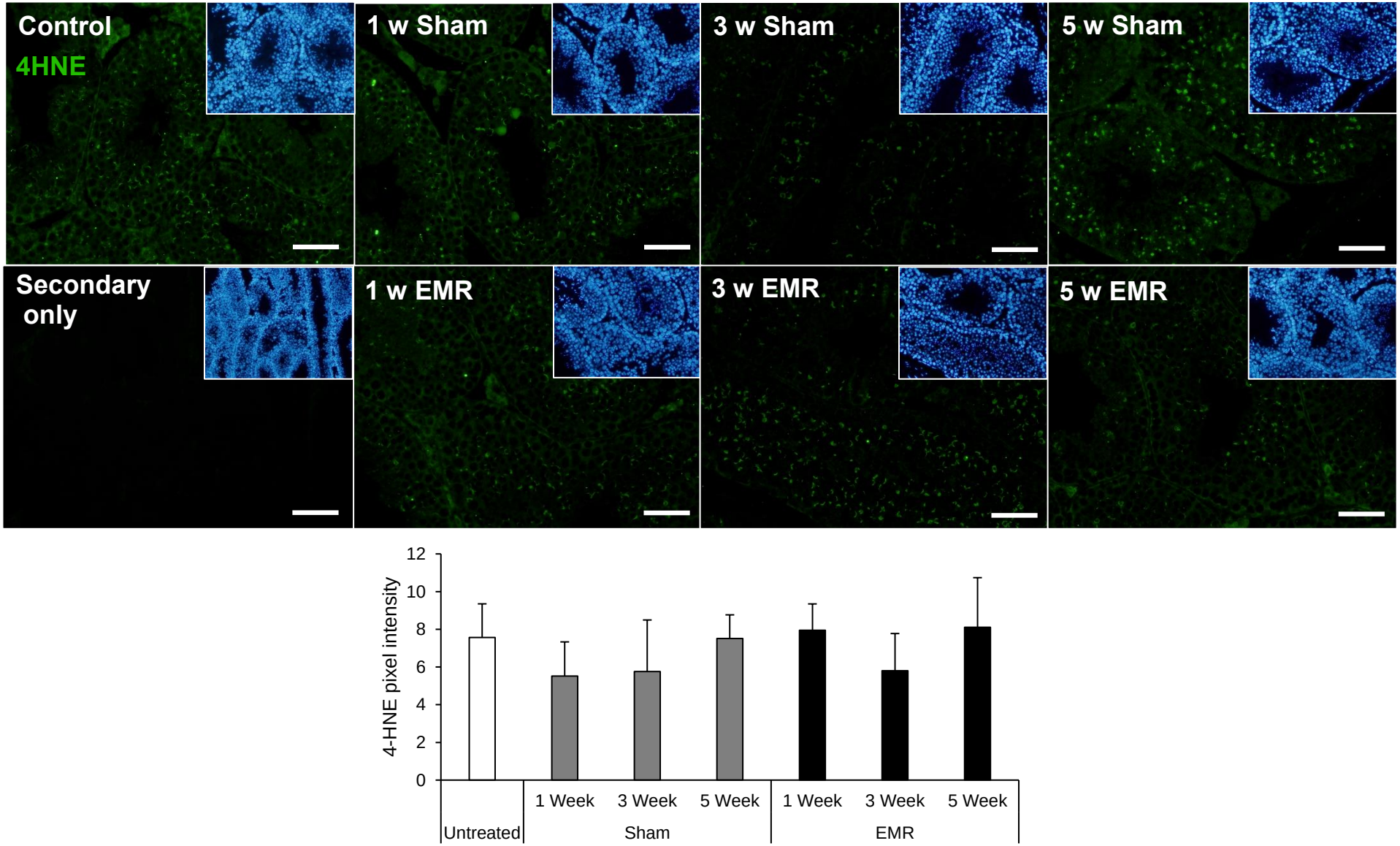


Figure 4. RF-EMR exposure does not induce elevated 4-hydroxynonenal formation in the testis. Testis sections were probed for 4-hydroxynonenal expression, for the untreated control, as well as sham and RF-EMR exposures across all time points. Scale bar represents 200 µM. Alongside this, pixel intensity analysis was performed on the germ cell population inside the seminiferous tubules to quantify expression levels across treatments. Error bars represent standard error values around the mean, n = 3.

To explore the effect of *in vivo* RF-EMR exposure on spermatozoa, we next investigated the outcomes of the radiation regime as describe above on sperm motility and vitality (Fig. 5). It was observed that the total number of live spermatozoa isolated from the cauda epididymis was diminished with RF-EMR exposure ($p < 0.05$) (Fig. 5A), an effect that was particularly evident after 5 weeks of exposure ($p < 0.001$); whereas no changes were observed in our sham exposed populations. In a similar fashion, we noted a significant reduction in the motility (Fig. 5B) of spermatozoa collected from RF-EMR exposed mice following a treatment regime of 5 weeks ($p < 0.05$). This reduction in sperm motility also extended to the objective measurements of progressive and rapid sperm motility (Fig. 5C, D) in exposed mice, but did not reflect reductions to sperm velocity following exposure to RF-EMR (data not shown). In this regard, the detrimental impact on both parameters was again most notable following 5 weeks of exposure ($p < 0.001$). Furthermore, in each of the assessed parameters, spermatozoa collected from each sham group produced measurements unchanged from the unexposed control group.

To confirm the demonstration that RF-EMR has the ability to induce oxidative stress in male germ cells and spermatozoa (Agarwal *et al*., 2009; De Iuliis *et al*., 2009), we next investigated the effect of our exposure regime at inducing elevated levels of ROS which may account for the perturbed sperm motility and vitality in spermatozoa of exposed mice (Fig. 6). The dihydroethidium (DHE) fluorescent probe was utilized to provide insight into levels of whole cell ROS production (Fig. 6A), which revealed a basal level of approximately 14% positive cells in control populations. Sham or EMR treatment conditions did not result in a significant deviation from this response. Next, mitochondrial ROS generation was specifically explored with use of the MitoSOX red (MSR) fluorescent probe (Fig. 6B). We documented a similar response between the control and sham exposed groups, where approximately 20% of cells tested positive for mitochondrial ROS production. However, treatment with RF-EMR for 1 or 3 weeks, produced a significant, two-fold elevation in mitochondrial ROS generation, compared to the control cell population ($p < 0.05$). However, this excessive production of mitochondrial ROS had normalized to basal levels following 5 weeks of EMR exposure, with no difference being measured with respect to the control population at this time.

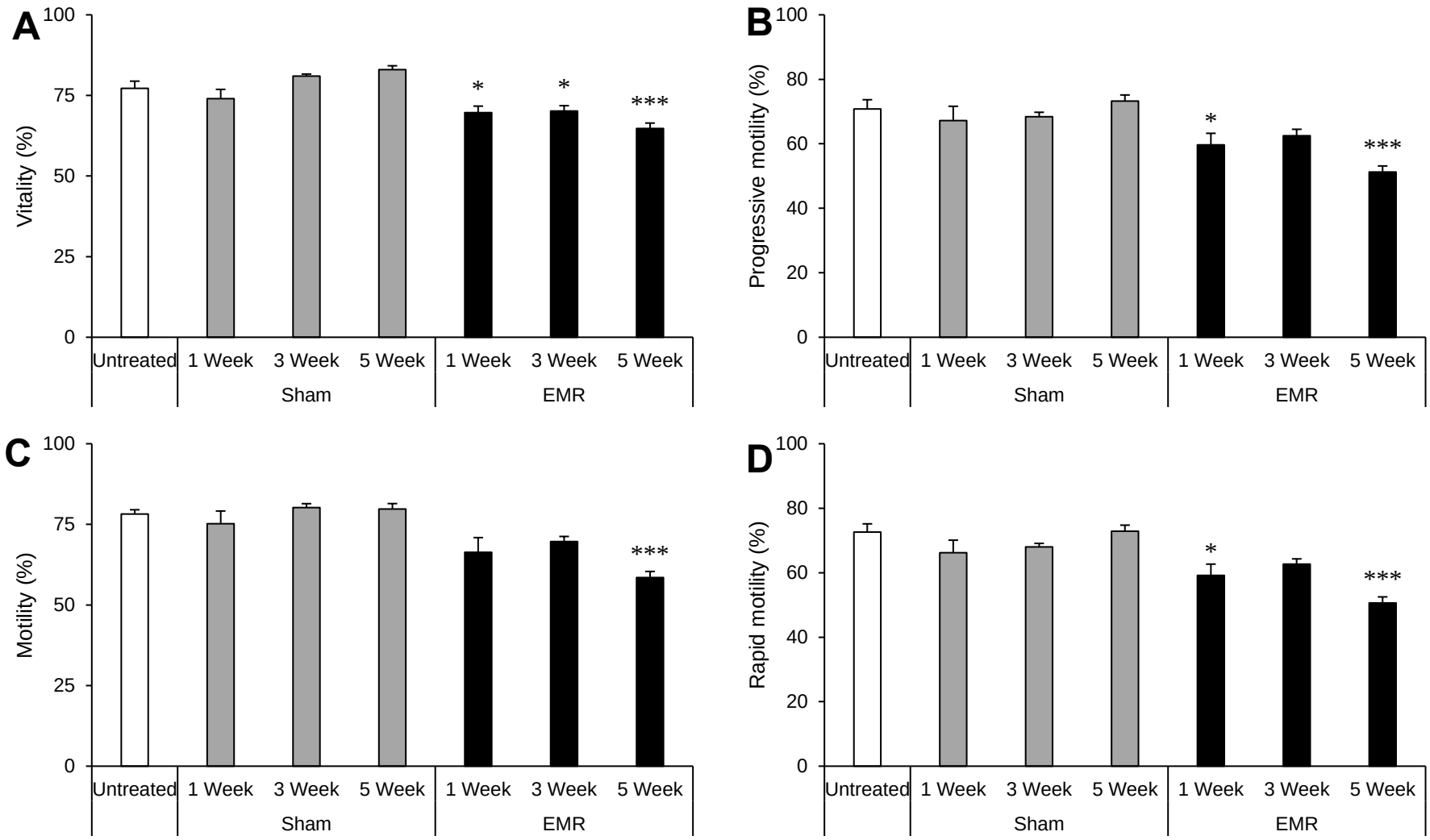


Figure 5. Sperm vitality and motility declines in response to RF-EMR exposure. Spermatozoa were collected from the cauda epididymis via a method of retrograde perfusion. (A) Sperm vitality assessed via the eosin-exclusion method. Next, sperm motility (B), progressive motility (C) and rapid motility (D) was evaluated using computer assisted semen analysis for all treatment groups and exposure time points. Error bars represent standard error values around the mean, n = 5.

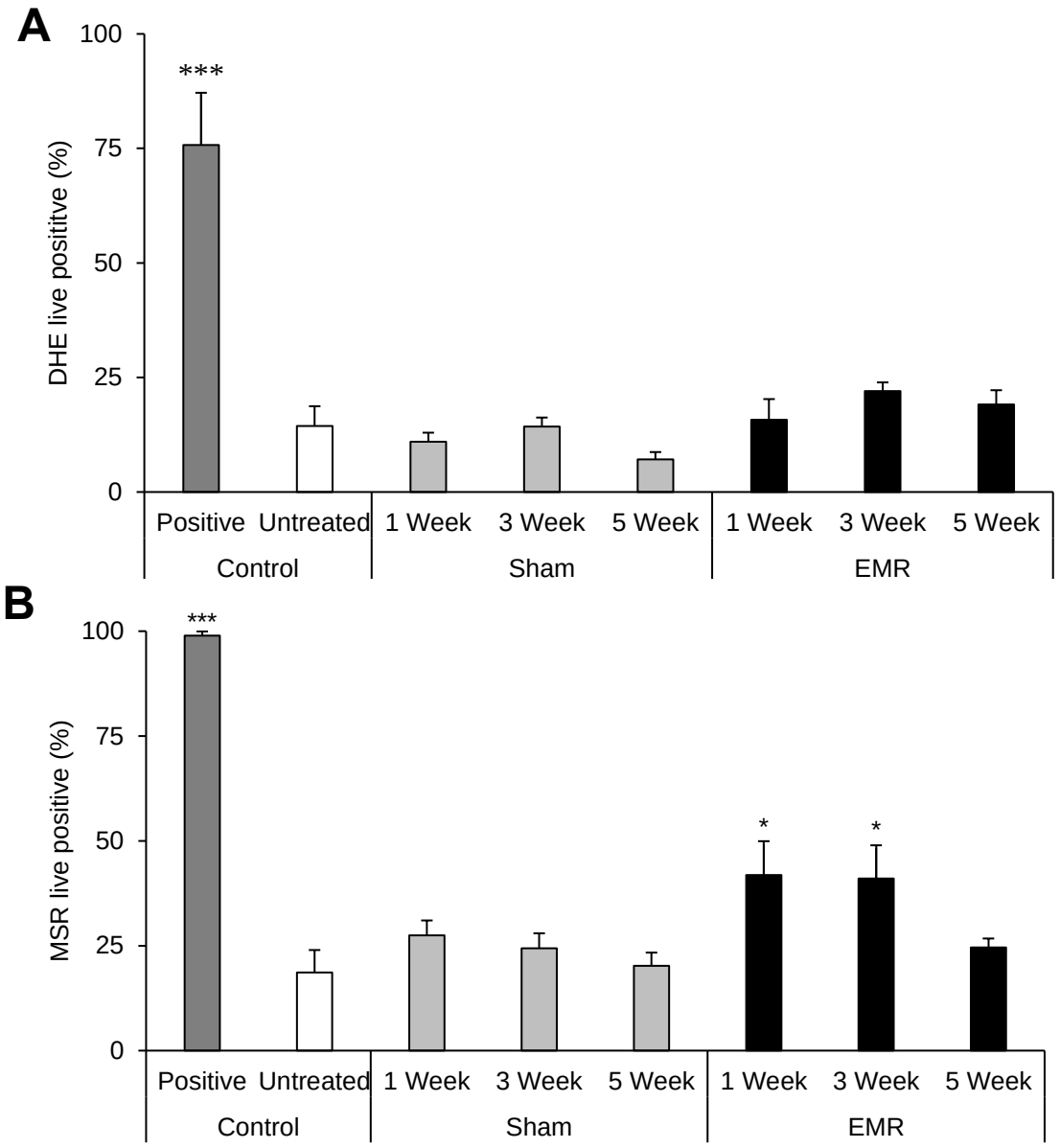


Figure 6. Exposure to RF-EMR stimulates the generation of mitochondrial reactive oxygen species. Spermatozoa were loaded with fluorescent probes and then analysed using flow cytometry to indicate reactive oxygen species (ROS) generation following exposure to sham and RF-EMR exposure conditions. (A) Global levels of ROS generated in the sperm cell was assessed with the dihydroethidium probe. (B) Mitochondrial ROS generation was investigated with the MitoSOX red probe. Error bars represent standard error values around the mean, n = 5.

Subsequently, DNA damage assays were employed to gain insight on the effect of RF-EMR induced ROS on the DNA integrity of mouse spermatozoa (Fig. 7). The halo assay (Fig. 7A), which determines whether DNA is intact by the presence of a halo-like glow around stained DNA, revealed a modest but significant decrease in halo positive spermatozoa following 3 (6%) and 5 (5%) weeks RF-EMR exposure ($p < 0.05$) indicating elevation in DNA fragmentation. To complement this finding, the alkaline comet assay (Fig. 7B) was also performed to assess sperm DNA fragmentation after RF-EMR exposure. In agreement with the halo assay, it was observed that exposure to RF-EMR stimulated DNA fragmentation after 1 week (18%; $p < 0.01$), climaxing at 5 weeks (23%; $p < 0.001$). We next demonstrated that this DNA damage was coincident with oxidative DNA damage, highlighted by an increase in the percentage of sperm displaying positive staining for 8-hydroxy-2-deoxyguanosine (8OHdG; Fig. 7C), a biomarker of oxidative DNA damage and oxidative stress. In all three exposure times, EMR induced a significant ($p < 0.05$) increase in 8OHdG labelling relative to control and sham exposed populations. Further, this labelling was localised to the nuclear compartment of the sperm head and was consistently more intense in EMR treated spermatozoa (Fig. 7D).

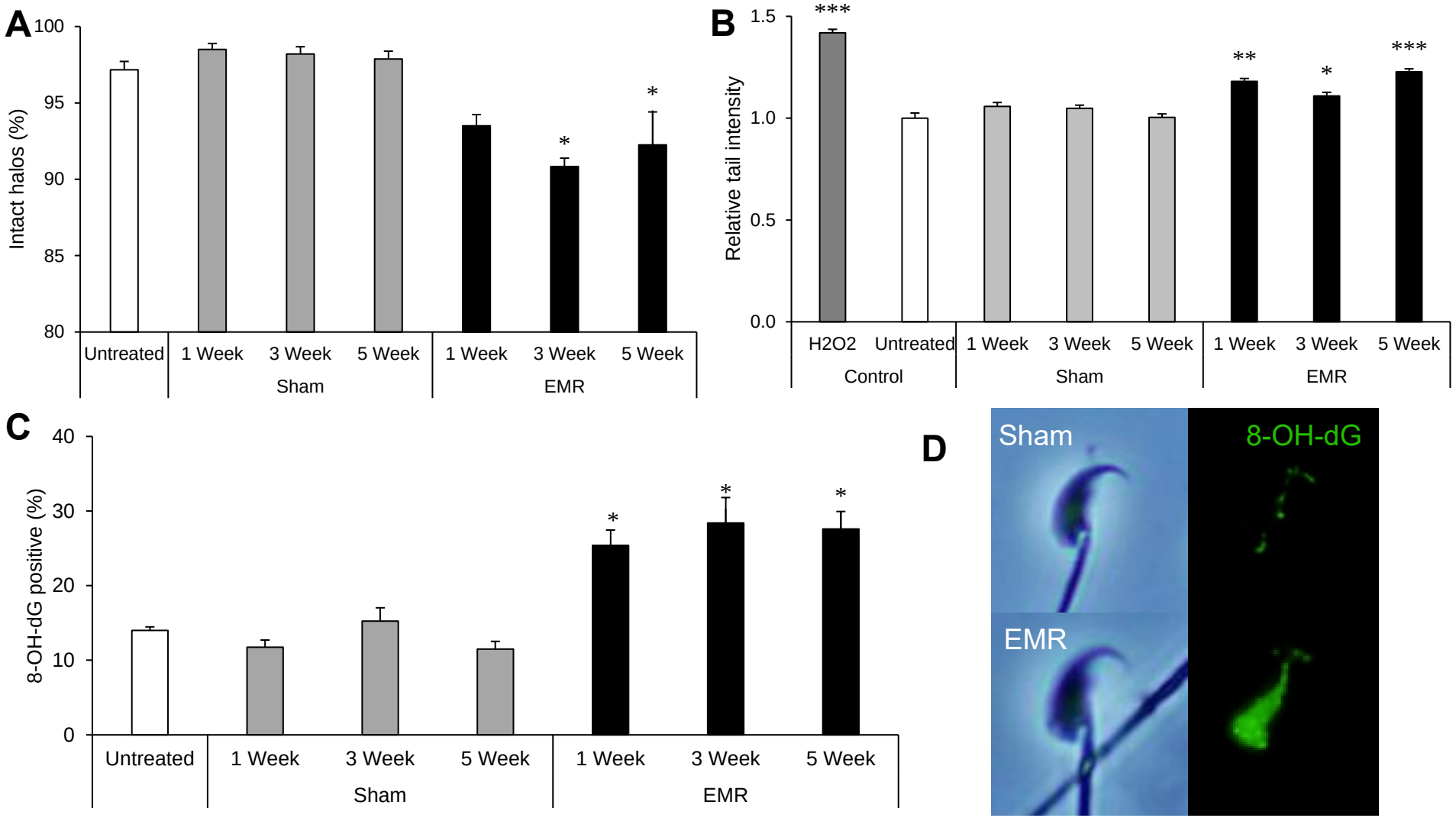


Figure 7. RF-EMR exposure induces widespread DNA damage in spermatozoa. DNA fragmentation was assessed by the halo assay (A) and then quantified by the alkaline comet assay (B). (C) Oxidative DNA damage was then evaluated with an 8-hydroxy-2-deoxyguanosine (8-OH-dG) antibody. (D) Representative images of spermatozoa stained with the 8-OH-dG antibody from the sham and RF-EMR exposed populations following 5 weeks of exposure. Error bars represent standard error values around the mean, n = 5.

In order to determine if there were any effects of compromised sperm motility (Fig. 5B) and elevated sperm DNA damage (Fig. 7) on the fertilization competence of RF-EMR exposed spermatozoa, we undertook an assessment of selected markers of sperm capacitation and sperm-zona pellucida binding with 5 week RF-EMR exposed spermatozoa (Fig. 8). As capacitation markers, levels of tyrosine phosphorylation (Fig. 8A) and acrosome reacted spermatozoa (Fig. 8B) between the control and EMR treatment groups remained unchanged with EMR exposure, with a modest but non-significant increase documented in acrosome reacted sperm. The average number of spermatozoa bound to the zona pellucida of fixed oocytes was also unchanged across our control (25), sham (25) and RF-EMR exposed (19) populations (Fig. 8C, D; $p = 0.99$). As an extension of this assessment of sperm function, the ability of spermatozoa from all three treatment groups to achieve fertilization and progression to the blastocyst stage of development was then investigated (Fig. 9). Exposure to RF-EMR continued to assert no observable differences with respect to fertilization rate (Fig. 9A), with all treatment groups resulting in the fertilization of between 83-87% of inseminated oocytes. When these zygotes were cultured through to the blastocyst development (Fig. 9B) stage, a modest increase was observed in the development rate of the EMR group, although no significance was seen across any of the treatment groups

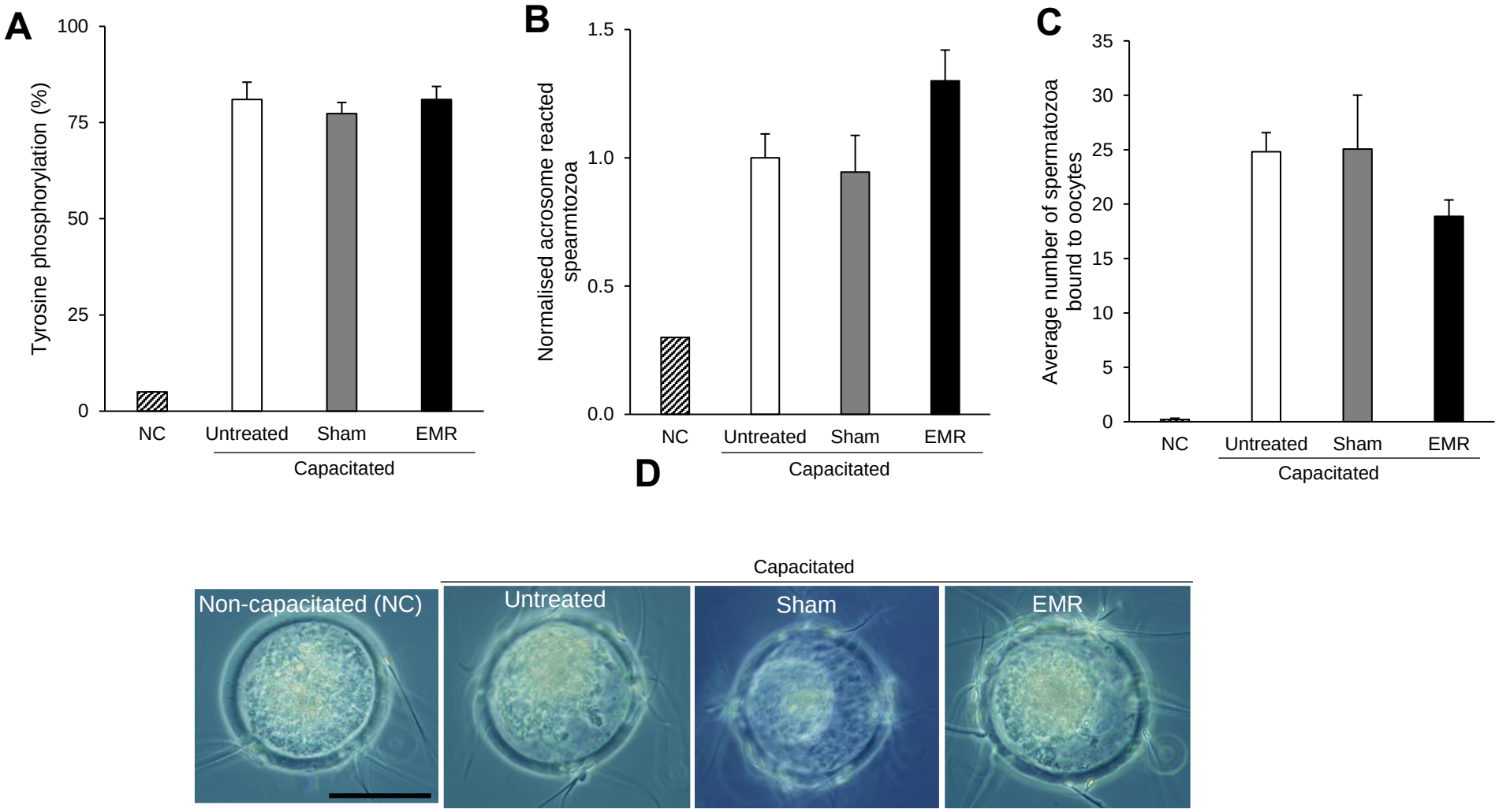


Figure 8. The effect of RF-EMR treatment on capacitation, and zona binding capacity. Spermatozoa were driven to capacitate in the presence of dbcAMP and the phosphodiesterase inhibitor pentoxifylline. (A) Tyrosine phosphorylation assessed via pt66 staining of the sperm tail. (B) The incidence of acrosome reacted spermatozoa, determined by peanut agglutinin binding to the sperm outer acrosomal membrane. (C) Zona pellucida binding ability of spermatozoa, assessed by counting the average number of spermatozoa bound to a collection of at least 8 oocytes. These interactions are demonstrated with representative images (D). Scale bar = 50 µm. Error bars represent standard error values around the mean, n = 3.

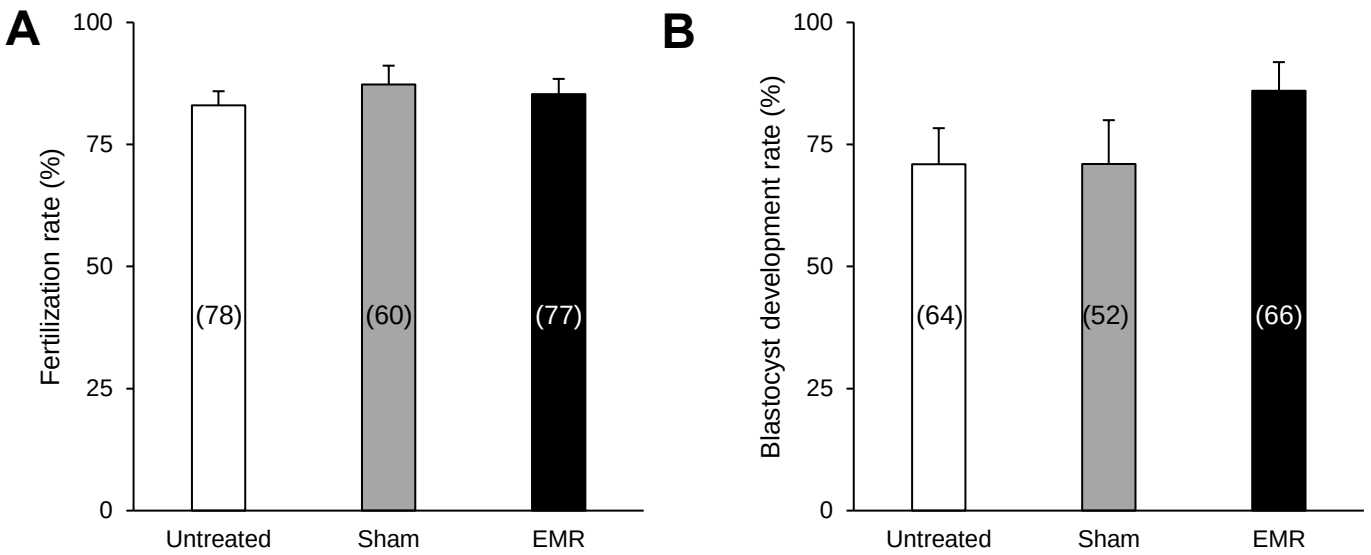


Figure 9. The effect of RF-EMR on sperm fertilizing ability and early embryonic development. Spermatozoa collected from RF-EMR treated mice were utilized for *in vitro* fertilization. (A) Fertilization rate and (B) Development rate of *in vitro* fertilized embryos to the blastocyst stage. Error bars represent standard error values around the mean, n = 3. Total egg/embryo numbers used for all three replicates are listed in brackets for each treatment group.

DISCUSSION

Several lines of evidence now propose RF-EMR to be capable of inducing a state of oxidative stress in a variety of cell types and systems (Avci *et al*., 2012; Irmak *et al*., 2002; Meral *et al*., 2007; Yurekli *et al*., 2006), including the male germ line (Mailankot *et al*., 2009; De Iuliis *et al*., 2009). It is well established that spermatozoa are particularly sensitive to oxidative insults, which is likely attributable to their surplus of polyunsaturated fatty acids and restricted supply of antioxidant defences (Aitken, 2013; Aitken *et al*., 2014). What is yet to be clearly demonstrated is how RF-EMR is capable of inducing such cellular responses in the absence of a thermal induction mechanism. While our previous, *in vitro,* investigation is consistent with similar studies that observe oxidative stress as having a central role, this study is designed to gain further insight into the mechanism by which RF-EMR may affect biology, in the male reproductive system. Consistent with our previous study, we here contribute data to support the dysregulation of sperm mitochondria, as a pivotal process for the driver of RF-EMR associated stresses (Fig. 6). Utilizing an *in vivo* exposure model, while better approximating the complexities around environmental exposures, also enables the dissection of the effects on discrete stages of male germ line development as these cells progress throughout the reproductive tract. Our analyses focused on the interaction of RF-EMR with: 1) epididymal spermatozoa, 2) spermatozoa during their progression via the testis through to epididymis, and 3) during the development of germ cells through to epididymal spermatozoa, with respect to the 1, 3 and 5 week exposure regimes (Zhu *et al*., 2004).

While limited reports in previous studies have identified RF-EMR capable of inducing defects to tubule structure in the testis of rats (Al-Damegh, 2012; Dasdag *et al*., 1999), here we observed no effects associated with exposure regarding morphological changes in the testis (Fig. 2C). Further investigation indicated that both DNA double strand breaks (Fig. 3) in developing germ cells within this tissue ($p = 0.07$) and the levels of lipid aldehyde by-product 4HNE in these tubules (Fig. 4; $p = 0.22$) were not elevated due to RF-EMR exposure. This finding is not surprising given the lack of robust and unanimous evidence presented in the

literature to date, which indicates that overt effects are unlikely at environmental exposure conditions. Given the lack of consistency in the field, if a potential health impact does exist, the biological effects may be subtle in nature (Houston *et al*., 2016). Our focus on the germ line within the male reproductive tract, as previously described (Aitken, 2013; Aitken *et al.*, 2014), affords us an acutely sensitive model cell type to further test our hypothesis that RF-EMR associated stress is linked to mitochondrial dysfunction and to further assess the parallels across our *in vitro* and *in vivo* studies.

Under this model we observed clear declines in motility and vitality in caudal epididymal sperm (Fig. 5), which arose in concert of the increases in mitochondrial ROS generation after 1 and 3 weeks of exposure ($p < 0.05$; Fig. 6), providing evidence that oxidative stress is a key outcome of RF-EMR exposure in the male germ line. Indeed, the increases in mitochondrial ROS in response to RF-EMR complements the body of literature implicating RF-EMR in the generation of a state of oxidative stress in a variety of cell types (De Iuliis *et al*., 2009; Hou *et al*., 2015; Kahya *et al*., 204; Yao *et al*., 2008) and is clearly not restricted to the male germ line. Importantly, the induction of mitochondrial ROS generation highlighted in this study was not exposure period dependent. Elevations in ROS at 1 and 3 weeks were trailed by a decline at 5 weeks, equivalent to levels that mirrored unexposed sham controls. The decline in active ROS production in spermatozoa with extended exposure to RF-EMR could be explained by responses that either reduce mitochondrial function in exposed cells or conceivably, an elevation of intrinsic antioxidant molecules arises. Evidence for either will be another important discovery implicating ROS in the aetiology of RF-EMR induced sperm damage. One line of evidence is supported by the morphology of the mitochondrion, which changes significantly during spermatogenesis (Kaye, 1958); most notably during the maturation of spermatogonia to spermatocytes, where these organelles undergo extensive vacuolarization (Meinhardt *et al*., 2000). Furthermore, the activity of the mitochondria is also heightened in spermatocyte and spermatid populations, whereas spermatozoa limit their investment to oxidative phosphorylation and utilize glycolysis primarily (Ramalho-Santos *et al*., 2009). In addition, the

mitochondria of caput epididymal spermatozoa are silenced (Aitken *et al*., 2007), which may protect them from mitochondrial perturbed ROS production (Suski *et al*., 2011). However, this suppressed state is lost throughout epididymal maturation (Aitken *et al*., 2007). On the other hand, this phenomenon of declining ROS generation over exposure time may also suggest a protective response with an upregulation of antioxidant molecules emerging from the male reproductive tract. This has previously been documented for vitamins A and E being concentrated in the testis following exposure to RF-EMR (Ozorak *et al*., 2013). Furthermore, enzymatic antioxidants such as superoxide dismutase and glutathione peroxidase are depleted in spermatozoa exposed to RF-EMR (Kesari *et al*., 2011). Despite this decline in ROS production, the motility of the spermatozoa collected in this study suffered most significantly after a 5 week exposure period (Fig. 5B-D). This suggests that while the real-time production of mitochondrial ROS (as detected by MitoSOX red) was ameliorated by the spermatozoa when collected at 5 weeks, they had already incurred oxidative damage earlier in the exposure period, consistent with increases in ROS observed at 1 and 3 weeks.

Suppression of sperm motility in association with elevated ROS has previously been very well defined by lipid peroxidation events yielding reactive aldehydes such as 4-hydroxynonenal, which in turn cause damage to the membrane and irreversible protein modifications and alkylation of the sperm axoneme (Baker *et al*., 2015). If there was indeed a history of oxidative stress at stages in the development of the spermatozoa that were collected at 5 weeks, then regardless of the lack of active detectable ROS, these cells should bear the hallmarks of oxidative stress and should express elevated levels of oxidised DNA in the form of 8-hydroxy-2-deoxyguanosine (8-OH-dG). In agreement with this notion, we detected the presence of the oxidative stress biomarker 8-OH-dG (Fig. 7C) with a striking increase in the staining of sperm nuclei collected from exposed mice at all time points, indicating abundant guanosine oxidation. This finding was in-line with Liu *et al*. (2013b), who documented a significant elevation in the formation of 8-OH-dG in spermatocytes exposed to RF-EMR. Furthermore, this localization was similar to that described previously in mouse spermatozoa subjected to oxidative stress

(Kocer *et al*., 2015; Noblanc *et al*., 2013). Meanwhile, the incidence of 8-OH-dG positive spermatozoa was also significantly increased ($p < 0.05$) following treatment, supporting RF-EMR as a mediator of oxidative stress. Accompanied with oxidative DNA damage, we observed elevated DNA fragmentation in the form of single strand breakage (Fig. 7B) following exposure to RF-EMR. Enhanced levels of DNA fragmentation have previously been documented following exposure to RF-EMR in spermatozoa (De Iuliis *et al.,* 2009; Gorpinchenko *et al*., 2014; Zalata *et al*., 2015) and spermatocytes (Liu *et al*., 2013), which may be generated through removal of oxidized guanosine residues to reflect the excision of oxidative DNA base adducts under the activity of OGG1 (Smith *et al.*, 2013b). Indeed, Liu *et al*. (2013a) documented that DNA damage induced by RF-EMR in spermatocytes can be ameliorated when these cells are pre-treated with the antioxidant compound melatonin, and De Iuliis *et al.* (2009) highlight a strong correlation between DNA fragmentation and oxidative DNA damage in RF-EMR treated spermatozoa, which further supports the potential for an oxidative nature of RF-EMR associated stress.

Utilizing the 1, 3 and 5-week exposure regime, has allowed us to isolate the different stages of development along the male reproductive tract that may be sensitive to RF-EMR stress. It was revealed that at all stages of sperm development, whether in the testis or epididymis, the male gamete was sensitive to DNA damage (Fig. 7). Sperm motility also suffered significantly following 5 weeks of treatment, which suggests that there may be a specific point over the germ cell maturation and/or developmental lifetime, where cells are uniquely sensitive to RF-EMR (Liu *et al*., 2013a). With one week of exposure, the spermatozoa collected were undergoing epididymal transit. Whereas in the three and five week exposures, germ cells in the testis would also form part of the sperm population on their maturation cycle to spermatozoa (Zhu *et al*., 2004). One explanation of the differential ROS profile in this study may be that different germ cell stages are more resistant to RF-EMR. This again raises the idea of mitochondrial susceptibility in germ cells and spermatozoa throughout spermatogenesis and epididymal transit. Most importantly, extended exposure to RF-EMR

likely allowed for the ROS generated by this insult to overwhelm cellular antioxidant supply and trigger lipid peroxidation within germ cells and spermatozoa, thereby impairing sperm motility as seen after 5 weeks of exposure. While future studies are required to identify vulnerable stages of germ cell development to RF-EMR, the detection of DNA damage at all periods assessed in this study suggests that this may exist in both the testis and epididymis.

Importantly, and in line with previous studies (De Iuliis *et al*., 2009), sperm motility was relatively unaffected during exposure times (1 and 3 weeks; Fig. 5) where oxidative DNA damage and DNA fragmentation was observed (Fig. 7). When these cells were utilized for *in vitro* fertilization the fertilized oocytes were capable of compensating for this damage, allowing the resulting embryos to develop to the blastocyst stage at statistically similar percentages to sham exposed and control spermatozoa (Fig. 9B). However, a major consequence of DNA damage transmission from the paternal genome to the offspring is the increased incidence of birth defects; including childhood cancers, and in extreme cases abortions of the foetus (Aitken *et al*., 2014). Even while the incidences of negative health impacts for the offspring would be expected to be rare, there is still a vital burden of the newly formed zygote to detect and repair any DNA lesions in order to generate a viable embryo. Where this repair fails, mutations may then be expressed in the offspring. It has been widely documented that transmission of DNA damage to the embryo leads to adverse clinical outcomes (Simon *et al*., 2017). Consistent with our hypothesis, the lack of obvious impairment of both sperm function and early embryo development in this study, is not unexpected. If any health risk exists with chronic exposure to low power RF-EMR, the pathways and biological perturbations potentially involved are likely to be understated. While the male germline is capable of adverse reaction to RF-EMR, the lack of effect observed in early embryo development is not evidence for zero health risk and further investigation is required to establish the precise nature of perturbation in male germline which could be used to further targeted studies and indeed, further animal exposure models. In particular, multi- and trans-generational studies such as that performed by Sommer *et al*. (2009) will shed light on the genomic and potential cumulative changes caused by RF-EMR

over generations, and will be important to direct the future of this field of research. With respect to trans-generational effects, a recent study has identified that extremely low frequency-EMR is capable of downregulating the expression of DNA (cytosine-5)-methyltransferase 1 (DNMT1) in the U2OS cell line (Kuzniar *et al*., 2017). Certainly, the genetic effects of RF-EMR require further investigation considering that aberrant DNMT1 activity has been linked to the formation of cancers and onset of developmental disorders (Kuzniar *et al*., 2017). While still controversial, data is mounting that indicates damaging impacts of RF-EMR exposure. At this time, there is a clear need to identify how these biological effects are precipitated. Using this knowledge, the significance of future clinical studies can be greatly elevated.

This study supplies further evidence to support the hypothesis that RF-EMR is capable of inducing a state of oxidative stress, and the mitochondria within the male germline a likely target. We unveiled a significant loss of sperm motility associated with elevated levels of mitochondrial ROS generation, which led to the acquisition of both oxidative DNA damage and DNA fragmentation following exposure to RF-EMR. Furthermore, these data lend support to our hypothesis that RF-EMR interferes with the mitochondrial electron transport chain, driving a state of oxidative stress. The next step in this field will likely require transgenerational studies utilizing exposures to mimic environmental conditions. This will aid in addressing whether these stress pathways can be stimulated at highly accurate physiological doses of RF-EMR.

REFERENCES

Agarwal A, Desai NR, Makker K, Varghese A, Mouradi R, Sabanegh E & Sharma R 2009 Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an in vitro pilot study. *Fertil Steril* **92** 1318-1325.

Aitken RJ 2013 Human spermatozoa: revelations on the road to conception. *F1000Prime Rep* **5** 39.

Aitken, R. J., Nixon, B., Lin, M., Koppers, A. J., Lee, Y. H. and Baker, M. A 2007 Proteomic changes in mammalian spermatozoa during epididymal maturation. *Asian J Androl* **9**, 554-64.

Aitken RJ, Smith TB, Jobling MS, Baker MA & De Iuliis GN 2014 Oxidative stress and male reproductive health. *Asian J Androl* **16** 31-38.

Al-Damegh MA 2012 Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E. *Clinics (Sao Paulo)* **67** 785-792.

Avci B, Akar A, Bilgici B & Tuncel OK 2012 Oxidative stress induced by 1.8 GHz radio frequency electromagnetic radiation and effects of garlic extract in rats. *Int J Radiat Biol* **88** 799-805.

Baker MA, Weinberg A, Hetherington L, Villaverde AI, Velkov T, Baell J & Gordon CP 2015 Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biol Reprod* **92** 108.

Biggers JD, Whitten WK and Whittingham DG 1971 The culture of mouse embryos in vitro. In 'Methods in Mammalian Embryology'. Eds J. C. Jnr Daniel. 86–116. Freeman: San Francisco.

Dasdag S, Akdag MZ, Ulukaya E, Uzunlar AK & Ocak AR 2009 Effect of mobile phone exposure on apoptotic glial cells and status of oxidative stress in rat brain. *Electromagn Biol Med* **28** 342-354.

Dasdag S, Ketani MA, Akdag Z, Ersay AR, Sari I, Demirtas OC & Celik MS 1999 Whole-body microwave exposure emitted by cellular phones and testicular function of rats. *Urol Res* **27** 219-223.

De Iuliis GN, Newey RJ, King BV & Aitken RJ 2009 Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. *PLoS One* **4** e6446.

Demirel S, Doganay S, Turkoz Y, Dogan Z, Turan B & Firat PG 2012 Effects of third generation mobile phone-emitted electromagnetic radiation on oxidative stress parameters in eye tissue and blood of rats. *Cutan Ocul Toxicol* **31** 89-94.

Erogul O, Oztas E, Yildirim I, Kir T, Aydur E, Komesli G, Irkilata HC, Irmak MK & Peker AF 2006 Effects of electromagnetic radiation from a cellular phone on human sperm motility: an in vitro study. *Arch Med Res* **37** 840-843.

Ghanbari M, Mortazavi SB, Khavanin A & Khazaei M 2013 The Effects of Cell Phone Waves (900 MHz-GSM Band) on Sperm Parameters and Total Antioxidant Capacity in Rats. *Int J Fertil Steril* **7** 21-28.

Hou Q, Wang M, Wu S, Ma X, An G, Liu H & Xie F 2015 Oxidative changes and apoptosis induced by 1800-MHz electromagnetic radiation in NIH/3T3 cells. *Electromagn Biol Med* **34** 85-92.

Houston BJ, Nixon B, King BV, De Iuliis GN & Aitken RJ 2016 The effects of radiofrequency electromagnetic radiation on sperm function. *Reproduction* **152** R263-R276.

Irmak MK, Fadillioglu E, Gulec M, Erdogan H, Yagmurca M & Akyol O 2002 Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits. *Cell Biochem Funct* **20** 279-283.

Kahya MC, Naziroglu M & Cig B 2014 Selenium reduces mobile phone (900 MHz)-induced oxidative stress, mitochondrial function, and apoptosis in breast cancer cells. *Biol Trace Elem Res* **160** 285-293.

Katen AL, Chambers CG, Nixon B & Roman SD 2016 Chronic Acrylamide Exposure in Male Mice Results in Elevated DNA Damage in the Germline and Heritable Induction of CYP2E1 in the Testes. *Biol Reprod* **95** 86.

Kaye JS 1958 Changes in the fine structure of mitochondria during spermatogenesis. *J Morph*. **102**(2) 347-399.

Kelsh MA, Shum M, Sheppard AR, McNeely M, Kuster N, Lau E, Weidling R, Fordyce T, Kuhn S & Sulser C 2011 Measured radiofrequency exposure during various mobile-phone use scenarios. *J Expo Sci Environ Epidemiol* **21** 343-354.

Kesari KK, Kumar S & Behari J 2011 Effects of radiofrequency electromagnetic wave exposure from cellular phones on the reproductive pattern in male Wistar rats. *Appl Biochem Biotechnol* **164** 546-559.

Khalil AM, Abu Khadra KM, Aljaberi AM, Gagaa MH & Issa HS 2014 Assessment of oxidant/antioxidant status in saliva of cell phone users. *Electromagn Biol Med* **33** 92-97.

Kocer A, Henry-Berger J, Noblanc A, Champroux A, Pogorelcnik R, Guiton R, Janny L, Pons-Rejraji H, Saez F, Johnson GD, Krawetz SA, Alvarez JG, Aitken RJ & Drevet JR 2015 Oxidative DNA damage in mouse sperm chromosomes: Size matters. *Free Radic Biol Med* **89** 993-1002.

Kuzniar A, Laffeber C, Eppink B, Bezstarosti K, Dekkers D, Woelders H, Zwamborn AP, Demmers J, Lebbink JH & Kanaar R 2017 Semi-quantitative proteomics of mammalian cells upon short-term exposure to non-ionizing electromagnetic fields. *PLoS One* **12** e0170762.

Liu C, Duan W, Xu S, Chen C, He M, Zhang L, Yu Z & Zhou Z 2013 Exposure to 1800 MHz radiofrequency electromagnetic radiation induces oxidative DNA base damage in a mouse spermatocyte-derived cell line. *Toxicol Lett* **218** 2-9.

Liu C, Gao P, Xu SC, Wang Y, Chen CH, He MD, Yu ZP, Zhang L & Zhou Z 2013 Mobile phone radiation induces mode-dependent DNA damage in a mouse spermatocyte-derived cell line: a protective role of melatonin. *Int J Radiat Biol* **89** 993-1001.

Lord T & Aitken RJ 2015 Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Dev Biol* **406** 1-13.

Mailankot M, Kunnath AP, Jayalekshmi H, Koduru B & Valsalan R 2009 Radio frequency electromagnetic radiation (RF-EMR) from GSM (0.9/1.8GHz) mobile phones induces oxidative stress and reduces sperm motility in rats. *Clinics (Sao Paulo)* **64** 561-565.

Marchionni I, Paffi A, Pellegrino M, Liberti M, Apollonio F, Abeti R, Fontana F, D'Inzeo G & Mazzanti M 2006 Comparison between low-level 50 Hz and 900 MHz electromagnetic stimulation on single channel ionic currents and on firing frequency in dorsal root ganglion isolated neurons. *Biochim Biophys Acta* **1758** 597-605.

Masuda H, Sanchez S, Dulou PE, Haro E, Anane R, Billaudel B, Leveque P & Veyret B 2006 Effect of GSM-900 and -1800 signals on the skin of hairless rats. I: 2-hour acute exposures. *Int J Radiat Biol* **82** 669-674.

Meinhardt A, McFarlane JR, Seitz J & de Kretser DM 2000 Activin maintains the condensed type of mitochondria in germ cells. *Mol Cell Endocrinol* **168** 111-117.

Meral I, Mert H, Mert N, Deger Y, Yoruk I, Yetkin A & Keskin S 2007 Effects of 900-MHz electromagnetic field emitted from cellular phone on brain oxidative stress and some vitamin levels of guinea pigs. *Brain Res* **1169** 120-124.

Narayanan SN, Kumar RS, Karun KM, Nayak SB & Bhat PG 2015 Possible cause for altered spatial cognition of prepubescent rats exposed to chronic radiofrequency electromagnetic radiation. *Metab Brain Dis*.

Noblanc A, Damon-Soubeyrand C, Karrich B, Henry-Berger J, Cadet R, Saez F, Guiton R, Janny L, Pons-Rejraji H, Alvarez JG, Drevet JR & Kocer A 2013 DNA oxidative damage in mammalian spermatozoa: where and why is the male nucleus affected? *Free Radic Biol Med* **65** 719-723.

Ozguner F, Altinbas A, Ozaydin M, Dogan A, Vural H, Kisioglu AN, Cesur G & Yildirim NG 2005 Mobile phone-induced myocardial oxidative stress: protection by a novel antioxidant agent caffeic acid phenethyl ester. *Toxicol Ind Health* **21** 223-230.

Ozorak A, Naziroglu M, Celik O, Yuksel M, Ozcelik D, Ozkaya MO, Cetin H, Kahya MC & Kose SA 2013 Wi-Fi (2.45 GHz)- and mobile phone (900 and 1800 MHz)-induced risks on oxidative stress and elements in kidney and testis of rats during pregnancy and the development of offspring. *Biol Trace Elem Res* **156** 221-229.

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P. C., Sousa, A. P. and Amaral, A 2009 Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Hum Reprod Update* **15**, 553-72.

Salford LG, Brun A, Sturesson K, Eberhardt JL & Persson BR 1994 Permeability of the blood-brain barrier induced by 915 MHz electromagnetic radiation, continuous wave and modulated at 8, 16, 50, and 200 Hz. *Microsc Res Tech* **27** 535-542.

Simon L, Zini A, Dyachenko A, Ciampi A & Carrell DT 2017 A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. *Asian J Androl* **19** 80-90.

Smith TB, Baker MA, Connaughton HS, Habenicht U & Aitken RJ 2013 Functional deletion of Txndc2 and Txndc3 increases the susceptibility of spermatozoa to age-related oxidative stress. *Free Radic Biol Med* **65** 872-881.

Smith TB, Dun MD, Smith ND, Curry BJ, Connaughton HS & Aitken RJ 2013 The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *J Cell Sci* **126** 1488-1497.

Sommer AM, Grote K, Reinhardt T, Streckert J, Hansen V & Lerchl A 2009 Effects of radiofrequency electromagnetic fields (UMTS) on reproduction and development of mice: a multi-generation study. *Radiat Res* **171** 89-95.

Suski, J. M., Lebiedzinska, M., Bonora, M., Pinton, P., Duszynski, J. and Wieckowski, M. R 2012 Relation between mitochondrial membrane potential and ROS formation. *Methods Mol Biol* **810**, 183-205.

Terras MM & Ramsay J 2016 Family Digital Literacy Practices and Children's Mobile Phone Use. *Front Psychol* **7** 1957.

World Health Organization 2010 *In WHO Laboratory Manual for the Examination and Processing of Human Semen* 7–114. *Fifth Edition*. Geneva: World Health Organization

Yao K, Wu W, Wang K, Ni S, Ye P, Yu Y, Ye J & Sun L 2008 Electromagnetic noise inhibits radiofrequency radiation-induced DNA damage and reactive oxygen species increase in human lens epithelial cells. *Mol Vis* **14** 964-969.

Yurekli AI, Ozkan M, Kalkan T, Saybasili H, Tuncel H, Atukeren P, Gumustas K & Seker S 2006 GSM base station electromagnetic radiation and oxidative stress in rats. *Electromagn Biol Med* **25** 177-188.

Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y & Mostafa T 2015 In vitro effect of cell phone radiation on motility, DNA fragmentation and clusterin gene expression in human sperm. *Int J Fertil Steril* **9** 129-136.

CHAPTER 4

Whole body heating induces oxidative stress and DNA damage in the male germ line

Accepted: Biology of Reproduction (modified to this manuscript slightly, through peer review process)

Authors: Brendan J Houston[1], Brett Nixon[1], Jacinta H Martin[1], Elizabeth G Bromfield[1], Kristen E McEwan[1], Geoffry N De Iullis[1] and R John Aitken[1]

[1] Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

Chapter 4: Overview

In this chapter, we move our focus to another environmental factor, ambient temperature heating and investigate its effects on sperm development and quality. Above the safe limits for RF-EMR exposure, a secondary effect in the form of heat begins to develop in association with the oscillation of the radio waves. Here, the effect of this insult on the male reproductive tract is investigated, to determine tolerance of this heat sensitive system to heat stress and the potential mechanism of action of this stress.

Here, we characterise how spermatogenesis is affected by acute heating, by collecting spermatozoa from mice from 1 day to 6 weeks post treatment, corresponding to the maturation of key stages of germ cells throughout development to spermatozoa in the cauda epididymis. A second model was utilized to determine if repetitive heat exposure regime resulted in more drastic effects to sperm quality and to give further insight into the effects of heating on later stage germ cells, specifically the round spermatids.

The data generated in this chapter strongly demonstrates that throughout spermatogenesis, specific germ cell types are vulnerable to heat stress. In particular, round spermatids demonstrate multiple forms of damage as they mature to spermatozoa, encapsulated by losses to sperm motility, severe onset of plasma membrane fluidity and impaired DNA integrity in the form of DNA fragmentation. These hallmarks suggest heating also induces oxidative stress in the male germ line. Interestingly, no significant reductions to sperm fertility were observed during *in vitro* fertilization and subsequent early embryo development. However, further investigation is required to determine mutational loads presented by this treatment regime.

TITLE: Heat exposure induces oxidative stress and DNA damage in the male germ line

AUTHORS: Brendan HOUSTON[1*], Geoffry DE IULIIS, Jacinta MARTIN[1], Elizabeth BROMFIELD[1], Kristen MCEWAN[1], Brett NIXON[1] and R. John AITKEN[1,]

AUTHOR AFFLIATIONS:

[1] Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, Discipline of Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

CORRESPONDENCE: Brendan HOUSTON, School of Environmental and Life Sciences, Discipline of Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

Tel: +61 24921 2043

Fax: +61 24921 6308

Email: brendan.houston@uon.edu.au

KEY WORDS:

Heating, spermatozoa, germ cells, DNA damage,

RUNNING TITLE: Impact of elevated ambient temperature on spermatogenesis

Abstract

The reproductive consequences of global warming are not currently understood. In order to address this issue we have examined the reproductive consequences of exposing male mice to a mild heat stress. For this purpose, adult male mice were exposed to an elevated ambient temperature of 35ºC under two exposure models. The first involved acute exposure for 24 hours, followed by recovery periods of between 1 day and 6 weeks. The alternative heating regimen involved a daily exposure of 8 hours for periods of 1 or 2 weeks. In our acute model, we identified elevated sperm mitochondrial ROS generation ($p < 0.05$), increased sperm membrane fluidity ($p < 0.05$) as well as DNA damage in the form of single strand breaks ($p < 0.001$) and oxidative DNA damage ($p < 0.05$); characteristic of an oxidative stress cascade. This DNA damage was detected in, and possibly originated from, pachytene spermatocytes ($p < 0.001$) and round spermatids ($p < 0.001$) isolated from testes after 1 day recovery. Despite these lesions, the spermatozoa of heat-treated mice exhibited no differences in their ability to achieve hallmarks of capacitation or to fertilize the oocyte and support development of embryos to the blastocyst stage (all $p > 0.05$). Collectively, our acute heat stress model supports the existence of heat susceptible stages of germ cell development, with the round spermatids being most perturbed and spermatogonial stem cells exhibiting resistance to this insult. Such findings were complemented by those generated from our chronic heat stress model, which further supported the vulnerability of the round spermatid population.

Introduction

It is well established that the testis and epididymis of a majority of mammalian species are adapted to operate at temperatures 2-7°C below core body temperature as a consequence of being accommodated within a scrotal sack (Hansen, 2009; Waites, 1991; Wechalekar *et al*., 2010). Although the adaptive significance of the cooler environment afforded by the scrotum is still being actively debated, it has been proposed that this temperature differential maintains optimal spermatogenesis, minimizes gamete mutation rates, and/or supports sperm maturation and storage in the epididymis (Gallup, 2009). Accordingly, these organs and the male germ line they support, are susceptible targets for heat stress arising from inguinal clothing and/or elevated ambient temperatures. Indeed, it is well documented that heat stress negatively impacts male reproduction, affecting multiple stages of spermatogenesis and driving an overall reduction in sperm count, motility and normal morphology (Hansen *et al*., 2009; Perez-Crespo *et al*., 2008; Zhang *et al*., 2015). It follows that men who are occupationally exposed to extreme heat conditions commonly experience spermatogenic arrest, characterised by the onset of azoospermia, oligozoospermia or teratozoospermia (Dada *et al*., 2003) and men exhibit reduced fertility and sperm counts in summer months across the world (Gyllenborg *et al*., 1999; Jorgensen *et al*., 2001; Levine *et al*., 1988; 1990). This situation is compounded in our species owing to relatively low fertility arising from reduced semen quality (Huang *et al*., 2017; Virtanen *et al*., 2017); a response that has been increasingly linked to a variety of adverse environmental exposures (Virtanen *et al*., 2017).

Interest in the effects of thermal stress on male fertility has spanned many decades, with the deleterious nature of this stress on testicular function first being identified in studies reported throughout the 1920s and 1940s in species such as the human, rabbit and rat (Walker, 1928; Carmichael, 1945; Jeffcoate, 1946). In seeking to account for the mechanistic basis of this damage, it has been proposed that the most heat sensitive stages of germ cell development correspond to the spermatids and the pachytene spermatocytes (Perez-Crespo *et al*., 2008; Setchell. 1998; Wettemann and Desjardins, 1979; Zhu *et al*., 2004). Both of these

germ cell populations exhibit elevated levels of DNA damage in response to acute heat stress, resulting in signification reductions in the success of embryonic development achieved following fertilisation of oocytes once these cells mature to spermatozoa. Heat stress also has the ability to impair the development of spermatocytes into spermatids (Wettemann and Desjardins, 1979). In marked contrast, preceding phases of germ cell development, such as type A spermatogonia, appear relatively resilient to heat stress (McLean *et al*., 2002). This leads to a situation whereby the immediate post-heating reduction in germ cell proliferation and sperm quality is progressively ameliorated as the unaffected type A spermatogonia mature to replenish the damaged pool of spermatids and pachytene spermatocytes (Zhu *et al*., 2004).

Notably, while many of the preceding studies were designed to investigate the effects of localised heating on the testis, and therefore mimic conditions experienced in response to inguinal clothing, the comparative impact of ambient temperature heating models where the whole body is subjected to thermal stress, remain less well studied. In those studies that have sought to address this paradigm, it has been shown that spermatozoa exhibit hallmarks of apoptosis (Wechalekar *et al*., 2010; 2016) and are significantly less competent at supporting embryo development (Zhu *et al*., 2004) following exposure to an elevated temperature of 35ºC for as little as 24 h. It has been identified that the production of reactive oxygen species is a common outcome of heat stress in male germ cells (Hansen *et al*., 2009), which suggests that this insult is capable of inducing a state of oxidative stress. However, a mechanistic explanation for the embryonic losses associated with such stress has not been established. Furthermore, an extensive investigation of spermatogenesis, sperm quality and function under conditions of ambient heat stress has not been undertaken. In this study we sought to refine our understanding of the molecular and functional changes induced in spermatozoa in relation to the decreased fertility observed after either acute or chronic elevation of ambient temperature.

Materials and methods

The chemicals and reagents used in this study were purchased from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA) unless stated otherwise, and were of research grade. The fluorescent probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA), unless otherwise stated. All fluorescent imaging was performed using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Heating exposure regimens

Male C57BL/6 mice were exposed to heated environmental temperature in an animal intensive care unit cage (Lyon Technologies, Chula Vista, CA, USA). These mice were at least 8 weeks of age, with food and water provided *ad libitum*. Exposure (see Figure 1) was performed for either 24 h at 35ºC and 30% humidity (acute heat stress), or for 8 h per day under the same conditions for one or two weeks (chronic heat stress). Following exposure, the 24 h treated mice were allowed to recover over a period of 1 day – 6 weeks and were then culled. This timing of recovery was selected to correspond to the maturation of each major stage of germ cell development through to the spermatozoa (see Figure 1A). For the chronic 8 h per day exposures, the mice were culled the following day after the final heat treatment, to gain insight into the effect of multiple heating treatments on sperm quality (see Figure 1B). This approach was designed to provide insight into these effects on the spermatozoa in the epididymis and also the spermatids, believed to be particularly heat vulnerable. Spermatozoa were collected from the cauda epididymis and assayed for impacts of heating on cell activity and function as described below. Five mice were utilized for each time point, unless otherwise stated.

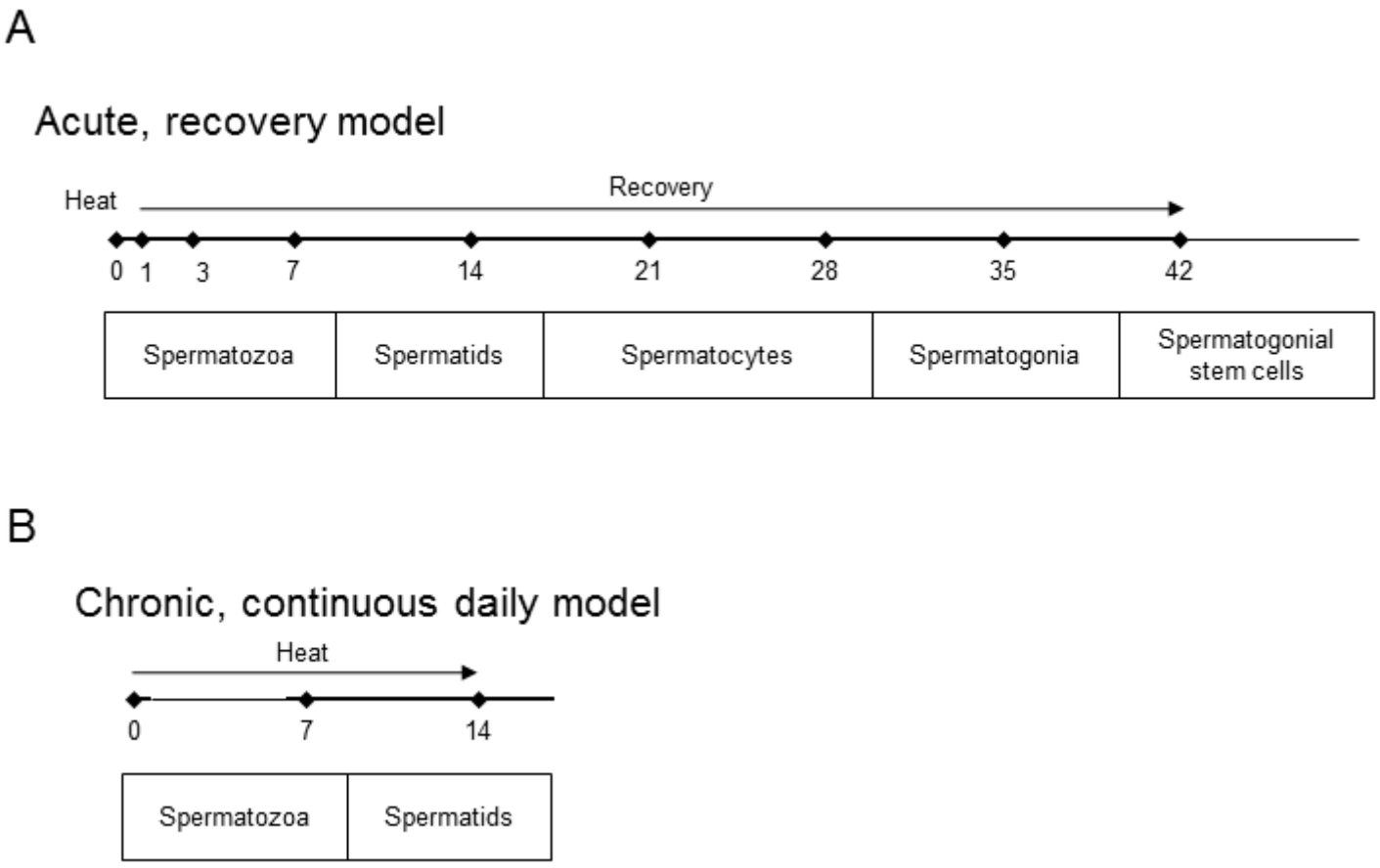


Figure 1. Ambient temperature heat treatment regimens. Mice were treated with heated environmental conditions at a temperature of 35ºC and 30% humidity under an acute or chronic exposure approach. Each major stage of germ cell development insulted by heat treatment is documented. This corresponds with specific recovery times taken to mature to spermatozoa, whereby cells were collected in the cauda epididymis. **A.** Acute exposure model. Mice were heated for 24 hours continuously, then removed from heating conditions and allowed to recover for 1 day – 6 weeks. **B.** Chronic-like model. Mice were heated for 8 hours per day for a period of 1 or 2 weeks, then assayed the following day.

Isolation of reproductive organs and spermatozoa

Dissection

Experimental protocols were approved by the University of Newcastle Animal Care and Ethics Committee (Ethics number 2014-447). Epididymides and testes were dissected from adult C57BL/6 mice immediately after being culled via CO_2 asphyxiation. Where required, mature spermatozoa were isolated from the cauda epididymis by retrograde perfusion via the vas deferens (Smith *et al*., 2013). One testis and one epididymis (fitted to a plastic grid) was placed in Bouin's fixative (9% formaldehyde, 5% acetic acid, 0.9% picric acid) for 6 h at 4ºC in a rotator. These organs were then resuspended in 70% ethanol overnight at 4ºC in a rotator. Finally, residual Bouin's fixative was washed out through resuspension in 70% ethanol and the organs were stored at 4ºC in preparation for sectioning. One section from each testis and epididymis was stained with hematoxylin and eosin to investigate testis and epididymal morphology. Three sections per treatment were assessed for morphological abnormalities in comparison to control tissue. Here, the presence of maturing germ cells and spermatozoa in the seminiferous tubules of the testis was evaluated. The presence of spermatozoa and morphologically normal tubules across the three principal regions of the epididymis was also evaluated.

Collection of spermatozoa

Spermatozoa were isolated from the cauda epididymis by a method of retrograde perfusion, into micro-capillary tubes. Upon collection, the spermatozoa were resuspended in 1 ml of modified Biggers, Whiting, Whittingham media (BWW; Biggers *et al*., 1971) and allowed to disperse for 15 min. Sperm concentration for each sample was determined using a haemocytometer and objective sperm motility was then assessed by computer assisted sperm analysis (CASA; IVOS, Hamilton Thorne, Danvers, MA, USA). A minimum of 100 spermatozoa in five fields were assessed using 2X-CEL slides (Hamilton Thorne) suspended on a pre-warmed stage (37ºC; Smith *et al*., 2013). The following settings were utilised: negative phase-contrast optics, 60 frames/sec recording rate, minimum cell size of 9 pixels, minimum contrast

of 80, low size gate of 0.3, high size gate of 1.95, low intensity gate of 0.5, high intensity gate of 1.3, nonmotile head size of 45 pixels, nonmotile head intensity of 75, average path velocity (VAP) threshold of 10 µm/sec, slow (static) cells VAP threshold of 5 µm/sec, slow (static) cells straight-line velocity (VSL) threshold of 0 µm/sec, and threshold straightness (STR) of 75%. Cells exhibiting a VAP of 10 µm/sec and a STR >0 were considered progressive. Cells with a VAP greater than that of the mean VAP of progressive cells were considered rapid. Sperm vitality was assessed via the eosin exclusion method (World Health Organization, 2010), using a dilution of 1:1.

Germ cell isolation

Enriched pachytene spermatocyte (81% purity) and round spermatid (89% purity) populations were isolated from 1 day acute heat treated testes as previously described (Baleato *et al*., 2005). Briefly, testes were dissected, removed of their tunica albuginea, and washed in DMEM at 600 × g, 4ºC for 5 min. Collagenase was then utilized to digest the seminiferous tubules for 15 min, which were then resuspended in 0.5% v/v trypsin-EDTA and rotated for 15 min at 21ºC. Each sample was then resuspended in DMEM and passed through a 70 µm filter to remove cell aggregates. Each suspension was then layered above a separate continuous 2-4% BSA/DMEM gradient for sedimentation over 3 h to enrich for each germ cell population. The bottom 10 ml layer of this gradient was discarded and the second 10 ml layer was collected, containing the spermatocytes. The following 25 ml was discarded and spermatids were collected as the next 10 ml fraction. Both cell suspensions were washed in DMEM, pelleted and stored at -80ºC.

Determination of oxidative stress in spermatozoa

Flow cytometry

Spermatozoa were assessed for oxidative stress levels via flow cytometry using the mitochondrial superoxide probe MitoSOX red (MSR) and the membrane fluidity marker Merocyanine 540 (M540) in conjunction with the Sytox Green (SYG) vitality stain. Cells were resuspended in either 2 µM MSR or 2.7 µM M540, in combination with 20 nM SYG in BWW

for 15 min in the dark at 37ºC. These cells were then centrifuged at 450 × g for 5 min and then resuspended in 400 µL BWW. Each sample was transferred to a flow cytometry tube for analysis with a FACS-Canto flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488-nm argon laser and 633 nm helium-neon laser. Analysis of these data was undertaken using CellQuest software (BD Biosciences, San Jose, CA, USA).

Oxidative DNA damage (8-OH-dG) immunofluorescence

Spermatozoa were snap frozen in liquid nitrogen and stored at -80ºC for the purpose of the 8-OH-dG assay. These cells were then resuspended in primary DNA/RNA damage antibody (Novus Biologicals, Littleton, CO, USA) (25 µg/ml in PBST) overnight at 4ºC. Following incubation, spermatozoa were washed in PBS and then incubated with AlexaFluor 488 goat α mouse secondary (5 µg/ml in PBS) for one h at 37ºC, washed twice in PBS and placed on slides for viewing with fluorescence microscopy. A sample of 100 cells were assessed for this analysis, scored positive by the presence of nuclear fluorescence.

Immunohistochemistry

Sectioned slides were dewaxed by suspension in three xylene baths for 5 min each, and then rehydrated in ethanol baths of decreasing concentration; twice in 100% for 5 min each, then once in 90%, 70% and 50% for 1 min each. Antigen retrieval was then performed by microwaving slides in a solution of 50 mM Tris (pH 10.5) for 9 min (tubulin). Each slide was blocked in a solution of 3% bovine serum albumin (BSA)-PBST for 1 h at room temperature and washed in PBS for 5 min. Following this, primary antibody incubation was performed DNA/RNA damage antibody (8-OH-dG; Novus, Littleton, CO, USA) (5 µg/ml) overnight at 4ºC. For tubulin, a conjugated primary antibody was used (2.5 µg/ml) for 1 h at 37ºC. Slides were then washed 3 times in PBS for 5 min. Secondary antibody incubation was undertaken in 1% BSA-PBST for DNA/RNA damage using AlexaFluor-594 (Thermo Fisher Scientific, MA, US) conjugated α-rabbit or α-goat antibodies (10 µg/ml) for 1 h at 37ºC. Slides were washed 3 times in PBS for 5 min and counterstained with DAPI (0.5 µg/ml) for 5 min at room temperature.

Finally, slides were washed twice in PBS for 5 min and mounted in Mowiol 4-88 (Millipore, Darmstadt, Germany) with antifade for viewing under a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). For quantification of 8-OH-dG quantification, a minimum of 15 seminiferous tubules or cauda epididymis tubules were utilized for mean pixel intensity assessment through ImageJ software (NIH, USA). This quantification was restricted to only the germ cells within the seminiferous tubules of the testis, or only the lumen of the cauda tubules containing the sperm population. Three replicates were performed for each treatment.

TUNEL (Apop-Tag kit, Millipore).

Tissues sections were dewaxed and rehydrated as detailed above. Antigen retrieval was then performed with 20 µg/ml proteinase-K/PBS for 10 min at room temperature. For the positive control, DNase buffer (Roche) was diluted 1:1 in PBS and applied to the slide for 5 min at room temperature, followed by DNase (Roche) diluted 1:1 in DNase buffer for 10 min at room temperature. Equilibration buffer was then applied to each slide for 5 min at room temperature, followed by TdT enzyme for 1 h at 37ºC. Stop solution was applied to complete the reaction and incubated for 10 min at room temperature. Slides were then washed in PBS three times. Next, slides were treated with anti-digoxigenin conjugate for 30 min at room temperature. All slides were washed three times in PBS and mounted in Mowiol 4-88 with antifade for viewing with fluorescence microscopy. This analysis was performed using mean pixel intensity within seminiferous tubules through ImageJ software (NIH, USA), excluding interstitial tissue and focusing on the germ cell population. Three replicates were performed for each treatment, and at least 10 tubules were analyzed for each slide.

Alkaline comet assay

The comet assay was performed as described previously (Katen *et al*., 2016). Spermatozoa and germ cells from samples pelleted and stored at -80ºC were resuspended in PBS to a final concentration of 4×10^4 cells/µl. A 10 µl aliquot of this cell suspension was mixed with 70 µl

agarose (Trevigen, Gaithersburg, MA, USA) and allowed to set on Dakin G376 slides pre-coated with 1% low melting point agarose (ProSciTech, Kirwan, QLD, Australia) sealed with a coverslip overnight at 4ºC. Briefly, coverslips were removed, and slides were treated with lysis solution 1 (0.8 M Tris-HCl, 0.8 M DTT, 1% SDS; pH 7.5) then lysis solution 2 (0.4 M Tris-HCl, 50 mM EDTA, 2 M NaCl, 0.4 M DTT; pH 7.5), while sealed with a coverslip for 30 min each at room temperature. Again, coverslips were detached and slides were washed in tris-boric acid buffer (0.445 M Tris-HCl, 10 mM EDTA, 0.445 M boric acid) for 10 min at room temperature. Each slide was then treated with alkaline solution (0.03 M NaOH, 1 M NaCl) for 15 min at 4ºC, followed by electrophoresis in alkaline buffer (0.03 M NaOH) at 1 V/cm for 3 min for spermatozoa, or 4 min for precursor germ cells. Slides were then washed in neutralization solution (0.4 M Tris-HCl; pH 7.5) for 5 min. SYBR green nucleic acid stain (Thermo Fisher Scientific) was applied to the slides immediately before viewing on the microscope, and a coverslip was added. The level of DNA damage was analyzed using Comet Assay IV software (Perceptive Instruments, Suffolk, UK). A collection of at least four replicates were used for each analysis, where a minimum of 30 comets were assessed and subsequently utilized for statistical analysis. For the positive control, spermatozoa were resuspended in 500 µM hydrogen peroxide for 5 min at room temperature. These cells were then washed in PBS, and then resuspended in PBS.

Sperm capacitation, oocyte binding and fertilization assays

Mature oocytes were retrieved from the distal ampullae of 3-4 week old C57BL/6 female mice following a superovulation regimen consisting of an intraperitoneal injection of 7.5 IU equine chorionic gonadotropin and human chorionic gonadotropin (Intervet, Sydney NSW, Australia). Oocytes were washed thrice in human tubal fluid (HTF) prior to being transferred into a droplet of HTF supplemented with 1 mM reduced glutathione (GSH) as previously described (Martin *et al*., 2016). Spermatozoa were simultaneously recovered and capacitated by incubation in modified BWW medium supplemented with 1 mg/ml polyvinyl alcohol and 1 mg/ml methyl-beta cyclodextrin for 1 h at 37˚C under an atmosphere of 5% O_2, 6% CO_2 in N_2. Finally, oocytes

and 2×10^5 capacitated spermatozoa were co-incubated for 4 h at 37˚C, after which signs of successful fertilization were analysed; extrusion of the second polar body and/or pronucleus formation. To support embryonic development, zygotes were subsequently cultured in unsupplemented HTF media overnight and transferred into G1 PLUS culture medium (Vitrolife, Gothenberg, Sweden) on the morning of day 2. After which a further media change into G2 PLUS medium (Vitrolife) was conducted on day 4. Importantly, embryos were monitored daily and developmental rates recorded. The percentage of fertilized oocytes and percentage of embryos that had reached the blastocyst stage by the morning of day 5 was calculated.

Immunofluorescence of spermatozoa

Spermatozoa were fixed in 4% paraformaldehyde for 15 min at room temperature, washed twice in 0.05 M glycine and then stored in this solution at 4ºC for peanut agglutinin (PNA) or phosphotyrosine (pt66) staining. A sample of 2×10^6 cells was then treated with 0.1% Triton-X100 in PBS for 10 min at room temperature, followed by washing in PBS. These cells were then labelled with primary antibody (4 µg/ml pt66) or conjugated PNA lectin (2.5 µg/ml) in PBS for 1 h at 37ºC. The cells were washed once in PBS, and pt66 samples were then treated with AlexaFluor 488 goat α mouse secondary antibody (5 µg/ml in PBS) for 1 h at 37ºC. After a final wash in PBS, cells were placed on slides and viewed via fluorescence microscopy.

Statistical analysis

JMP version 11 (SAS Institute Inc., Cary, NC) was used to analyze the data in each experiment, which were performed with at least 5 independent replicates (unless stated otherwise). Normality of datasets was assessed with the Shapiro-Wilks test ($\alpha = 0.05$). Following this, a one-way ANOVA was used to compare normally distributed treatments, with a post-hoc Tukey's honest significant difference test ($\alpha = 0.05$). For data that was not normally distributed, the Wilcoxon test was used ($\alpha = 0.05$), with a post-hoc Dunn's test. Error bars represent mean values ± standard error of the mean.

Results

In order to confirm the stability of the ambient temperature environment generated by the heating apparatus employed in these studies, we first assessed the ambient temperature within the device over a 24 h time-course using a sensitive temperature probe (Figure 2A). Here, we observed a very consistent heating output of 35°C, with only small fluctuations of + 0.5°C at 30, 1 min, instances during this time course. Presented with a reliable heating treatment, we proceeded with our experimental exposure regimen. During the initial, acute exposure treatment, we documented the weight of all mice during their recovery period of up to 6 weeks, revealing no significant changes in weight associated with heat stress (Figure 2B). Furthermore, investigation of the testis:body weight ratios across all treatment methods confirmed no gross fluctuations (Figure 2C).

The effects of heating on spermatogenesis and testis structure

Next, we probed the effect of acute heating on spermatogenesis via an initial examination of the gross morphology of the testis (Figure 3). Testes from mice subjected to recovery periods of either 1 day, 2 weeks or 6 weeks (encompassing the beginning, middle and end of our recovery periods) were assessed, revealing no dramatic changes in any of the treatment groups. Indeed, each testis section was characterized by equivalent morphology (Figure 3A) and encompassed all stages of germ cell maturation including the presence of spermatozoa in the centre of the seminiferous tubules. To complement this analysis of the morphology of the germ cells post-heating, we explored the expression of α-tubulin in the testes of these mice (Figure 3B). As anticipated, tubulin was expressed widely throughout the seminiferous tubules, with predominant labelling detected at the periphery of the early stage germ cells, within the developing acrosomal vesicle and within the flagellum of spermatozoa. In this regard, we did not observe any apparent modifications to the expression of this protein, or the structural elements in which it resides, across any of the treatments.

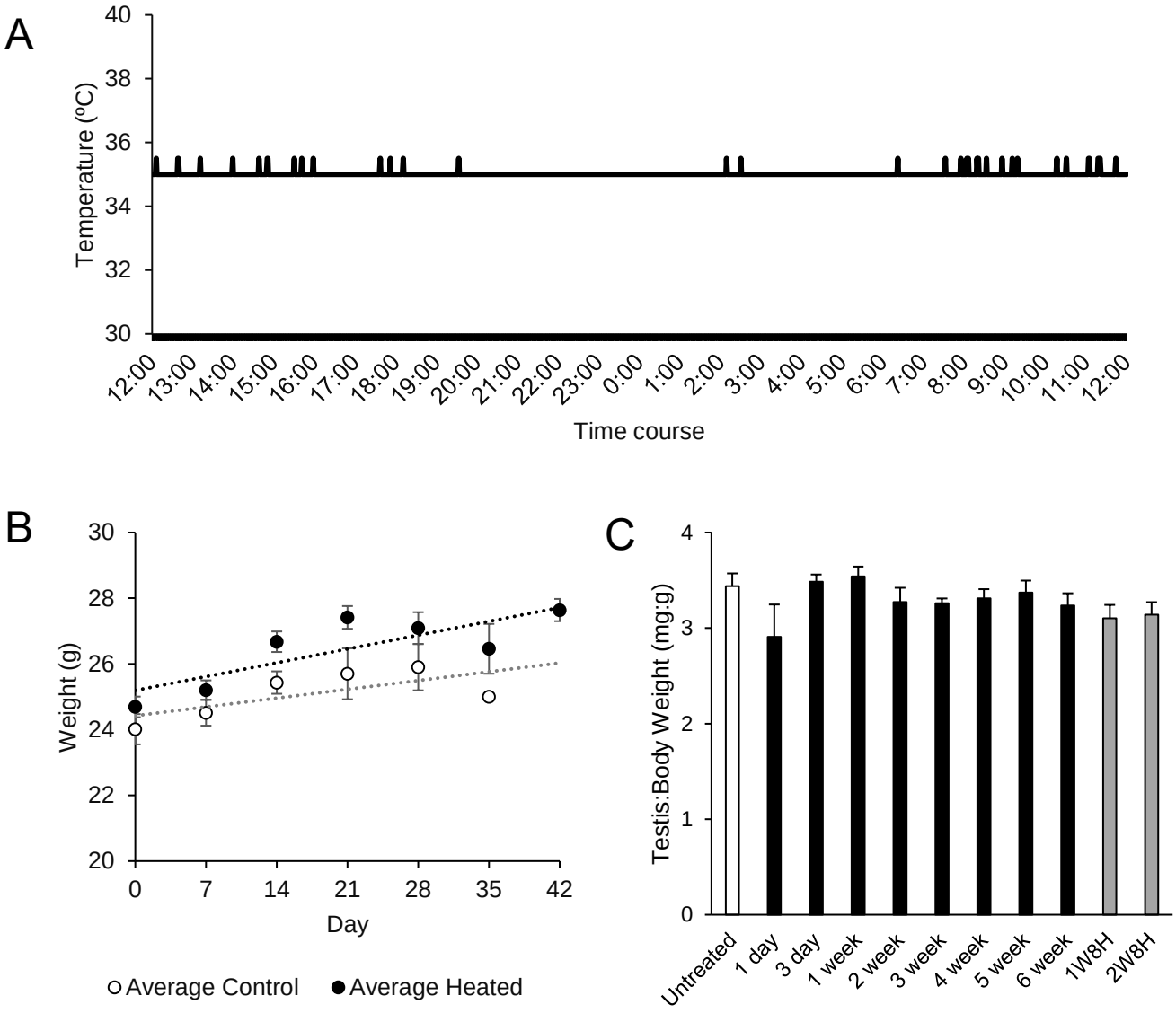
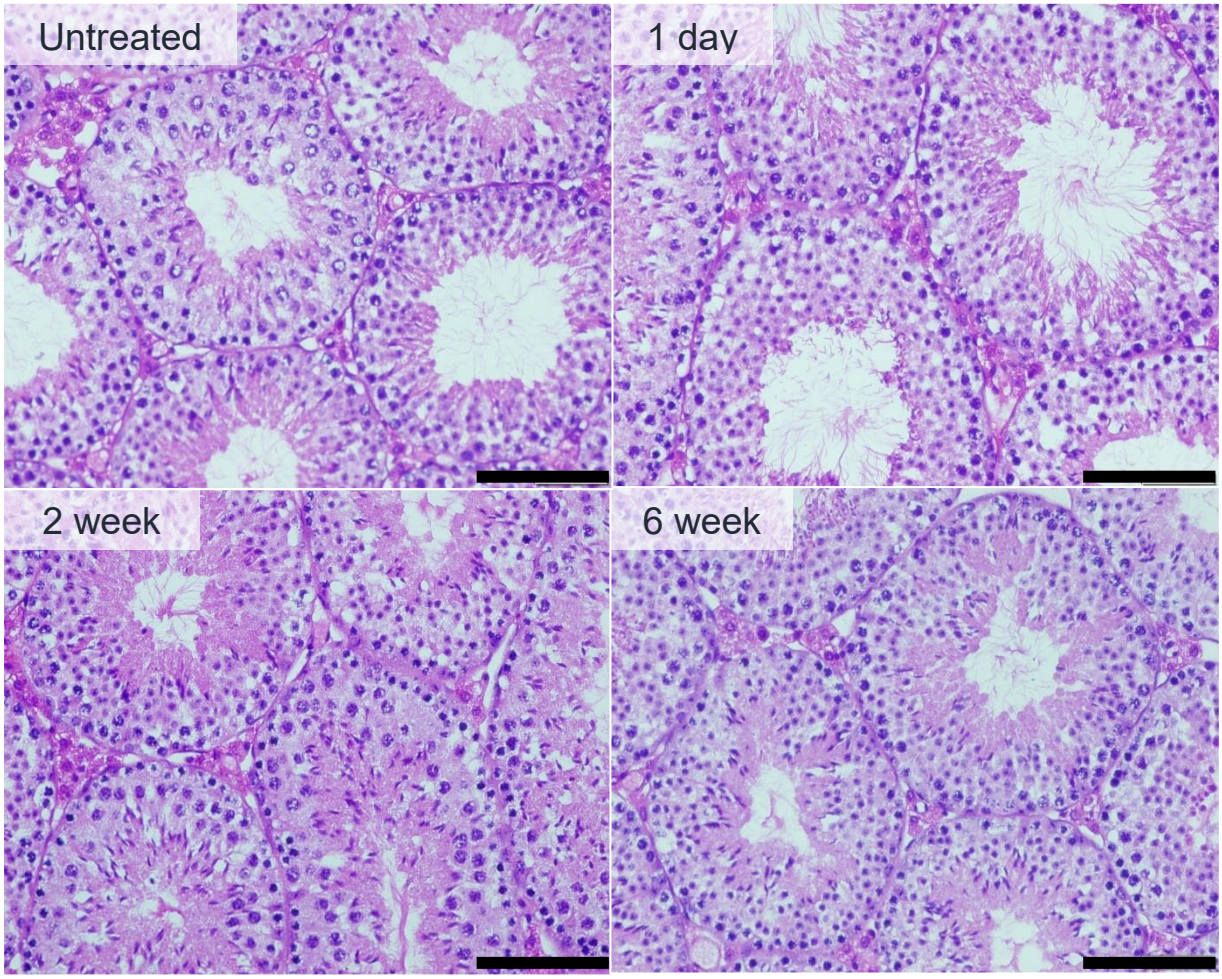


Figure 2. Heating machine stability and growth of mice with heat treatment. A. Temperature reading of heating apparatus over a 24 hour period. **B.** Mice were weighed weekly to determine growth of untreated and acute heat treated populations over the 6 week recovery period. **C.** Testis:body weight of mice from all treatment groups. Panel A n=1, B-C n=5.

A



B

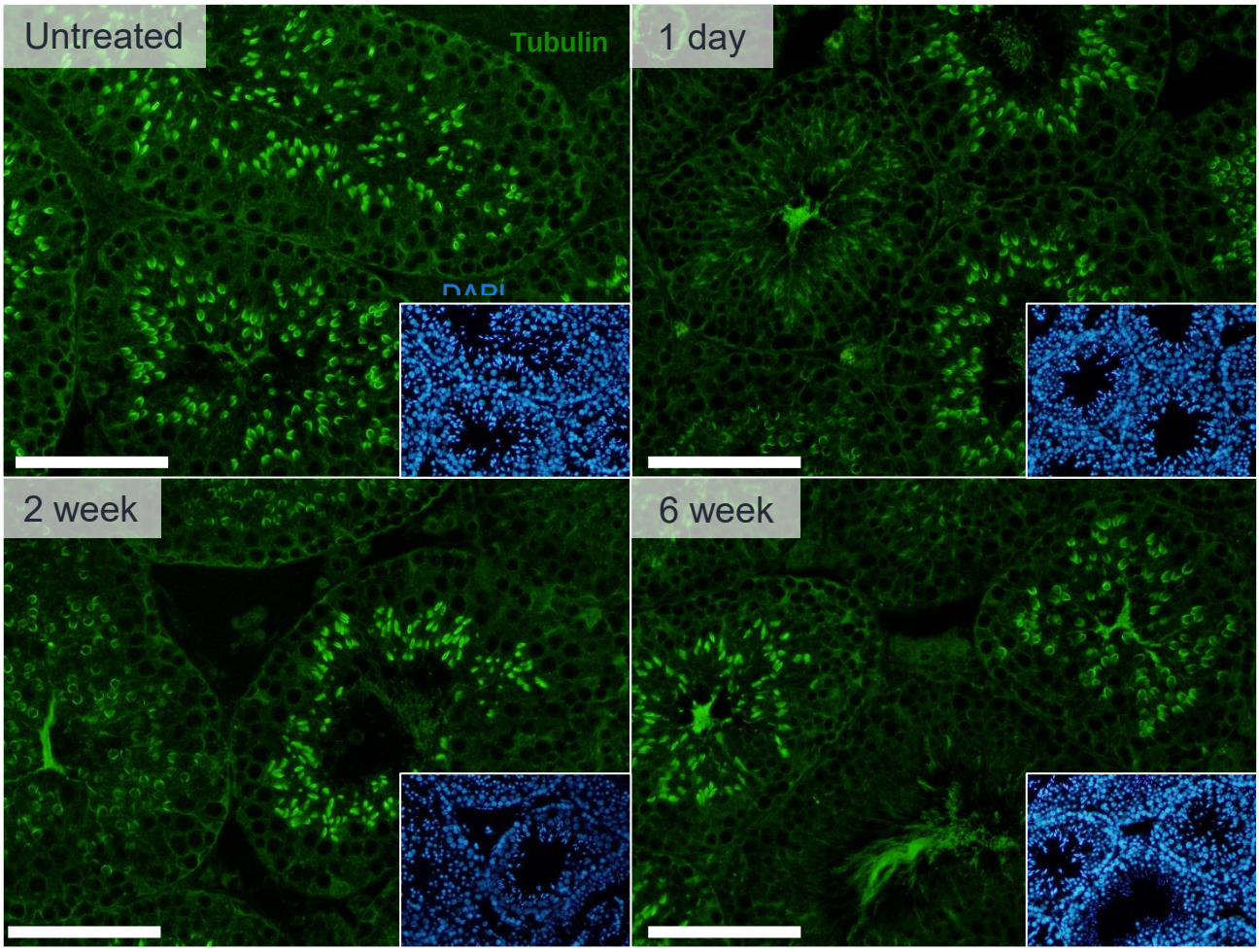


Figure 3. The effect of whole body heating on spermatogenesis and testis structure. Mouse testes from 1 day, 2 week and 6 week heat recovery treatments were fixed and sectioned for staining with a range of stress and structural markers. **A.** Haematoxylin and eosin stained testis sections. **B.** Alpha-tubulin testis staining as a structural marker. Scale bar = 200 µm, n=3

To extend these data, we next investigated whether acute heating was capable of inducing DNA damage to germ cells and spermatozoa residing in the testis of exposed males. First, the testis sections were probed for single strand DNA breakage using an Apop-Tag TUNEL kit (Figure 4). This analysis revealed a significant increase in TUNEL positive germ cells in the testis of heat-treated animals that were allowed 1 day recovery post-treatment ($p < 0.001$), in a similar fashion to that of our positive control ($p < 0.001$). Notably, by 2 and 6 weeks post-heat stress, the bulk of TUNEL positive cells had apparently been resolved such that the intensity of this labelling was indistinguishable from that of untreated controls. Finally, to investigate the induction of oxidative stress in response to heat stress, testis sections were probed with an antibody capable of detecting 8-hydroxy-2'-deoxyguanosine (8-OH-dG), a mutagenic base byproduct arising from oxidative DNA damage (Figure 5). Through quantification of pixel intensity, it was observed that the incidence of this marker in testicular germ cells was modestly elevated 1 day post heating. This marker peaked in expression 2 weeks post heating, to a significant degree ($p < 0.05$), and subsequently returned to basal, untreated, levels with a period of 6 weeks of recovery.

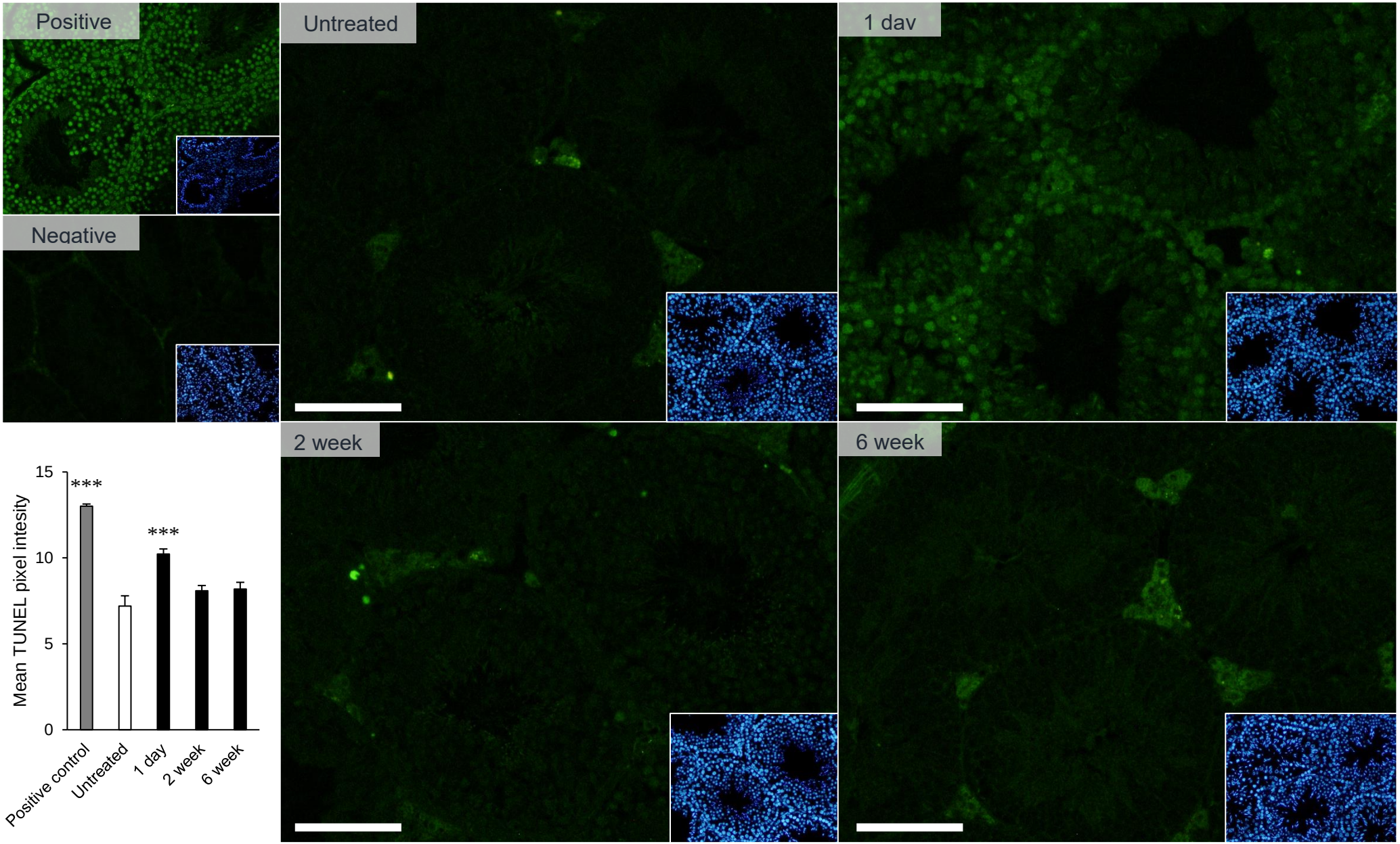


Figure 4. Whole body heating induced testis TUNEL staining as a marker of DNA damage. Mouse testes from 1 day, 2 week and 6 week heat recovery treatments were fixed and sectioned for staining with an ApopTag TUNEL kit. Pixel intensity was used to quantify staining, which is displayed as a graph alongside the images. DNase was used as the positive control. *** $p < 0.001$ compared to untreated control. Staining was performed on three independent replicates (n=3). Scale bar = 200 µm, n=3.

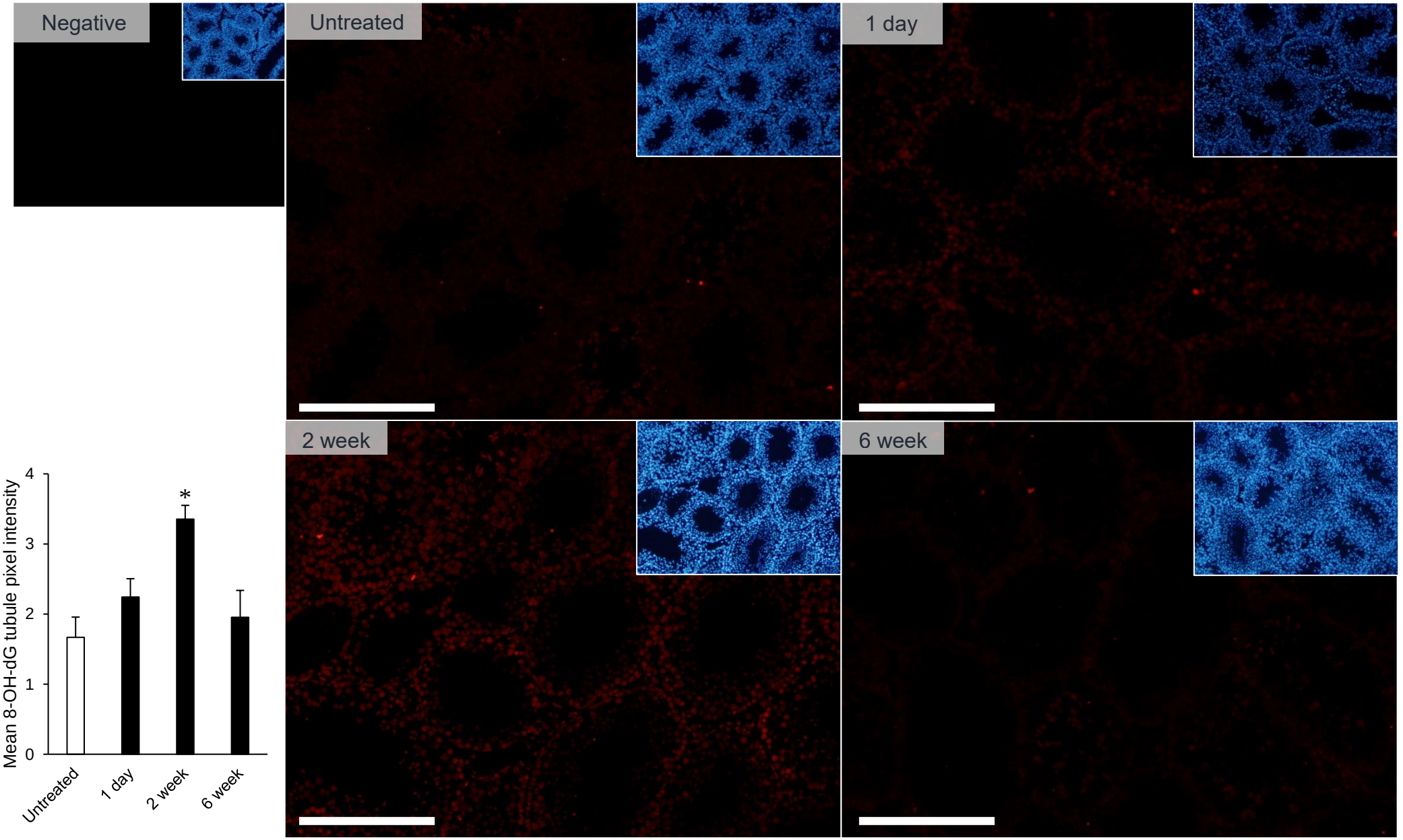
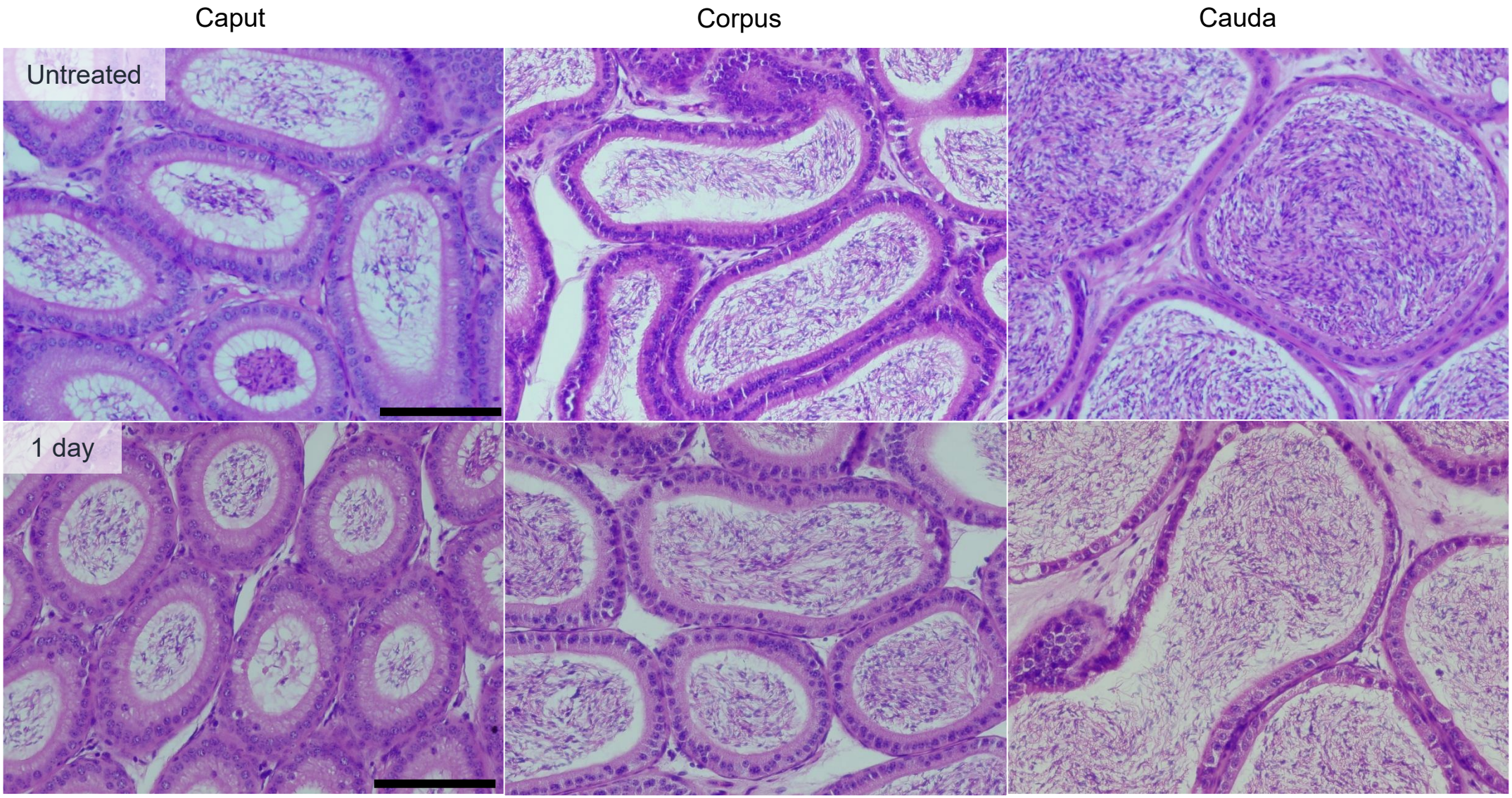


Figure 5. Whole body heating induced oxidative DNA damage in testicular germ cells. Mouse testes from 1 day, 2 week and 6 week heat recovery treatments were fixed and sectioned for staining with an antibody recognizing oxidative damage. The pixel intensity of the germ cells within the tubules was quantified and is displayed alongside images. * $p < 0.05$ compared to untreated control. Staining was performed on three independent replicates (n=3). Scale bar = 200 µm. Staining without the primary antibody was used for the negative control, n=3.

The effects of heating on the epididymal structure and epididymal sperm maturation

To complement the analysis of testicular response to heat stress, we undertook a similar evaluation of the impact of heating on the epididymis (Figure 6), an organ with a pivotal role in regulating the post-testicular maturation of spermatozoa. Consistent with the testes, we observed no overt abnormalities in the gross epididymal morphology following exposure of males to acute heat stress. Indeed, at each recovery timepoint examined, epididymal sections exhibited similar epithelial cell morphology and the lumen of all epididymal sections was replete with spermatozoa (Figure 6). Finally, to investigate oxidative DNA damage levels of the spermatozoa in the epididymis, these sections were incubated with anti-8-OH-dG antibodies and again assessed via mean pixel intensity with respect to caudal spermatozoa (Figure 7). Here, the levels of spermatozoa bearing signatures of oxidatively damaged DNA remained constant from the untreated control to 1 day heat recovery. Following this, the incidence of oxidative damage rose in 2 week recovery spermatozoa and remained elevated in 6 week recovery caudal spermatozoa, but neither achieved statistical significance (p = 0.12, 0.31, respectively).


Caput
Corpus
Cauda
Untreated
1 day

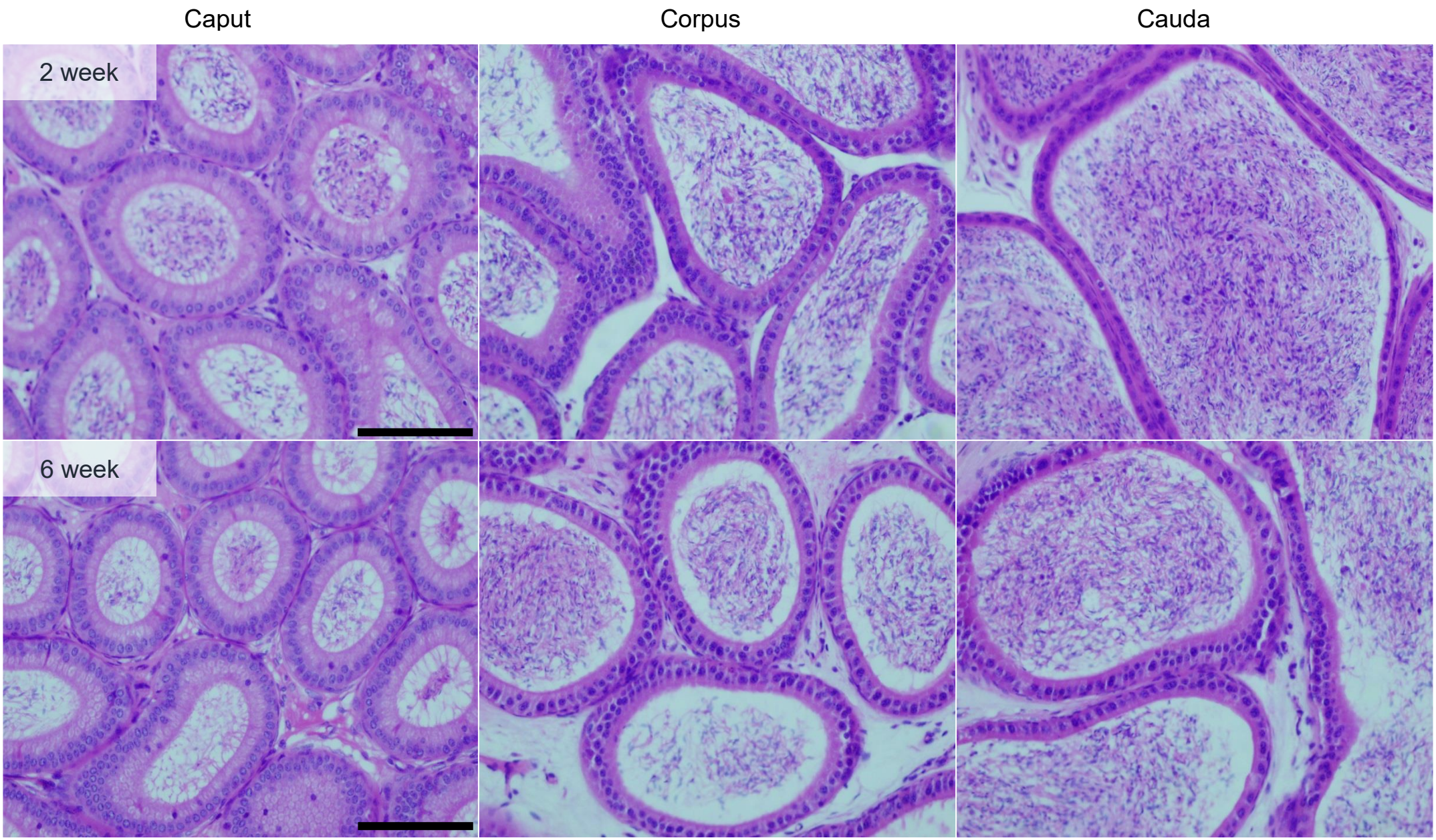


Figure 6. The effects of whole body heating on epididymal structure. Mouse epididymides from 1 day, 2 week and 6 week heat recovery treatments were fixed and sectioned for staining to investigate morphological abnormalities using haematoxylin and eosin staining. Images were taken for the three principal regions of this organ; the caput, corpus and cauda. Scale bar = 100 µm, n=3.

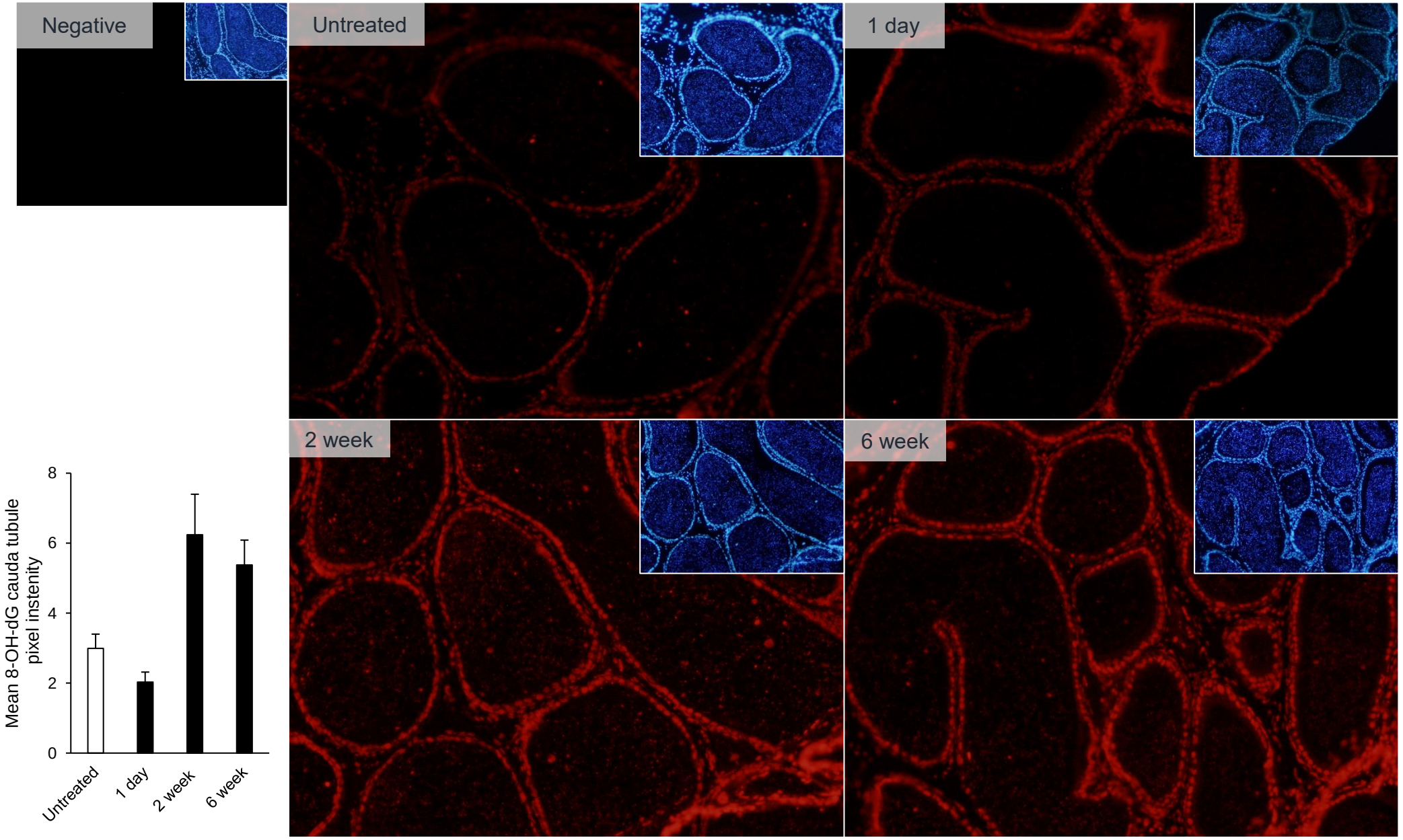


Figure 7. Whole body heating induced oxidative DNA damage in epididymal spermatozoa. Mouse epididymides from 1 day, 2 week and 6 week heat recovery treatments were fixed and sectioned for staining with an antibody recognizing oxidative damage. The pixel intensity of the spermatozoa within the lumen was quantified and is displayed alongside images. Staining was performed on three independent replicates (n=3). Scale bar = 200 µm. Staining without the primary antibody was used for the negative control, n=3.

Heating induced declines in sperm quality

In contrast to the minimal effects acute heat stress on the overall structure of the testes and epididymis, such treatment induced an immediate, highly significant impairment of overall and progressive motility ($p < 0.001$) (Figure 8A and B). The negative impact on these parameters was evident at 1 and 3 days post-treatment, in our acute heat-exposure model. However, this apparent reduction in overall and progressive sperm motility was ameliorated over time, with as little as 1 week recovery resulting in a return of both motility parameters to a level that was not significantly different from the spermatozoa of untreated animals (Figure 8A and B). Furthermore at 2 weeks post-heating, corresponding to the time at which heat exposed round spermatids would be expected to occupy the cauda epididymis, the overall levels of sperm motility were again observed to significantly decline ($p < 0.05$) (Figure 8A). Thereafter (i.e. 3 - 6 weeks post-heating), all sperm motility parameters consistently attained a comparable level to that of the untreated controls.

To confirm these findings and additionally understand the effect of extended heating on sperm motility, we employed our chronic heating model of 8 hours per day for 1 - 2 weeks (grey bars). This chronic exposure model was used to investigate the effects of continuous heat exposure on the epididymal spermatozoa (1 week recovery) and particularly, the maturing spermatids (2 week recovery) as we detected a potential susceptibility of the latter cell population to heat stress in our acute model, above. The chronic heating regimen was also associated with a significant reduction of sperm motility ($p < 0.05$), with both exposure periods (1 and 2 weeks) generating similar results (both 49% vs 70% in untreated controls). When comparing progressive sperm motility (Figure 8B), we again observed a similar decline across both exposure lengths. This was comparable to our acute model, where both 1 and 3 days post-acute heating (black bars) and 1 and 2 weeks of daily heating (grey bars) elicited significant declines in this key motility parameter ($p < 0.001$; $p < 0.05$, respectively). However, when the velocity of the spermatozoa was analysed it was found that their average path velocity (VAP; Figure 8C), straight line velocity (VSL; Figure 8D) and linearity (LIN; Figure 8E)

were all significantly compromised in the chronic exposure model ($p < 0.01$) but not acute, recovery treatment regimes. One final measure of sperm motility, amplitude of lateral head displacement (ALH; Figure 8E), remained constant across all treatment types.

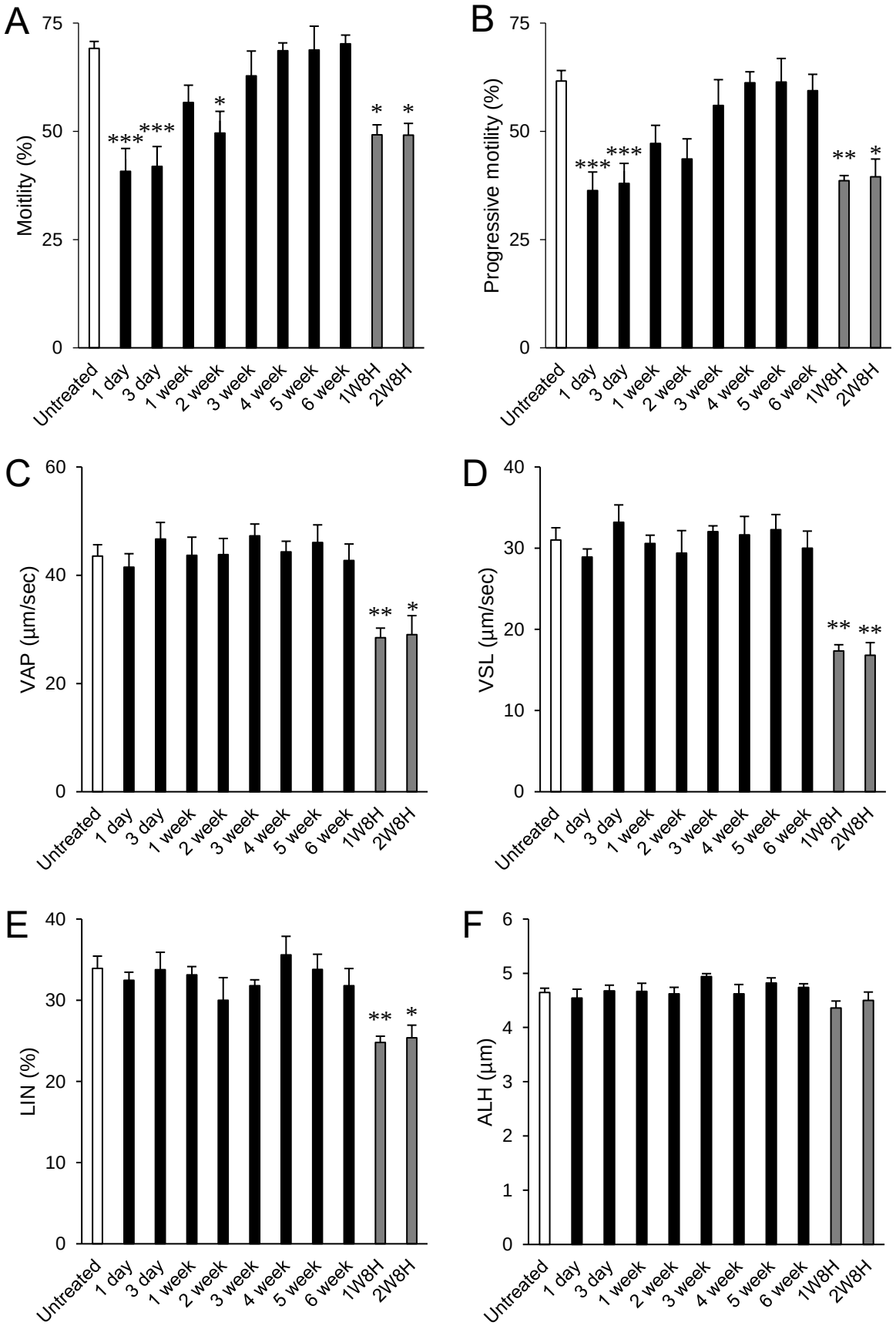


Figure 8. Motility parameters of spermatozoa collected from heat treated mice. Objective sperm motility and velocity was assessed via computer assisted sperm analysis (CASA) on spermatozoa from all treatment groups. **A.** Motility, **B.** Progressive motility, **C.** Average path velocity, **D.** Straight line velocity, **E.** Linearity and **F.** Amplitude of lateral head displacement. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control, n=5.

The involvement of oxidative stress in the heat stress response of spermatozoa

To ascertain the involvement of oxidative stress in accounting for the heat associated decrease in sperm quality, we next investigated the effect of heating on ROS production, lipid membrane structure and oxidative DNA damage (Figure 9). With respect to the number of vital cells collected from heated males (Figure 9A), we observed a heat induced decline, similar to that demonstrated in our sperm motility data (Figure 8A) for our acute, recovery model. A significant decline was observed for this parameter at 1 day ($p < 0.001$), 3 days ($p < 0.01$), and 2 weeks ($p < 0.05$) post-heating, but no such decline was associated with the chronic regimen in which mice were heated daily for 1 – 2 weeks. Investigation of mitochondrial ROS using the MitoSOX red probe (Figure 9B) identified a substantive, 1.5-fold increase in oxidant generation ($p < 0.05$) 3 days post-acute heating. Next we investigated sperm membrane fluidity using merocyanine 540 (M540; Figure 9C) to potentially explain the profile of heat induced modifications to motility. While, a majority of the heat treatments induced a similar response to our untreated control, following 2 weeks recovery from the acute heat insult again generated spermatozoa with a pronounced increase in M540 staining ($p < 0.05$); achieving a similar level of staining to that of our positive control ($p < 0.01$). As a final marker of oxidative stress, we assessed the number of spermatozoa presenting with 8-hydroxy-2'-deoxyguanosine staining (8-OH-dG; Figure 9D). Here, we observed a decline in the presence of this marker in spermatozoa recovered 3 days ($p < 0.05$) and 1 week ($p < 0.01$) post-heating, followed by a substantive increase in the expression of 8-OH-dG following 2 weeks recovery ($p < 0.05$).

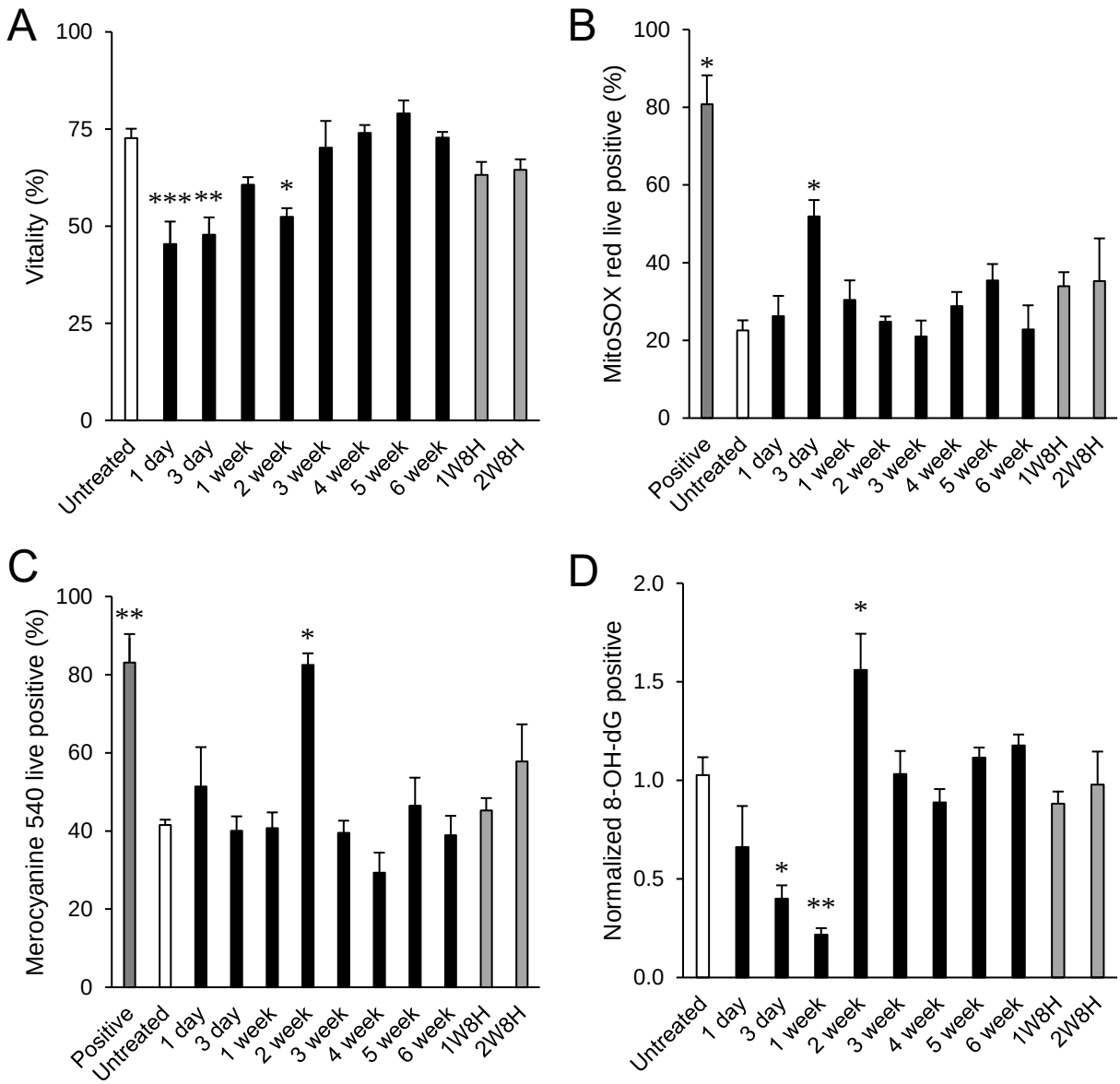


Figure 9. The ability of ambient heat treatment to induce oxidative stress in the spermatozoa of mice. A. Sperm vitality, assessed by the eosin exclusion method. **B.** Mitochondrial ROS generation in spermatozoa, detected with the MitoSOX red probe via flow cytometry. **C.** Membrane fluidity of spermatozoa, measured with the merocyanine 540 probe via flow cytometry. Arachidonic acid was used as a positive control for panels B and C. **D.** Oxidative DNA damage levels determined by 8-hydroxy-2'-deoxyguanosine antibody labelling to the sperm nuclear DNA (n=3). The percentage of positive cells were normalized to the untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated control, n=5

DNA fragmentation in heat-treated germ cells and spermatozoa

Of major importance to sperm function is the integrity of sperm DNA which is known to be vulnerable to oxidative stress. To investigate the existence of such damage, we utilized the alkaline comet assay to quantify the effects of heating on the incidence of sperm DNA damage (Figure 10A). Here, we observed an immediate significant elevation in the occurrence of DNA single strand breaks ($p < 0.001$), 1 day post-acute heat exposure. Additional vulnerable stages encompassed 2 ($p < 0.001$), 3 ($p < 0.01$), 4 ($p < 0.001$) and 5 weeks ($p < 0.001$) post-acute heating recovery, and 2 weeks ($p < 0.001$), but not 1 week, of daily heat exposure. Again, both exposure models suggested that the round spermatid stage of spermatogenesis was particularly vulnerable to the induction of DNA damage, as well as proliferating spermatogonia as proposed with 5 weeks of recovery in the acute model. To further investigate whether the appearance of such damage reflected the differential sensitivity of different stages of spermatogenesis to the effects of heat, we next isolated pachytene spermatocytes and round spermatids from 1 day acute heating recovery testes, using specialized density gradients (Figure 10B). In agreement with the sperm DNA damage data, we observed the DNA of both cell types to be particularly sensitive to heat, exhibiting significant elevations in the levels of DNA fragmentation ($p < 0.001$) when isolated from heat treated mice. Furthermore, with respect to spermatozoa collected from the cauda, these stages of germ cell development correspond to the 2 (round spermatids) and 3 (pachytene spermatocytes) week recovery samples, in the acute setting (Figure 1).

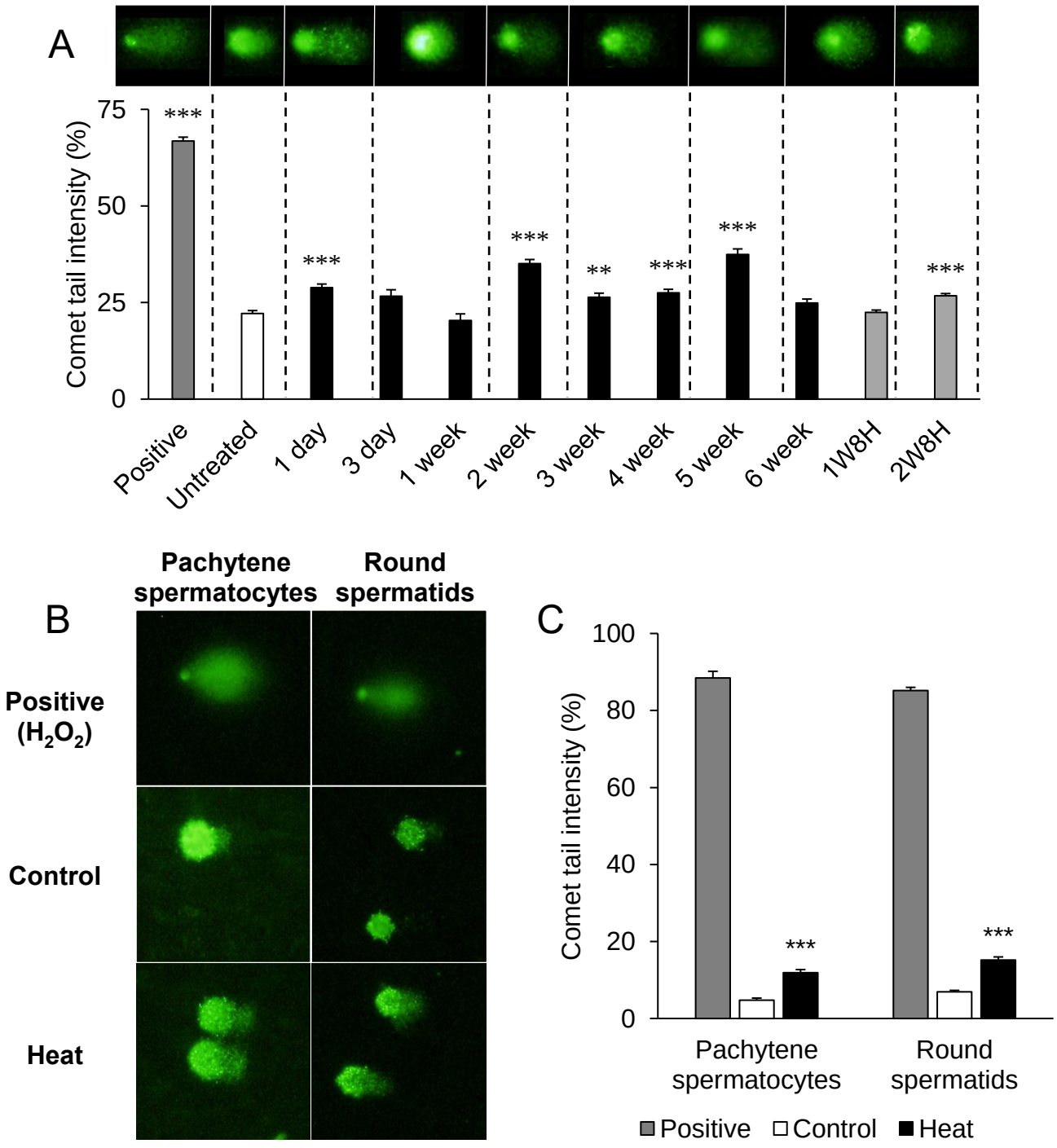
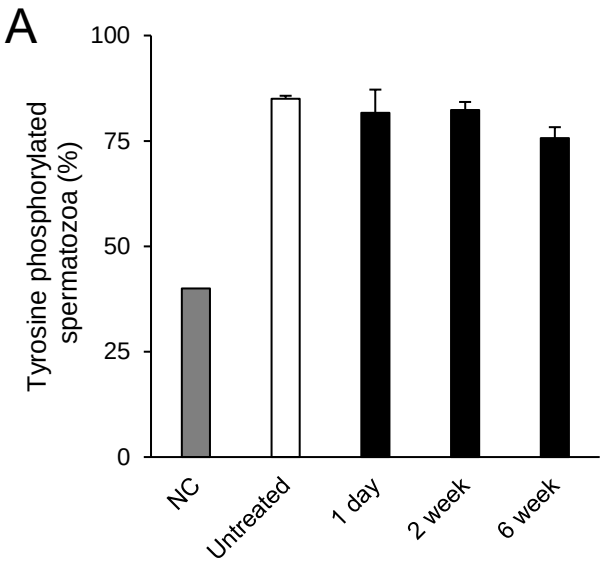
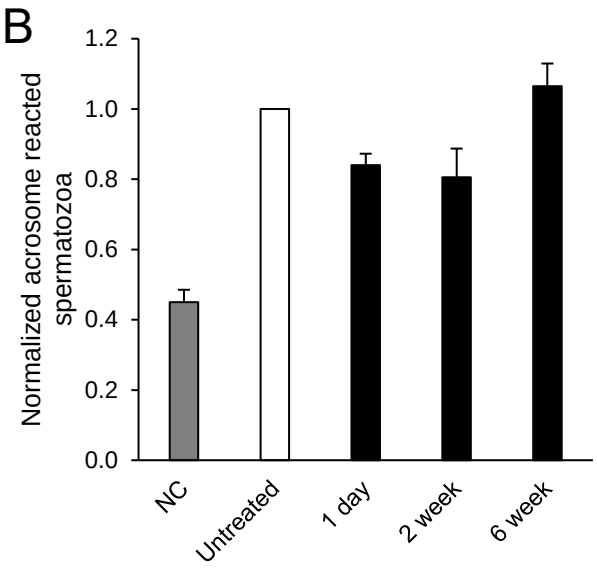


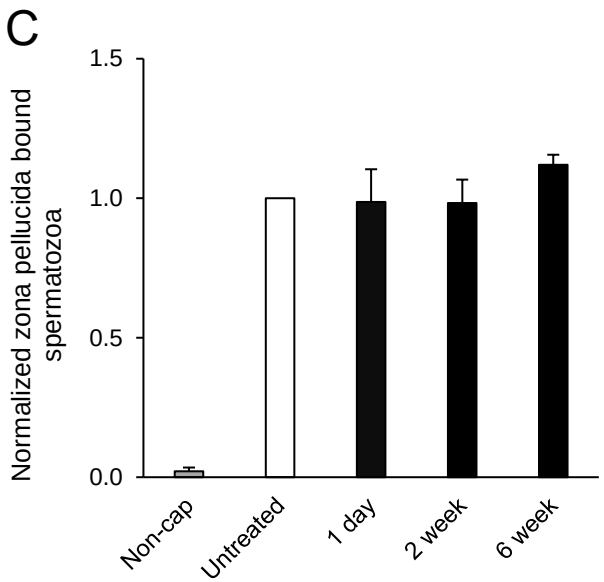
Figure 10. DNA fragmentation in heat treated germ cells and spermatozoa. A. DNA fragmentation in the form of single strand breaks was assessed in the spermatozoa of heat treated mice with the alkaline comet assay. Representative comets are shown above each of the treatments types for both exposure models. Hydrogen peroxide treatment was used as the positive control for this assay. **B.** Images of populations of pachytene spermatocytes and round spermatids isolated from 1 day heat recovery testes that were utilized again for the alkaline comet assay. **C.** Levels of DNA fragmentation, again in the form of single strand breaks, were then quantified in these cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control. Panel A n=5, B-C n=3.

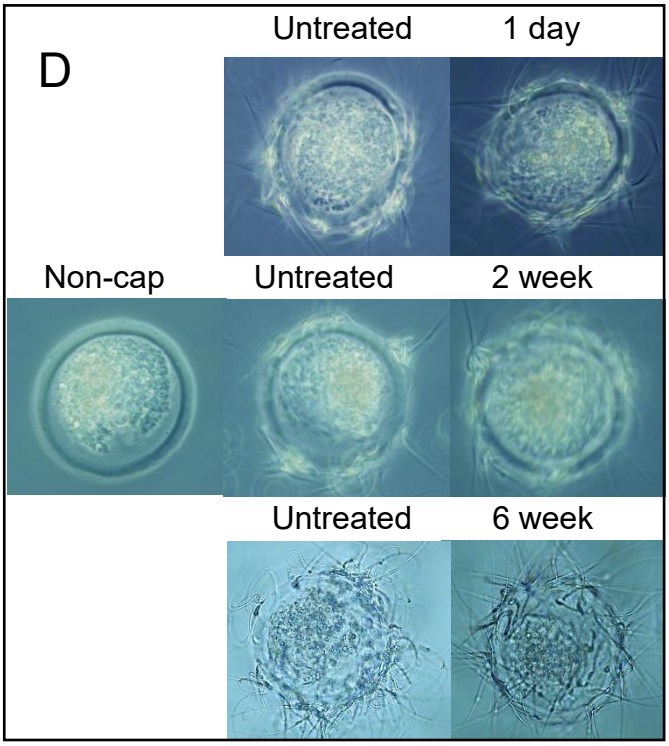
Fertilization capacity of acute heat treated spermatozoa

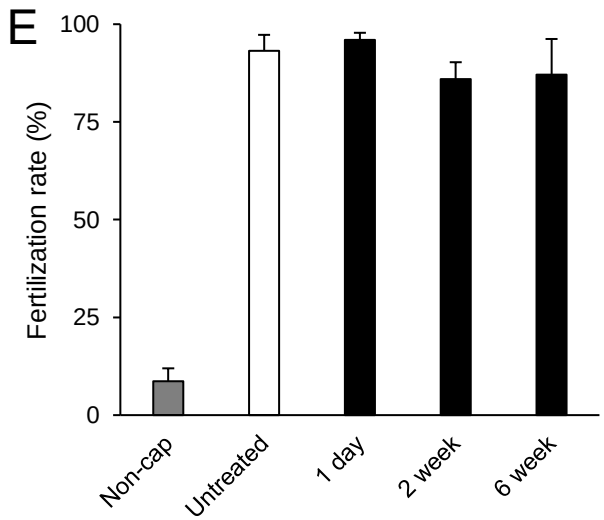
As a final approach at determining the effects of heating on sperm function, we conducted capacitation and fertility assays, focusing on phosphotyrosine expression, acrosome integrity, zona pellucida binding capacity and fertilization rates achieved through conventional IVF (Figure 11). Again, we utilized our 1 day, 2 week and 6 week recovery spermatozoa to investigate the early, middle and late periods of heat recovery in our acute model. Following stimulation of capacitation, we found no difference in the number of spermatozoa exhibiting complete tail tyrosine phosphorylation (Figure 11A) or those which had undergone a spontaneous acrosome reaction (Figure 11B). The ability of these spermatozoa to bind to the zona pellucida of salt stored oocytes (Figure 11C) was also unchanged compared to the untreated sample at all recovery time points, visually represented in Figure 11D. We next used *in vitro* fertilization (IVF) to ascertain how the downstream effects of impaired motility (Figure 8) and elevated DNA damage (Figures 9, 10) impacted the functionality of the heat-exposed spermatozoa. Here, we found that all recovery groups generated spermatozoa capable of fertilizing oocytes at statistically similar rates to untreated spermatozoa (Figure 11D). With regard to the development of these zygotes throughout early embryogenesis (Figure 11E), we detected a decrease in blastocyst formation rate in 2 week heat recovery spermatozoa, but this parameter did not achieve statistical significance (p = 0.25). Embryos generated from spermatozoa collected from 1 day and 6 week recovery mice, also displayed comparable developmental potential to those conceived with spermatozoa from untreated control animals.


A
Tyrosine phosphorylated spermatozoa (%)
100
75
50
25
0
NC
Untreated
1 day
2 week
6 week


B
Normalized acrosome reacted spermatozoa
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NC
Untreated
1 day
2 week
6 week


C
Normalized zona pellucida bound spermatozoa
1.5
1.0
0.5
0.0
Non-cap
Untreated
1 day
2 week
6 week


D
Untreated
1 day
Non-cap
Untreated
2 week
Untreated
6 week


E
Fertilization rate (%)
100
75
50
25
0
Non-cap
Untreated
1 day
2 week
6 week

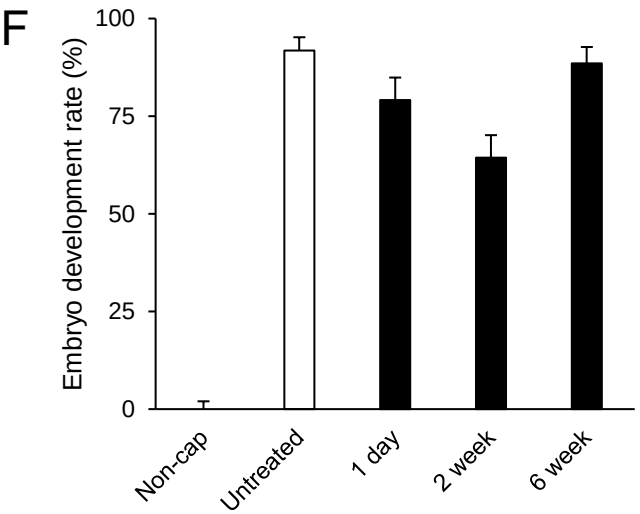

F
Embryo development rate (%)
100
75
50
25
0
Non-cap
Untreated
1 day
2 week
6 week

Figure 11. The capacitation and fertilization capability of spermatozoa collected from whole body heat treated mice. Spermatozoa was collected from heat treated mice at 1 day, 2 weeks and 6 weeks post insult and exposed to pro-capacitation conditions. These groups of spermatozoa were then assessed for their ability to undergo hallmarks of capacitation, achieve binding to the zona pellucida and, when utilized for IVF, fertilize oocytes and achieve embryonic blastocyst development. **A.** Protein tyrosine phosphorylation, a marker of the induction to sperm capacitation. **B.** The incidence of the acrosome reaction, normalized to the untreated control. **C.** The number of spermatozoa observed bound to the zona pellucida of oocytes, normalized to the untreated control. **D.** Representative images of all treatments utilized for the zona binding assay. **E.** Oocyte fertilization rate of these spermatozoa when used for IVF. **F.** Blastocyst development rate of these zygotes generated via IVF. Each experiment was performed three times (n=3), except panels E + F 6 week recovery (n=2).

Discussion

In this study, we have demonstrated that acute and chronic exposure to elevated ambient temperature can have significant negative effects on certain aspects of sperm quality. Further, these data provide evidence to support the existence of an oxidative stress mediated pathology in ambient heat stress. This oxidative stress manifested most significantly in spermatozoa rising from heat stressed round spermatids, but was also clearly established in spermatozoa rising from proliferating spermatogonia, and spermatocytes, but did not adversely affect the spermatogonial stem cell population itself. With respect to sperm function, these impacts did not have overt effects on the fertilizing capacity of these spermatozoa or the resulting embryos throughout early embryonic development to the blastocyst stage.

Historically, studies investigating the effect of heating on spermatogenesis have utilized temperatures in excess of 40ºC, by directly heating the testis or modifying the animal's environment. Outcomes commonly recorded in these studies include a clear impairment of the spermatogenic cycle, abnormal germ cell morphology and loss of germ cells populating the seminiferous tubules within the testis (Chirault *et* al., 2015; Chowdhury and Steinberger, 1970; Liu *et al*, 2011; Li *et al*., 2013a, b). In contrast, the acute heating regimen used in this study whereby mice were continuously exposed to an elevated temperature of 35ºC for 24 hours, failed to elicit equivalent overt changes in the development or morphology of testicular germ cells, or the overall structure of the testis (Figure 3). Such findings nevertheless accord with those of Comish *et al*. (2015), who have provided evidence that pronounced modifications of the male germline are only induced once the testis is subjected to a temperature threshold of at least 36ºC. Despite this, we did detect the presence of elevated levels of oxidative DNA damage in germ cells as well as enhanced TUNEL staining in the testis of mice 1 day post-heating. In agreement with previous studies (Gao *et al*., 2012; Li *et al*., 2011; Wechalekar *et al*., 2016), these data suggest that even relatively mild heat stress can induce DNA fragmentation and/or signal apoptosis within the testis. The existence of sperm oxidative DNA damage was also suggested by a 100% increase in the intensity of 8-OH-dG fluorescence

staining within spermatozoa residing in the cauda epididymal sections of mice 2 weeks after they were initially exposed to heat stress. A similar elevation in oxidative DNA damage in these sections was also documented following 6 weeks of recovery, a result that may be attributed to the retention of damaged spermatozoa in this epididymal segment for multiple weeks (Jones, 2004).

In seeking to assess the ontogeny of male germ cell vulnerability to heat stress we provided evidence that acute heat exposure elicited a negative impact on the motility parameters of those spermatozoa residing in the epididymis at the time of insult. Thus acute heat treatment led to a rapid (1 and 3 day recoveries post-acute heat exposure), significant decline ($p < 0.001$) in the overall percentage of motile cauda spermatozoa, as well as compromising the number of these cells capable of exhibiting progressive motility. Additionally, when germ cells were exposed as round spermatids, the resulting populations of spermatozoa they went on to generate (i.e. at 2 weeks recovery post-heat exposure) also suffered significant impairment of their overall motility ($p < 0.05$). These results mirror those obtained in a previous study involving a single, intense, exposure to heat stress at 42ºC, whereby the spermatozoa matured from heat-shocked round spermatids also exhibited impaired motility characteristics (Perez-Crespo *et al*., 2008). In the case of the rat, acute heat exposure has been shown to elicit a complete loss of sperm motility at 25 days after insult, which continues to be impacted beyond a full, 56-day, cycle of spermatogenesis (Creasy, 1997; Gao *et al*., 2012). This loss of motility then only exhibited modest signs of improvement once the reproductive system was allowed 79 days to recover. Moreover, clinical studies exploring the consequences of direct scrotal heating of the human testis, using a regimen consisting of biweekly exposure over a three month period, have also documented significant negative impacts on sperm motility (Zhang *et al*., 2015). Similar outcomes have been described in the agriculturally important bovine model (Meyerhoeffer *et al*., 1985). In both settings, a decline in motility during heat stress was documented, followed by a gradual recovery at the conclusion of this exposure. Further, this trend was also seen for sperm output

in men (Zhang *et al*., 2015). A common theme emerging from these independent studies, as well as our own, is that given sufficient time for recovery the heat-impairment of sperm motility is eventually repaired. Such reversibility is probably due to the enhanced resistance of the type-A spermatogonia to heat stress (Zhu *et al*., 2004), thus enabling these precursor germ cells to act as a buffering or defence mechanism to mitigate the impact of environmental threats upon spermatogenesis. This phenomenon may be explained by elevated concentrations of Cu/Zn superoxide dismutase present in spermatogonia in comparison to later stage germ cells (Celino *et al*., 2011), in concert with their highly efficient DNA repair activity in comparison to somatic and late stage germ cells, particularly spermatids (Rube *et al*., 2011).

The use of an alternative, chronic heat exposure regimen also led to a loss of sperm motility, irrespective of whether the insult was maintained for 1 or 2 weeks ($p < 0.05$). This finding is entirely commensurate with that of Wechalekar *et al*. (2010; 2016), who reported reductions in sperm motility of between 30-50% following 8 hours of ambient temperature heating for 3 consecutive days. Furthermore, our complete motility analysis identified a susceptibility to chronic heat exposure over 7-14 days. This was highlighted by impairment of sperm progressive motility ($p < 0.01$), velocity ($p < 0.01$) and linearity ($p < 0.01$), with the latter two parameters having previously remained unaffected in acutely exposed mice. During chronic heat exposure, associated heat stress is least damaging when an animal is capable of dissipating thermal energy, generally during the night (Gaughan *et al*, 2008). When individuals are not capable of undergoing this process, the heat stress propagates as an accumulated heat load and incites cellular stress (Hahn and Mader, 1997). This phenomenon is a leading hypothesis to explain chronic thermal cellular stress. Furthermore, our detection of significant motility reductions in both exposure models after 2 weeks, firmly implicates the round spermatid population as being a particularly heat-susceptible phase of germ cell development.

In addition to altered motility, we also detected a DNA damage response in spermatozoa exposed to heat treatment. Indeed, coincident with the sudden decline in sperm motility, we observed a significant increase in DNA fragmentation 1 day post-heat treatment ($p < 0.001$). These data raise the prospect that even in their most mature and conceivably protected form, spermatozoa are vulnerable to heat stress while residing within the epididymis. Similarly, we also identified that spermatozoa collected 2 - 5 weeks post-heating carried an elevated burden of oxidative DNA damage ($p < 0.001$). These 2 and 5 week recovery time points corresponded to the maturation of the heat stressed round spermatids and type B spermatogonia. As highlighted in previous studies of testicular heat stress (Meyerhoeffer *et al*., 1985; Perez-Crespo *et al*., 2008; Zhu *et al*, 2004), we documented a reversion of damage once the spermatogonial stem cells had progressed through a new round of spermatogenesis; which adds further support for the concept that this population of stem cells bear a high resistance to heat stress. By quantifying the levels of DNA damage in 1 day heat recovery round spermatids and pachytene spermatocytes, we confirmed the existence of DNA damage in these cell types prior to, and persisting through, their maturation to spermatozoa ($p < 0.001$).

As oxidative stress has previously been implicated in the heat-induced damage of germ cells (Hansen *et al*., 2009; Paul *et al*., 2009), we investigated markers of this phenomenon in the spermatozoa of our heat-treated mice. While the vitality of the acutely exposed spermatozoa declined with an identical profile to that of sperm motility, this response was absent within the specimens subjected to daily chronic exposure. Accompanying the loss of sperm vitality, we detected a significant elevation in generation of mitochondrial ROS at 3 days post-heating (Figure 9). Aligning with our findings, acute heat stresshas been found to induce mitochondrial ROS generation in chicken liver (Yang *et al*., 2010) and skeletal muscle (Mujahid *et al*., 2009) through mechanisms believed to involve mitochondrial electron transport chain impedance and altered uncoupler protein expression. Furthermore, acute heat exposure in the sea anemone significantly elevates the activity of electron transport chain complexes 1 and 2 (Hawkins and Warner, 2017), and it should be noted that both these sites are capable

of inducing significant ROS production via electron leakage (Quinlan *et al*., 2013; 2014). Collectively, these independent studies reinforce the concept that heat stress alters mitochondrial activity, leading to ROS generation and stimulating a state of oxidative stress. It is likely that this enhanced ROS generation is, at least in part, causally responsible for the heat-induced decline in sperm motility (Aitken *et al*., 2012). An additional signature of oxidative stress in this setting was unveiled in the form of an elevated 8-OH-dG profile in spermatozoa after 2 weeks of recovery. This base adduct forms upon oxidative damage to DNA, persisting as a biomarker of oxidative stress until acted upon by oxoguanine glycosylase 1 (Smith *et al*. 2013).

It is apparent from our data that spermatozoa sampled 2 weeks after heat-stress experience significant cellular changes, including modifications to the plasma membrane, altered motility profiles, and DNA lesions. These spermatozoa correspond to the round spermatid population at the time of heating (Hansen *et al*., 2009) and this particular stage of germ cell development has been previously noted as the most susceptible to heat stress (Perez-Crespo *et al*., 2008; Hansen, 2009). These cells may be particularly sensitive to the propagation of oxidative stress due to their abundance of readily oxidized substrates, including a vast assortment of RNAs (Nixon *et al*., 2015); the open chromatin conformation of these cells as they ready their DNA for extreme condensation (Perez-Crespo *et al*., 2008), and the declining DNA repair activity in these cells as they mature to elongating spermatids (Marchetti *et al*., 2015). The more tightly compacted DNA characteristic of elongating spermatids aids in protecting the later stage of germ cell development from oxidative DNA damage (Aitken and Curry, 2011; Zini and Libman, 2006). However, this process of compaction, in accompaniment with deficient DNA repair activity, also places maturing round spermatids in a vulnerable position by allowing these cells to carry damaged DNA throughout their development into spermatozoa (Leduc *et al*., 2008). These lesions will then be transmitted to the zygote via fertilization creating the potential for aberrant embryonic development leading to miscarriage or birth defects (Aitken *et al*., 2009). However, immediately following fertilization, the zygote

has a vital role to play in repairing the DNA damage brought into the egg by the fertilizing spermatozoon. This is particularly important in light of the elevated 8-OH-dG presence detected in 2 week recovery spermatozoa. This base lesion has been highlighted as remarkably mutagenic, and, in the event it evades zygotic repair, can result in mutations that impact the integrity of the offspring's genome (Lord and Aitken, 2015). In this study, we highlighted a non-significant trend of reduced embryonic development success when utilizing spermatozoa from the 2 week recovery period (round spermatid) for IVF. Studies by Zhu *et al*. (2004) support this finding by documenting that embryo development of *in vitro* fertilized oocytes is highly significantly impaired when utilizing spermatozoa recovered from heat treated mice.

It should also be noted that we detected significant DNA damage in type B spermatogonia (Figure 10), a phenomenon that has also been observed previously (Perez-Crespo *et al*., 2008). Thus, these cells are clearly also susceptible to heat stress in terms of DNA damage, yet the spermatozoa they generate do not display any apparent defects in motility, vitality or membrane fluidity. While the extended developmental lifetime these cells may have been sufficient to enable the excision of 8-OH-dG mutations, they nevertheless presented with significant levels of DNA fragmentation upon maturation to spermatozoa, and therefore, these cells also pose a risk to the transmission of DNA damage to the offspring.

In this study we have extended the analysis of ambient temperature on reproductive competence by documenting the vulnerability of the male germ line to heat stress and demonstrating that acute heat exposure results in significant modifications to germ cell development, impacting the quality of the spermatozoa produced thereafter. This thermal insult stresses a range of male germ cell types, including spermatogonia, spermatocytes, the terminal spermatozoa and most notably, the round spermatids. In spermatozoa that were developmentally exposed to heat at the round spermatid stage, significant increases in membrane fluidity and levels of DNA damage were detected. A differential profile was observed with a chronic exposure, which appears to primarily impair multiple aspects of sperm

motility and velocity, but also elicits sperm DNA fragmentation after 2 weeks of treatment. As with many studies investigating stresses to spermatogenesis, the damage we uncovered in the spermatozoa was ameliorated when the spermatogonial stem cells matured to gametes. Given that this damage was elicited at temperatures that humans and livestock are routinely exposed to, additional research is required to accurately model real-life conditions and assess the impact rising ambient temperature on fertility and the developmental normality of the offspring.

References

Aitken RJ & Curry BJ 2011 Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* **14** 367-381.

Aitken RJ, De Iuliis GN & McLachlan RI 2009 Biological and clinical significance of DNA damage in the male germ line. *Int J Androl* **32** 46-56.

Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS & De Iuliis GN 2012 Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biol Reprod* **87** 110.

Baleato RM, Aitken RJ & Roman SD 2005 Vitamin A regulation of BMP4 expression in the male germ line. *Dev Biol* **286** 78-90.

Carmichael CP 1945 Cryptorchidism. *J Natl Med Assoc* **37** 3-8.

Celino FT, Yamaguchi S, Miura C, Ohta T, Tozawa Y, Iwai T & Miura T 2011 Tolerance of spermatogonia to oxidative stress is due to high levels of Zn and Cu/Zn superoxide dismutase. *PLoS One* **6** e16938.

Chirault M, Lucas C, Goubault M, Chevrier C, Bressac C & Lecureuil C 2015 A combined approach to heat stress effect on male fertility in Nasonia vitripennis: from the physiological consequences on spermatogenesis to the reproductive adjustment of females mated with stressed males. *PLoS One* **10** e0120656.

Comish PB, Liang LY, Yamauchi Y, Weng CC, Shetty G, Naff KA, Ward MA & Meistrich ML 2015 Increasing testicular temperature by exposure to elevated ambient temperatures restores spermatogenesis in adult Utp14b (jsd) mutant (jsd) mice. *Andrology* **3** 376-384.

Creasy DM 1997 Evaluation of testicular toxicity in safety evaluation studies: the appropriate use of spermatogenic staging. *Toxicol Pathol* **25** 119-131.

Dada R, Gupta NP & Kucheria K 2003 Spermatogenic arrest in men with testicular hyperthermia. *Teratog Carcinog Mutagen* **Suppl 1** 235-243.

Gao J, Zuo Y, So KH, Yeung WS, Ng EH & Lee KF 2012 Electroacupuncture enhances spermatogenesis in rats after scrotal heat treatment. *Spermatogenesis* **2** 53-62.

Gaughan JB, Mader TL, Holt SM & Lisle A 2008 A new heat load index for feedlot cattle. *J Anim Sci* **86** 226-234.

Gibbs SD, Sattianayagam PT, Pinney JH, Gilbertson JA, Lachmann HJ, Wechalekar AD, Hawkins PN & Gillmore JD 2010 Renal biopsy is a relatively safe procedure in cases of suspected amyloidosis and a valuable tool in excluding non-AL forms of the disease. *Intern Med J* **40** 167-168.

Gillmore JD, Lachmann HJ, Wechalekar A & Hawkins PN 2010 Hereditary fibrinogen A alpha-chain amyloidosis: clinical phenotype and role of liver transplantation. *Blood* **115** 4313; author reply 4314-4315.

Gallup GG Jr, Finn MM & Sammis B 2009 On the Origin of Descended Scrotal Testicles: The Activation Hypothesis. *Evolutionary Psychology* **7** 517-526.
Grunfeld JP 2010 Systemic diseases: from amyloidosis to cryoglobulinemia. *Clin J Am Soc Nephrol* **5** 1912-1915.
Gyllenborg J, Skakkebaek NE, Nielsen NC, Keiding N & Giwercman A 1999 Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977-1995. *Int J Androl* **22** 28-36.
Hahn, GL & Mader TL 1997. Heat waves in relation to thermoregulation, feeding behavior and mortality of feedlot cattle. 563–571 in *Proc. 5th Int. Livest. Environ. Symp*., Bloomington, MN. Am. Soc. Agric. Eng., St. Joseph, MI.
Hansen PJ 2009 Effects of heat stress on mammalian reproduction. *Philos Trans R Soc Lond B Biol Sci* **364** 3341-3350.
Houston B, Curry B & Aitken RJ 2015 Human spermatozoa possess an IL4I1 l-amino acid oxidase with a potential role in sperm function. *Reproduction* **149** 587-596.
Huang C, Li B, Xu K, Liu D, Hu J, Yang Y, Nie H, Fan L & Zhu W 2017 Decline in semen quality among 30,636 young Chinese men from 2001 to 2015. *Fertil Steril* **107** 83-88 e82.
Jeffcoate TN 1946 Male Infertility. *Br Med J* **2** 185-191.
Jones R 2004 Sperm survival versus degradation in the Mammalian epididymis: a hypothesis. *Biol Reprod* **71** 1405-1411.
Jorgensen N, Andersen AG, Eustache F, Irvine DS, Suominen J, Petersen JH, Andersen AN, Auger J, Cawood EH, Horte A, Jensen TK, Jouannet P, Keiding N, Vierula M, Toppari J & Skakkebaek NE 2001 Regional differences in semen quality in Europe. *Hum Reprod* **16** 1012-1019.
Kastritis E, Wechalekar AD, Dimopoulos MA, Merlini G, Hawkins PN, Perfetti V, Gillmore JD & Palladini G 2010 Bortezomib with or without dexamethasone in primary systemic (light chain) amyloidosis. *J Clin Oncol* **28** 1031-1037.
Katen AL, Chambers CG, Nixon B & Roman SD 2016 Chronic Acrylamide Exposure in Male Mice Results in Elevated DNA Damage in the Germline and Heritable Induction of CYP2E1 in the Testes. *Biol Reprod* **95** 86.
Leduc F, Nkoma GB & Boissonneault G 2008 Spermiogenesis and DNA repair: a possible etiology of human infertility and genetic disorders. *Syst Biol Reprod Med* **54** 3-10.
Levine RJ, Bordson BL, Mathew RM, Brown MH, Stanley JM & Star TB 1988 Deterioration of semen quality during summer in New Orleans. *Fertil Steril* **49** 900-907.
Levine RJ, Mathew RM, Chenault CB, Brown MH, Hurtt ME, Bentley KS, Mohr KL & Working PK 1990 Differences in the quality of semen in outdoor workers during summer and winter. *N Engl J Med* **323** 12-16.
Li L, Han ZY, Li CM, Jiang XQ & Wang GL 2013 Upregulation of heat shock protein 32 in Sertoli cells alleviates the impairments caused by heat shock-induced apoptosis in mouse testis. *Cell Stress Chaperones* **18** 333-351.
Li W, Wu ZQ, Zhao J, Guo SJ, Li Z, Feng X, Ma L, Zhang JS, Liu XP & Zhang YQ 2011 Transient protection from heat-stress induced apoptotic stimulation by metastasis-associated protein 1 in pachytene spermatocytes. *PLoS One* **6** e26013.
Li Y, Huang Y, Piao Y, Nagaoka K, Watanabe G, Taya K & Li C 2013 Protective effects of nuclear factor erythroid 2-related factor 2 on whole body heat stress-induced oxidative damage in the mouse testis. *Reprod Biol Endocrinol* **11** 23.
Lord T & Aitken RJ 2015 Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Dev Biol* **406** 1-13.
Marchetti F, Bishop J, Gingerich J & Wyrobek AJ 2015 Meiotic interstrand DNA damage escapes paternal repair and causes chromosomal aberrations in the zygote by maternal misrepair. *Sci Rep* **5** 7689.
Martin JH, Nixon B, Lord T, Bromfield EG & Aitken RJ 2016 Identification of a key role for permeability glycoprotein in enhancing the cellular defense mechanisms of fertilized oocytes. *Dev Biol* **417** 63-76.
McLean DJ, Russell LD & Griswold MD 2002 Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. *Biol Reprod* **66** 1374-1379.
Meyerhoeffer DC, Wettemann RP, Coleman SW & Wells ME 1985 Reproductive criteria of beef bulls during and after exposure to increased ambient temperature. *J Anim Sci* **60** 352-357.

Mujahid A, Akiba Y & Toyomizu M 2009 Olive oil-supplemented diet alleviates acute heat stress-induced mitochondrial ROS production in chicken skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* **297** R690-698.
Nixon B, Bromfield EG, Dun MD, Redgrove KA, McLaughlin EA & Aitken RJ 2015 The role of the molecular chaperone heat shock protein A2 (HSPA2) in regulating human sperm-egg recognition. *Asian J Androl* **17** 568-573.
Paul C, Teng S & Saunders PT 2009 A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death. *Biol Reprod* **80** 913-919.
Perez-Crespo M, Pintado B & Gutierrez-Adan A 2008 Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev* **75** 40-47.
Pollack HN, Huang S & Shen PY 1998 Climate change record in subsurface temperatures: A global perspective. *Science* **282** 279-281.
Rube CE, Zhang S, Miebach N, Fricke A & Rube C 2011 Protecting the heritable genome: DNA damage response mechanisms in spermatogonial stem cells. *DNA Repair (Amst)* **10** 159-168.
Sattianayagam PT, Gibbs SD, Pinney JH, Wechalekar AD, Lachmann HJ, Whelan CJ, Gilbertson JA, Hawkins PN & Gillmore JD 2010 Solid organ transplantation in AL amyloidosis. *Am J Transplant* **10** 2124-2131.
Setchell BP 1998 The Parkes Lecture. Heat and the testis. *J Reprod Fertil* **114** 179-194.
Shah AD, Watts AJ, Mehta AB & Wechalekar AD 2010 An unusual case of transient dermatological reaction to bortezomib in AL amyloidosis. *Int J Hematol* **91** 121-123.
Smith TB, Dun MD, Smith ND, Curry BJ, Connaughton HS & Aitken RJ 2013 The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *J Cell Sci* **126** 1488-1497.
Virtanen HE, Jorgensen N & Toppari J 2017 Semen quality in the 21st century. *Nat Rev Urol*.
Waites GM 1991 Thermoregulation of the scrotum and testis: studies in animals and significance for man. *Adv Exp Med Biol* **286** 9-17.
Walker K 1928 Diagnosis and Treatment of Sterility in the Male. *Br Med J* **2** 652-655.
Wechalekar H, Setchell BP, Peirce EJ, Ricci M, Leigh C & Breed WG 2010 Whole-body heat exposure induces membrane changes in spermatozoa from the cauda epididymidis of laboratory mice. *Asian J Androl* **12** 591-598.
Wechalekar H, Setchell BP, Pilkington KR, Leigh C, Breed WG & Peirce E 2016 Effects of whole-body heat on male germ cell development and sperm motility in the laboratory mouse. *Reprod Fertil Dev* **28** 545-555.
Wettemann RP & Desjardins C 1979 Testicular function in boars exposed to elevated ambient temperature. *Biol Reprod* **20** 235-241.
Yang L, Tan GY, Fu YQ, Feng JH & Zhang MH 2010 Effects of acute heat stress and subsequent stress removal on function of hepatic mitochondrial respiration, ROS production and lipid peroxidation in broiler chickens. *Comp Biochem Physiol C Toxicol Pharmacol* **151** 204-208.
Zhang MH, Shi ZD, Yu JC, Zhang YP, Wang LG & Qiu Y 2015 Scrotal heat stress causes sperm chromatin damage and cysteinyl aspartate-spicific proteinases 3 changes in fertile men. *J Assist Reprod Genet* **32** 747-755.
Zhu B, Walker SK, Oakey H, Setchell BP & Maddocks S 2004 Effect of paternal heat stress on the development in vitro of preimplantation embryos in the mouse. *Andrologia* **36** 384-394.
Zini A & Libman J 2006 Sperm DNA damage: clinical significance in the era of assisted reproduction. *CMAJ* **175** 495-500.

CHAPTER 5

Final discussion

Chapter 5: Overview

This final chapter consolidates the findings of this thesis and explains our current understanding of how the contemporary issues of RF-EMR exposure and environmental heating affect male fertility. The studies described in thesis have provided considerable direction to the understanding of how RF-EMR may be affecting biology, implicating the mitochondria as a major site of perturbation by this form of stress. Interestingly, similar mechanisms also appear to underpin the impact of heat stress on male reproductive physiology. Collectively, this body of work highlights the susceptibility of the male reproductive tract to physical environmental factors, particularly during spermatogenesis and epididymal maturation, which results in a state of oxidative stress. As such, these results raise the idea that antioxidant interventions may play an important role in treatment of perturbed male fertility to combat the pressures imposed by environmental factors.

5.1 Introduction

Our species has relatively low fertility and over the last two decades a trend of consistently reduced semen quality has presented itself (Huang *et al*., 2017; Virtanen *et al*., 2017). Among the various factors implicated in this phenomenon, environmental exposures are believed to be a major driver (Tahmasbpour *et al*., 2014; Virtanen *et al*., 2017). It has now been well established that male factor infertility is equally as prevalent as that of female factor infertility. Accordingly, more emphasis is now being placed on treatment of male infertility, which currently affects around 5-7% of the population (Aitken *et al*., 2014; Lotti and Maggi, 2015). However, due to the idiopathic nature of a majority of the cases of male infertility, it has proven quite difficult to treat this condition. This is further complicated by the variety of factors that may be contributing to this aetiology (Tahmasbpour *et al*., 2014). Many infertile men are capable of producing semen samples that meet fertility criteria developed by the World Health Organization (World Health Organization, 2010), with respect to sperm counts, morphology and motility, yet are still unable to achieve fertilization (Aitken *et al*., 2014; Kumar and Singh, 2015). This is why a majority of male infertility cases are unable to be treated, thus forcing couples to seek assisted reproductive technologies such as *in vitro* fertilization (IVF) and, more recently, intracytoplasmic sperm injection (ICSI). While these technologies do achieve fertilization and subsequent pregnancies, they also bypass the natural selective pressures of the fertilization process that are in place to ensure the transmission of 'healthy DNA' to the offspring. Only recently have we been able to assess the effects of these technologies on the reproductive fitness of the next generation. On average, male offspring born via ICSI exhibit significantly lower sperm concentration, total sperm count and sperm motility than their naturally conceived counterparts (Belva *et al*., 2016). Furthermore, it has been identified that conception via ART increases the incidence of birth defects (Hansen *et al*., 2005), autism (Fountain *et al.*, 2015) childhood illnesses and required surgeries during childhood (Bonduelle *et al*., 2005). It is likely that the more ARTs are used, the more we will require them in the future (Kurinczuk *et al*., 2003; Belva *et al*., 2016). Therefore, it is essential to address the underlying, multiple, causes of male infertility, and investigate the contribution of contemporary environmental factors to which males are now almost ubiquitously exposed, such as RF-EMR and, in some continents, the consequences of global warming.

We live in a world where we are now constantly exposed to radiofrequency-electromagnetic radiation (RF-EMR) emitted for a variety of devices, in particular mobile phones. As has been detailed previously in this thesis, the precise impact of this form of stress on biological systems remains largely unknown (Houston *et al*., 2016). However, in the past decade many studies have improved our understanding of the biological effects of this radiation to the point where there is now a large body of evidence to suggest that, under certain conditions, RF-EMR is capable of eliciting cellular stress. On the other hand, environmental heating has much more obvious effects on the spermatogenic process, with an underlying explanation that the testis operates at a few degrees below core body temperature, and is therefore sensitive to thermal stress (Hansen, 2009; Waites, 1991). When considering the prospect of rising global temperatures, it is clear that the exposure of humans and many animal species to hotter climates may result in serious repercussions for male fertility. While the effects of heating on spermatogenesis have been well studied, investigations have mainly placed the emphasis on direct testicular exposure (Perez-Crespo *et al*, 2008; Paul *et al*., 2009; Zhang *et al*., 2015; Liu *et al*., 2012). However, many of these effects can be recapitulated in ambient exposure approaches such as in the investigations described in this thesis (Chapter 4).

5.2 RF-EMR and heating induce oxidative stress in the male reproductive system

It has already been established that the effects of RF-EMR on biological systems are controversial, owing to the fact that many studies have failed to uncover the modest cellular changes elicited by this form of radiation. However, as we have reviewed in Chapter 1, these effects can be detected in many cell types, represented by modified branching of brain nerves (Narayanan *et al*., 2015), blood brain barrier degeneration (Salford, 1993), oxidative stress (De Iuliis *et al*., 2009; Hou *et al*., 2014; Kahya *et al*., 2014; Ozguner *et al*., 2005), micronuclei formation (d'Ambrosio *et al*., 2002; Balode, 1996) and DNA fragmentation (De Iuliis et al., 2009; Zalata *et al*., 2015). Furthermore, when regarding the male reproductive system, as many as 78% of former studies have highlighted some form of RF-EMR mediated perturbation within the reproductive tract (Houston *et al*., 2016). This potential susceptibility of the male reproductive tract, and in particular the spermatozoa, to RF-EMR warranted our investigations described in Chapters 2 and 3. In recognition that one of the major controversies in this field of research is how RF-EMR may

impact biological systems to cause damage, a major aim of our studies was to provide novel insight into the mechanism(s) through which RF-EMR could interfere with cell function. Here, we add evidence to the body of literature demonstrating oxidative stress as a key mediator of the damage initiated by RF-EMR exposure. In support of the overarching hypothesis presented in Chapter 1, the mitochondria appear to be a key target of this form of radiation. Accordingly, both isolated immature germ cells (Chapter 2) and mature spermatozoa (Chapter 3; De Iuliis *et al*., 2009) demonstrate elevated levels of mitochondrial ROS generation following exposure to RF-EMR. Moreover, our utilization of selective mitochondrial electron transport chain (ETC) inhibitors, provided evidence that Complex III of the ETC is a likely focus for perturbation by RF-EMR (Chapter 2). This finding accords with the idea that the effects of RF-EMR are subtle, as interfering with mitochondrial respiration at this site results in the generation of ROS in the mitochondrial intermembranous space. This domain is, in turn, supplied with antioxidants that are capable of purging the initial stream of oxygen radicals (Koppers *et al*., 2008). As a consequence, this elevated ROS production must first overwhelm this frontline of defense before widespread effects are expected to be seen throughout the cell.

The mature spermatozoon is quite a unique cell type as it is a transcriptionally and translationally silent cell. Further, on account of its limited source of antioxidant defenses, the mature sperm cell is also quite susceptible to exogenous stresses, which often result in the propagation of oxidative stress through a variety of readily oxidized substrates; foremost among these are the vast supply polyunsaturated fatty acids housed within the sperm membrane, and necessary for supporting functional attributes such as motility. Furthermore, cytotoxic aldehydes generated as a consequence of lipid peroxidation have been shown to suppress sperm motility by binding to dynein heavy chain of the sperm axoneme (Baker, 2015). It is for this reason that sperm motility is compromised in response to oxidative stress, providing a clear and unambiguous readable output to demonstrate functional inhibition of these cells. Accordingly, we documented a reduction in sperm motility in both RF-EMR exposure models (*in vitro*, Chapter 2; *in vivo*, Chapter 3). This, in line with the presence of sperm DNA damage in the form of fragmentation and oxidative base adduct formation, strongly supports the existence of oxidative stress in these cells. Such damage is believed to have originated from the propagation of ROS generated via dysfunction of the mitochondrial ETC, observed in both isolated precursor germ cells (Chapter 2)

and spermatozoa (Chapter 3) exposed to RF-EMR. This result complements independent findings that RF-EMR is capable of stimulating the production of antioxidants in the testis (Al-Damegh, 2012), and inducing the fortification of spermatozoa with these compounds by the testicular environment following *in vivo* whole body exposure (Kesari *et al*., 2011). Furthermore, when rats treated with RF-EMR are supplemented with antioxidant compounds such as vitamins C and E, markers of oxidative stress are ameliorated (Kesari *et al*., 2011). While RF-EMR has been implicated in a decline in semen quality, it has been widely disproven that this form of radiation is capable of eliciting germ cell death that could account for reduced sperm counts (Adams *et al*., 2014). However, this does not discount the reduced viability of sperm collected from the cauda epididymis. This parameter was modestly reduced in spermatozoa from mice treated with RF-EMR immediately upon collection (Chapter 3), but was also achieved after directly exposing spermatozoa to RF-EMR *in vitro* for a period of 4 hours (Chapter 2). Again, this outcome may correlate with the increased susceptibility of the spermatozoa to oxidative insults. This interpretation is supported by the existence of oxidative DNA damage in the exposed spermatozoa (Chapters 2 and 3), but not cultured germ cells (Chapter 2); presumably owing to the heightened antioxidant defense capacity present in the latter cell population.

The fact that a similar outcome was observed using the alternative environmental insult of heat stress, identifies oxidative stress as a common biological consequence of varied forms of external stressors acting on the male reproductive system. A rich literature exists documenting the sensitivity of the male reproductive tract to heat stress, with the majority of these studies utilizing direct scrotal heating methods to mimic heating induced by inguinal clothing (reviewed by Hansen, 2009). As with RF-EMR, heating has been shown to elicit a state of oxidative stress in the male germ line, and this insult can lead to apoptosis of germ cells, resulting in a reduced sperm output (Wechalekar *et al*., 2010). In Chapter 4 of this thesis, we explored the impact of a mild ambient temperature heating model to determine if we could recapitulate these aforementioned effects under a whole body heating regimen. We identified no major structural disruptions in the testis or epididymis under acute heat exposure (Chapter 4). However, we did detect increased expression of TUNEL positive testicular germ cells as soon as 1 day after exposure. This was accompanied by elevated oxidative DNA damage in mature spermatozoa sampled 2 weeks after the imposition of heat stress. Furthermore, both isolated germ cells

(pachytene spermatocytes (PSs) and round spermatids (RSs); Chapter 4) and mature spermatozoa demonstrated elevated DNA fragmentation after acute heat exposure. Such findings support the notion that key stages of sperm cell development differ in their susceptibility to heat stress. In particular, the RS population appears to represent the most sensitive developmental window; an observation that has been attributed to a combination of the cells possessing an open chromatin structure (Perez-Crespo *et al*., 2008) and declining DNA repair capacity (Marchetti *et al*., 2015). The corresponding sperm population arising from RS exposed to acute heat shock exhibited a combination of membrane defects, reduced motility and oxidative DNA damage; responses that appeared to be linked to elevated mitochondrial ROS production in these cells (Chapter 4). While the RS appear to be most susceptible to heat stress, elevated DNA damage also develops in heat treated spermatozoa, as well as spermatozoa matured from type B spermatogonia and spermatocytes subjected to heat stress *in vivo* (Chapter 4; Hansen *et al*., 2009; Perez-Crespo *et al*., 2008). In fact, the only germ cell population that appears to remain resilient to heat stress are those within the spermatogonial stem cell (SSC) niche (Chapter 5; Perez-Crespo *et al*., 2008; Zhang *et al*., 2015). As such, the dysfunctional sperm characteristics observed upon heat treatment are generally recovered once the SSCs have achieved a new round of spermatogenesis; a period of approximately 6 weeks in the mouse and 3 months in the human (accounting for progression of these cells through to the cauda epididymis). Furthermore, this has been modelled in men by heat stressing the testis and then allowing a recovery period (Zhang *et al*., 2015). In this case, the damage elicited by heat exposure is not detectable after the SSCs mature through to spermatozoa at the conclusion of heat stress. This finding is fortunate for men wanting to conceive that are also exposed to elevated environmental or occupational temperatures, as removal of the heat stress should allow for spermatogenesis to continue with the production of healthy spermatozoa.

While our data suggest that spermatozoa produced under heat stress retain the functional competence to achieve fertilization, previous research has identified that resulting embryos suffer premature death, at the blastocyst stage just prior to implantation (Zhu *et al*., 2004). In our study, the burden of damage carried by the fertilizing spermatozoon did not significantly reduce embryonic development to the blastocyst stage (Chapter 5). This may be explained by the higher quality spermatozoa produced under these conditions participating in fertilization, as we did not

detect a complete disruption to all spermatozoa generated during or post-heat treatment. In future studies it will clearly be important to test the genetic integrity of the offspring generated under such circumstances.

Irrespective, it is apparent that heat stress is clearly a problem for the fertility of our species, as it is for alternative mammalian species of agricultural and/or ecological significance. As a case in point, livestock species such as cattle are often raised in warmer climates where they may be particularly vulnerable to thermal stress. Indeed, the attendant rise in global temperatures associated with climate change mean that the effects of heating on fertility is now a very timely issue (Hansen, 2009). The phenomenon of heat load, particularly in cattle, has been identified as an important factor in determining the ability of an animal to cope with heat stress. This heat load takes effect after successive hot days, when the animal is unable to dissipate heat into its environment (Chen *et al*., 2015), particularly during night time. At this time, the consolidated heat stress takes effect and poor reproductive outcomes are observed (De Rensis and Scaramuzzi, 2003; Pegorer *et al*., 2007; Morton *et al*., 2007).

5.3 Limitations and future directions

At this point of time, our species is not only being constantly exposed to RF-EMR from mobile phones, but more so at a range of frequencies, from a host of sources. Firstly, RF-EMR communication on mobile phone devices utilizes a broad range of frequencies (Chapter 1; Houston *et al*., 2016), the most common being the 900 MHz (880-915 MHz uplink and 925-960 MHz downlink) or 1800 MHz (1710-1785 MHz uplink and 1805 MHz-1880 MHz downlink) bands, which is dependent on country. These two major frequencies were investigated in this thesis (Chapters 3 and 2, respectively). While the penetrance of these waves into biological material is increased at lower frequencies (Chapter 1; Houston *et al*., 2016), the effects of RF-EMR seem to be independent of wavelength (which corresponds inversely to frequency). For example, we identified a disruption of mitochondrial function in isolated germ cells exposed *in vitro* at 1800 MHz and also in spermatozoa exposed *in vivo* at 905 MHz. This phenomenon is widely supported by the principle that RF-EMR can induce cellular stress at a range of frequencies as demonstrated in the male reproductive system (reviewed by Houston *et al*., 2016), but also in brain tissue at 50 Hz (Reale *et al*., 2014), 835 Hz (Kim *et al*., 2017) and 1800 MHz (Chen *et al*., 2014).

Secondly, there are other sources of EMR at the radiofrequency level, including the use of wave modulation, Wi-Fi (2400 MHz), as well as the emerging mobile data bands, 3G, 4G and now 5G. Notably, the biological impact of these more recent mobile data bands have yet to be subjected to scientific scrutiny, and are being used under the premise that no considerable clinical outcomes of RF-EMR have been documented. Considering that mobile phone RF-EMR can induce cellular modifications throughout its wide range of frequencies, it is likely that these forms of RF-EMR are also capable of inducing these changes. Some research has been undertaken to investigate the effects of Wi-Fi on biology, which, like classical mobile phone radiation, has been identified to induce oxidative stress in the liver and kidney (Salah *et al*., 2013), brain (Othman *et al*., 2017) and in the embryo, affecting implantation (Shahin *et al*., 2013). Wi-Fi has also been shown to modify cognitive behaviour (Banaceur *et al*., 2013), induce reductions in histological dimensions of the male reproductive tract (Dasdag *et al*., 2015), and may affect heart rate in a subset of the population (Havas and Marrongelle, 2013). However, again, these studies must be considered against others that have unveiled no effects of Wi-Fi, such as brain oxidative stress parameters (Ait-Aissa *et al*., 2013), and those regarding the immune system (Laudisi *et al*., 2012; Sambucci *et al*., 2010, 2011). Finally, a similar story is to be told for the influence of 3G data RF-EMR on biology, which is capable of inducing multiple apoptotic hallmarks in astrocytes (Liu *et al*., 2012), and stimulating the phosphorylation of members of the heat shock and MAP-kinase pathways in brain tissue (Kesari *et al*., 2014), but has no effect on glioblastoma cells *in vitro* (Liu *et al*., 2015). As we are exposed to a cocktail of these radiation forms, it is therefore important to determine how they affect biology in combination, as well as discrete entities. Furthermore, while our studies represent significant progress in characterizing the mechanism of action through which RF-EMR can impact sperm cell physiology, we did not fully address the biological relevance of RF-EMR exposures that play out in a real-world scenario. Addressing this question is only truly relevant once a robust mechanism of action has been accepted, but is clearly the most important route to follow.

Both RF-EMR and environmental heating were capable of inducing significant levels of DNA damage in spermatozoa *in vivo*, which has clear implications for negative clinical outcomes. An interesting future study may investigate the effect of these insults on models of reduced fertility to determine the effects of compounded environmental stresses on the male reproductive system.

It is expected that in models where oxidative stress is in play, that RF-EMR and heating would induce more severe defects to sperm function and spermatogenesis. For example the SAMP8 mouse line, which generates spermatozoa with reduced progressive motility and DNA integrity due to accelerated ageing and poor oxidative DNA repair mechanisms, would likely be highly susceptible to environmental heating and potentially RF-EMR exposure. Meanwhile, it is unknown how RF-EMR and heating would affect sperm quality in models of perturbed fertility arising from genetic or other causes. It is also important to investigate the potential of epigenetic changes in the spermatozoa of these mice. This is clearly an interesting prospect that these insults could control the activation or repression of certain genes during embryonic development. It has been recently suggested that male infertility may be linked to abnormal DNA methylation profiles in spermatozoa (Olszewska *et al*., 2017) and, even more pertinent, that this methylation is influenced by the onset of oxidative stress (Valinluck *et al*, 2004), which further supports the potential for epigenetic changes. Finally, these data lend support to the continued development and application of antioxidant therapies tailored to combat the detrimental impact of environmental factors on the male reproductive system. While this form of therapy is a popular approach to treating idiopathic male infertility, it is important to determine its effect in the context of the insults studied in this thesis. However, it is also imperative that there is cautionary use of these therapies as there needs to be a delicate balance of ROS and antioxidants for normal sperm production and function.

5.4 Final remarks

Through studying the effects of RF-EMR and ambient temperature heating on the male reproductive tract, we have identified important mechanistic features accounting for the pathology of these stresses. While the effects of RF-EMR on biology are still not widely accepted, we have generated evidence to support Complex III of the mitochondrial electron transport chain as a likely target of RF-EMR in isolated male germ cells. Our understanding of this mechanism, which appears to be ROS mediated, will direct future studies, which should focus on multigenerational approaches conducted at real-life exposure levels to gain further understanding of the clinical effects of RF-EMR. However, this will require identifying the involvement of the variety of RF-EMR sources as mentioned above; from mobile phones, Wi-Fi and data origins. With respect to

ambient temperature heating, we unveiled a profile of impaired sperm quality resulting from heat vulnerable germ cell stages. In particular, the round spermatids, but also spermatocytes, proliferating spermatogonia and spermatozoa exhibited susceptibility to this form of stress. Furthermore, the attendant reduction in sperm quality resulting from elevated ambient temperatures was demonstrated in both acute and chronic exposure models. Importantly, both RF-EMR and ambient temperature heating can stimulate a state of oxidative stress in the male reproductive tract, hallmarked by mitochondrial ROS generation, reduced sperm motility and the subsequent formation of oxidative DNA damage and DNA fragmentation. However, the spermatozoa produced under these conditions in this study were readily capable of undergoing capacitation and fertilizing oocytes in an *in vitro* setting. Furthermore, the resulting embryos were not overtly inhibited in their development to the blastocyst stage. Nevertheless, such findings do not discount the possibility that damaged spermatozoa may have been excluded from the fertilization cascade in favour of their undamaged counterparts. Alternatively, damage harboured by fertilizing spermatozoa may have been sufficiently repaired by the DNA repair mechanisms present in the oocyte, thus enabling embryo development to proceed. In any case, it is imperative that the genetic and epigenetic integrity of the resultant embryos are investigated in more detail in future studies. In conclusion, the convergent detrimental outcomes elicited by factors such as RF-EMR and heat on the male reproductive tract, has identified that generating a state of oxidative stress is a key susceptibility of this biological system.

5.5 References

Adams JA, Galloway TS, Mondal D, Esteves SC & Mathews F 2014 Effect of mobile telephones on sperm quality: a systematic review and meta-analysis. *Environ Int* **70** 106-112.

Ait-Aissa S, de Gannes FP, Taxile M, Billaudel B, Hurtier A, Haro E, Ruffie G, Athane A, Veyret B & Lagroye I 2013 In situ expression of heat-shock proteins and 3-nitrotyrosine in brains of young rats exposed to a WiFi signal in utero and in early life. *Radiat Res* **179** 707-716.

Aitken RJ, Smith TB, Jobling MS, Baker MA & De Iuliis GN 2014 Oxidative stress and male reproductive health. *Asian J Androl* **16** 31-38.

Al-Damegh MA 2012 Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E. *Clinics (Sao Paulo)* **67** 785-792.

Baker MA, Weinberg A, Hetherington L, Villaverde AI, Velkov T, Baell J & Gordon CP 2015 Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biol Reprod* **92** 108.

Balode Z 1996 Assessment of radio-frequency electromagnetic radiation by the micronucleus test in bovine peripheral erythrocytes. *Sci Total Environ* **180** 81-85.

Banaceur S, Banasr S, Sakly M & Abdelmelek H 2013 Whole body exposure to 2.4 GHz WIFI signals: effects on cognitive impairment in adult triple transgenic mouse models of Alzheimer's disease (3xTg-AD). *Behav Brain Res* **240** 197-201.

Belva F, Bonduelle M, Roelants M, Michielsen D, Van Steirteghem A, Verheyen G & Tournaye H 2016 Semen quality of young adult ICSI offspring: the first results. *Hum Reprod* **31** 2811-2820.

Chen JM, Schutz KE & Tucker CB 2015 Cooling cows efficiently with sprinklers: Physiological responses to water spray. *J Dairy Sci* **98** 6925-6938.

d'Ambrosio G, Massa R, Scarfi MR & Zeni O 2002 Cytogenetic damage in human lymphocytes following GMSK phase modulated microwave exposure. *Bioelectromagnetics* **23** 7-13.

Dasdag S, Tas M, Akdag MZ & Yegin K 2015 Effect of long-term exposure of 2.4 GHz radiofrequency radiation emitted from Wi-Fi equipment on testes functions. *Electromagn Biol Med* **34** 37-42.

De Iuliis GN, Newey RJ, King BV & Aitken RJ 2009 Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. *PLoS One* **4** e6446.

De Rensis F & Scaramuzzi RJ 2003 Heat stress and seasonal effects on reproduction in the dairy cow--a review. *Theriogenology* **60** 1139-1151.

Fountain C, Zhang Y, Kissin DM, Schieve LA, Jamieson DJ, Rice C & Bearman P 2015 Association between assisted reproductive technology conception and autism in California, 1997-2007. *Am J Public Health* **105** 963-971.

Hansen PJ 2009 Effects of heat stress on mammalian reproduction. *Philos Trans R Soc Lond B Biol Sci* **364** 3341-3350.

Havas M & Marrongelle J 2013 Replication of heart rate variability provocation study with 2.4-GHz cordless phone confirms original findings. *Electromagn Biol Med* **32** 253-266.

Hou Q, Wang M, Wu S, Ma X, An G, Liu H & Xie F 2015 Oxidative changes and apoptosis induced by 1800-MHz electromagnetic radiation in NIH/3T3 cells. *Electromagn Biol Med* **34** 85-92.

Houston BJ, Nixon B, King BV, De Iuliis GN & Aitken RJ 2016 The effects of radiofrequency electromagnetic radiation on sperm function. *Reproduction* **152** R263-R276.

Kahya MC, Naziroglu M & Cig B 2014 Selenium reduces mobile phone (900 MHz)-induced oxidative stress, mitochondrial function, and apoptosis in breast cancer cells. *Biol Trace Elem Res* **160** 285-293.

Kesari KK, Kumar S & Behari J 2011 Effects of radiofrequency electromagnetic wave exposure from cellular phones on the reproductive pattern in male Wistar rats. *Appl Biochem Biotechnol* **164** 546-559.

Kesari KK, Meena R, Nirala J, Kumar J & Verma HN 2014 Effect of 3G cell phone exposure with computer controlled 2-D stepper motor on non-thermal activation of the hsp27/p38MAPK stress pathway in rat brain. *Cell Biochem Biophys* **68** 347-358.

Kim JH, Yu DH, Huh YH, Lee EH, Kim HG & Kim HR 2017 Long-term exposure to 835 MHz RF-EMF induces hyperactivity, autophagy and demyelination in the cortical neurons of mice. *Sci Rep* **7** 41129.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA & Aitken RJ 2008 Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* **93** 3199-3207.

Kumar N & Singh AK 2015 Trends of male factor infertility, an important cause of infertility: A review of literature. *J Hum Reprod Sci* **8** 191-196.

Kurinczuk JJ 2003 Safety issues in assisted reproduction technology. From theory to reality--just what are the data telling us about ICSI offspring health and future fertility and should we be concerned? *Hum Reprod* **18** 925-931.

Laudisi F, Sambucci M, Nasta F, Pinto R, Lodato R, Altavista P, Lovisolo GA, Marino C & Pioli C 2012 Prenatal exposure to radiofrequencies: effects of WiFi signals on thymocyte development and peripheral T cell compartment in an animal model. *Bioelectromagnetics* **33** 652-661.

Liu M, Qi L, Zeng Y, Yang Y, Bi Y, Shi X, Zhu H, Zhou Z & Sha J 2012 Transient scrotal hyperthermia induces lipid droplet accumulation and reveals a different ADFP expression pattern between the testes and liver in mice. *PLoS One* **7** e45694.

Liu YX, Li GQ, Fu XP, Xue JH, Ji SP, Zhang ZW, Zhang Y & Li AM 2015 Exposure to 3G mobile phone signals does not affect the biological features of brain tumor cells. *BMC Public Health* **15** 764.

Liu YX, Tai JL, Li GQ, Zhang ZW, Xue JH, Liu HS, Zhu H, Cheng JD, Liu YL, Li AM & Zhang Y 2012 Exposure to 1950-MHz TD-SCDMA electromagnetic fields affects the apoptosis of astrocytes via caspase-3-dependent pathway. *PLoS One* **7** e42332.

Lotti F & Maggi M 2015 Ultrasound of the male genital tract in relation to male reproductive health. *Hum Reprod Update* **21** 56-83.

Marchetti F, Bishop J, Gingerich J & Wyrobek AJ 2015 Meiotic interstrand DNA damage escapes paternal repair and causes chromosomal aberrations in the zygote by maternal misrepair. *Sci Rep* **5** 7689.

Morton JM, Tranter WP, Mayer DG & Jonsson NN 2007 Effects of environmental heat on conception rates in lactating dairy cows: critical periods of exposure. *J Dairy Sci* **90** 2271-2278.

Narayanan SN, Kumar RS, Karun KM, Nayak SB & Bhat PG 2015 Possible cause for altered spatial cognition of prepubescent rats exposed to chronic radiofrequency electromagnetic radiation. *Metab Brain Dis*.

Olszewska M, Barciszewska MZ, Fraczek M, Huleyuk N, Chernykh VB, Zastavna D, Barciszewski J & Kurpisz M 2017 Global methylation status of sperm DNA in carriers of chromosome structural aberrations. *Asian J Androl* **19** 117-124.

Othman H, Ammari M, Sakly M & Abdelmelek H 2017 Effects of prenatal exposure to WIFI signal (2.45GHz) on postnatal development and behavior in rat: Influence of maternal restraint. *Behav Brain Res* **326** 291-302.

Ozguner F, Altinbas A, Ozaydin M, Dogan A, Vural H, Kisioglu AN, Cesur G & Yildirim NG 2005 Mobile phone-induced myocardial oxidative stress: protection by a novel antioxidant agent caffeic acid phenethyl ester. *Toxicol Ind Health* **21** 223-230.

Paul C, Teng S & Saunders PT 2009 A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death. *Biol Reprod* **80** 913-919.

Pegorer MF, Vasconcelos JL, Trinca LA, Hansen PJ & Barros CM 2007 Influence of sire and sire breed (Gyr versus Holstein) on establishment of pregnancy and embryonic loss in lactating Holstein cows during summer heat stress. *Theriogenology* **67** 692-697.

Perez-Crespo M, Pintado B & Gutierrez-Adan A 2008 Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev* **75** 40-47.

Reale M, Kamal MA, Patruno A, Costantini E, D'Angelo C, Pesce M & Greig NH 2014 Neuronal cellular responses to extremely low frequency electromagnetic field exposure: implications regarding oxidative stress and neurodegeneration. *PLoS One* **9** e104973.

Salah MB, Abdelmelek H & Abderraba M 2013 Effects of olive leave extract on metabolic disorders and oxidative stress induced by 2.45 GHz WIFI signals. *Environ Toxicol Pharmacol* **36** 826-834.

Salford LG, Brun A, Sturesson K, Eberhardt JL & Persson BR 1994 Permeability of the blood-brain barrier induced by 915 MHz electromagnetic radiation, continuous wave and modulated at 8, 16, 50, and 200 Hz. *Microsc Res Tech* **27** 535-542.

Sambucci M, Laudisi F, Nasta F, Pinto R, Lodato R, Lopresto V, Altavista P, Marino C & Pioli C 2011 Early life exposure to 2.45GHz WiFi-like signals: effects on development and maturation of the immune system. *Prog Biophys Mol Biol* **107** 393-398.

Tahmasbpour E, Balasubramanian D & Agarwal A 2014 A multi-faceted approach to understanding male infertility: gene mutations, molecular defects and assisted reproductive techniques (ART). *J Assist Reprod Genet* **31** 1115-1137.

Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A & Sowers LC 2004 Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). *Nucleic Acids Res* **32** 4100-4108.

Virtanen HE, Jorgensen N & Toppari J 2017 Semen quality in the 21st century. *Nat Rev Urol* **14** 120-130.

Waites GM 1991 Thermoregulation of the scrotum and testis: studies in animals and significance for man. *Adv Exp Med Biol* **286** 9-17.

Wechalekar H, Setchell BP, Peirce EJ, Ricci M, Leigh C & Breed WG 2010 Whole-body heat exposure induces membrane changes in spermatozoa from the cauda epididymidis of laboratory mice. *Asian J Androl* **12** 591-598.

World Health Organization 2010 In *WHO Laboratory Manual for the Examination and Processing of Human Semen* 7–114. *Fifth Edition*. Geneva: World Health Organization.

Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y & Mostafa T 2015 In vitro effect of cell phone radiation on motility, DNA fragmentation and clusterin gene expression in human sperm. *Int J Fertil Steril* **9** 129-136.

Zhang MH, Shi ZD, Yu JC, Zhang YP, Wang LG & Qiu Y 2015 Scrotal heat stress causes sperm chromatin damage and cysteinyl aspartate-spicific proteinases 3 changes in fertile men. *J Assist Reprod Genet* **32** 747-755.

Zhu B, Walker SK, Oakey H, Setchell BP & Maddocks S 2004 Effect of paternal heat stress on the development in vitro of preimplantation embryos in the mouse. *Andrologia* **36** 384-394.