Consequences for the bovine embryo of being derived from a spermatozoon subjected to oxidative stress

KEM Hendricks and PJ Hansen*

Objective To determine whether oxidative damage of ejaculated frozen–thawed sperm prior to oocyte insemination in vitro affects the competence of the resultant embryo to develop to the blastocyst stage.

Method Extended frozen semen from bulls was thawed, subjected to Percoll gradient purification to obtain motile spermatozoa and mixed with medium containing the pro-oxidants menadione or tert-butyl hydroperoxide. After 3 h at 38.5°C, the sperm were washed and used to inseminate oocytes in vitro. Embryo development proceeded until 8 days after insemination.

Results Treatment of sperm with 15 or 30 μmol/L menadione reduced the proportions of oocytes that cleaved and those that developed to the blastocyst stage; 30 μmol/L menadione reduced the proportion of cleaved embryos that developed to the blastocyst stage at day 8 after insemination. Oocytes inseminated with sperm treated with 150 or 300 μmol/L tert-butyl hydroperoxide had lower proportions of cleavage and blastocyst development, and the proportion of cleaved embryos becoming blastocysts was also reduced.

Conclusion Oxidative damage to ejaculated sperm can compromise the ability of the sperm to cause oocyte cleavage and leads to formation of embryos with reduced competence for development.

Keywords cattle; embryo; menadione; reactive oxygen species; sperm; tert-butyl hydroperoxide

Cattle embryos derived from different sires can have disparate abilities to develop in vitro or survive after transfer to recipients. The sperm contribution to the embryo includes the paternal DNA, plasma membrane, centriole and RNA molecules. Damage to these components could theoretically compromise the developing embryo. In humans, sperm DNA damage, in particular, has been associated with failure of spermatozoal pronuclear decondensation, reduction in embryo developmental potential and reduced implantation rates.

The degree to which damage to sperm after ejaculation actually reduces embryo competence is unclear, but there is some evidence that molecules that increase sperm oxidative stress can reduce embryo competence. In one study, the proportion of cleaved bovine embryos that developed to the blastocyst stage was reduced for embryos treated with hydrogen peroxide. In the current study, we tested the hypothesis that oxidative stress of ejaculated bull sperm after freeze–thawing would damage sperm, leading to the formation of embryos with reduced developmental competence. The effects of two pro-oxidant chemicals were examined: menadione and tert-butyl hydroperoxide. Menadione is a vitamin K2 precursor that generates superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂) and other reactive oxygen species in its conversion from quinine to semiquinone and back to quinine in the presence of molecular oxygen. In boar sperm, menadione increases reactive oxygen species production, reduces motility, depresses mitochondrial transmembrane potential and reduces ATP content. Tert-butyl hydroperoxide is an organic peroxide that reportedly decreased sperm count and motility and reduced litter size following intraperitoneal injection in male mice, and it also causes mid-piece lipid peroxidation in fresh and frozen–thawed stallion sperm.

Materials and methods

Culture media for in vitro production of embryos were prepared as described elsewhere. Menadione (2-methyl-1,4-naphthoquinine) was purchased from Sigma (St Louis, MO, USA) and dissolved in absolute ethanol to make a 10 mmol/L stock solution. Fresh stock solutions of menadione were made every 2 weeks. Tert-butyl hydroperoxide solution was purchased from Sigma and diluted to 300 and 600 μmol/L (0 % (v/v) ethanol) in a sperm medium called Sp-TALP on the day of use. Hoechst 33342 was from Calbiochem (San Diego, CA, USA). All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated.

Sperm preparation

Extended frozen semen from bulls was obtained from Select Sires Inc. (Plain City, OH, USA), Southeast Semen (Wellborn, FL, USA) and ABS Global (DeForest, WI, USA). Semen was thawed, subjected to Percoll gradient purification to obtain motile spermatozoa, diluted in Sp-TALP to 40 x 10⁶ spermatozoa/mL and mixed 1:1 with Sp-TALP containing the treatment chemicals prior to incubation at 38.5°C in air for 3 h using a water bath. At the end of the incubation period, treated sperm were washed and resuspended in Sp-TALP.

Sperm motility

The percent of sperm exhibiting motility after the incubation period was assessed by visual examination. Briefly, 20 μL of sperm suspension were placed on a glass slide pre-warmed at 37°C and examined under ×200 magnification. Motility was estimated for 100 spermatozoa in 10 different fields.

In vitro production of embryos

Embryo production was performed as previously described. Briefly, cumulus–oocyte complexes were obtained by slicing 2- to 10-mm
follcles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from a local abattoir. Cumulus–oocyte complexes containing at least one layer of compact cumulus cells were selected for maturation and fertilisation. They were washed twice in oocyte collection medium and placed in groups of 10 in 50-µL drops of oocyte maturation medium overlaid with mineral oil and matured for 20 to 22 h at 38.5°C and 5% CO₂ in humidified air. Matured oocytes were then washed once and transferred in groups of 30 or 60 to 4-well plates containing 425 µL of fertilisation medium and fertilised with 30 µL (6 × 10⁶) Percoll-purified spermatozoa from a single bull. After 8 to 9 h of co-incubation at 38.5°C and 5% CO₂ in humidified air, putative zygotes were removed from fertilisation wells and denuded of cumulus cells by vortexing in 100 µL of 1000 U/mL hyaluronidase. Denuded putative zygotes were cultured in groups of 25 to 35 in 50-µL drops of a culture medium called KSOM-BE215 overlaid with mineral oil at 38.5°C in a humidified atmosphere of 5% CO₂, 5% O₂ and the balance of nitrogen. Cleavage was assessed on day 3 after insemination and the presence of blastocysts was determined on days 7 and 8 after insemination.

**Experiments**

Effects of menadione and tert-buty]l hydroperoxide were evaluated in two experiments. The concentrations of pro-oxidants used were based on preliminary experiments to determine effective concentrations. In the first experiment, oocytes were fertilised with sperm that had been incubated in control medium (0.3% (v/v) ethanol in Sp-TALP) or 15 µmol/L or 30 µmol/L menadione. The experiment was replicated four times with a separate bull for each replicate and with 278 to 347 oocytes/group. In the second experiment, oocytes were fertilised with sperm that had been incubated with control medium (Sp-TALP), or 150 µmol/L or 300 µmol/L tert-buty]l hydroperoxide. The experiment was replicated five times with a different bull for each replicate and with 266 to 464 oocytes/group.

The developmental competence of embryos formed from menadione-treated sperm was assessed by measuring the percentage of cleaved embryos becoming blastocysts.

**Statistical analysis**

For each replicate, the percentages of oocytes that cleaved, oocytes that became blastocysts and cleaved oocytes (i.e. embryos) that became blastocysts were calculated for all embryos within the same treatment. Thus, the group of embryos treated alike within each replicate was the experimental unit. Data for these embryo traits and for sperm motility were subjected to least-squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS for Windows, Release 9.0, SAS Institute, Inc., Cary, NC, USA). Data were analysed without transformation and again after arcsin transformation to correct for any non-normality associated with percentage data. All main effects and interactions were included in the mathematical models. Replicate was considered random and other main effects were considered fixed. Hence, treatment × replicate was the error term for treatment. The CONTRAST statement of SAS was used to compare treatments (tert-buty]l hydroperoxide and menadione) against their respective controls. Data reported for P values are from the analysis of transformed data, while least-squares means are from the analysis of non-transformed data.

**Results**

**Menadione**

Incubation of sperm with menadione caused a concentration-dependent decrease in both sperm motility (P < 0.05) and the proportion of oocytes that underwent cleavage after insemination (P < 0.001) (Table 1). At 15 µmol/L, there was no significant reduction in the proportion of cleaved embryos that became blastocysts, although values were numerically lower than for oocytes inseminated with control sperm. At 30 µmol/L, the percent of cleaved embryos that became blastocysts was lower (P < 0.05) than for controls; none of the cleaved embryos derived from sperm treated with 30 µmol/L menadione became a blastocyst.

**Tert-buty]l hydroperoxide**

Treatment of sperm with tert-buty]l hydroperoxide had significant deleterious effects on motility, oocyte cleavage and development (Table 2). Treatment with 300 µmol/L tert-buty]l hydroperoxide decreased both sperm motility (P < 0.01) and cleavage of oocytes (P < 0.05) following insemination. The lower concentration (150 µmol/L) tended to reduce motility (P < 0.10), but did not affect the percentage of oocytes that cleaved after insemination. The proportion of oocytes that developed into blastocysts by day 7 or 8 after

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**Table 1. Effect of treatment of sperm with menadione (0, 15 and 30 µmol/L) on sperm motility, embryo cleavage and blastocyst formation following insemination of matured oocytes**

<table>
<thead>
<tr>
<th>Menadione (µmol/L)</th>
<th>n*</th>
<th>Motility (%)</th>
<th>Cleavage (%)</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Blastocysts/oocyte (%)</td>
<td>Blastocysts/cleaved (%)</td>
<td>Blastocysts/oocyte (%)</td>
</tr>
<tr>
<td>0</td>
<td>4 (301)</td>
<td>53.8 ± 5.9</td>
<td>77.4 ± 3.1</td>
<td>26.0 ± 1.9</td>
<td>34.2 ± 8.4</td>
</tr>
<tr>
<td>15</td>
<td>4 (347)</td>
<td>32.5 ± 5.9*</td>
<td>7.4 ± 3.1***</td>
<td>2.6 ± 1.9***</td>
<td>22.8 ± 8.4</td>
</tr>
<tr>
<td>30</td>
<td>4 (285)</td>
<td>5.8 ± 5.9**</td>
<td>3.9 ± 3.1***</td>
<td>0.0 ± 1.9***</td>
<td>0.0 ± 8.4*</td>
</tr>
</tbody>
</table>

Data are least-squares means ± SEM.

Means that differ from 0 µmol/L are indicated by *P < 0.05, **P < 0.01, ***P < 0.001.

*Number of replicates (total number of embryos evaluated per treatment).
insemination was less for oocytes inseminated with sperm treated with 150 or 300 μmol/L tert-butyl hydroperoxide (P < 0.05) than for oocytes inseminated with sperm incubated with control medium. The proportion of cleaved embryos that were blastocysts at day 7 or 8 after insemination was less for oocytes inseminated with sperm treated with 150 μmol/L (P < 0.01 at day 7 and P < 0.05 at day 8) or 300 μmol/L tert-butyl hydroperoxide (P < 0.01) compared with sperm incubated with control medium.

### Discussion

Exposure of ejaculated frozen–thawed sperm to the pro-oxidants used in this study significantly reduced the cleavage rate. Such an effect, which is most likely the result of damage to the fertilising ability of the sperm, as indicated here by decreased motility, is not surprising because similar effects have been seen for other molecules causing oxidative stress, such as hypoxanthine-xanthine oxidase and hydrogen peroxide. Menadione reduces sperm motility, mitochondrial membrane potential and ATP content, whereas tert-butyl hydroperoxide causes mid-piece lipid peroxidation in fresh and frozen–thawed stallion sperm. The present results also demonstrate that high concentrations of oxidative stress to ejaculated sperm can cause the formation of embryos with reduced competence for developing to the blastocyst stage. Thus, damage to the sperm not only reduces fertilising ability, but also causes changes in the resultant embryo that reduce embryonic function.

One potential cause of the reduced developmental competence of embryos derived from sperm exposed to oxidative stress is damage to one or more of the components of the sperm cell that are incorporated in the embryo. Alternatively, the reduction in motility and fertilising ability caused by pro-oxidants could lead to ageing of the oocyte (because it takes longer to be fertilised) and reduced developmental competence. That ageing can compromise oocytes is indicated by the observation that inseminated bovine oocytes that matured for 28 or 34 h are less likely to produce cleaved embryos that develop to the blastocyst stage than inseminated oocytes aged for 22 h.

In conclusion, the observation that oxidative stress can lead to a reduction in both the cleavage rate and embryo competence demonstrates that changes in sperm function can effect the embryo formed from such sperm. These effects could be physiologically relevant, because sperm may encounter oxidative stress in the female and male reproductive tracts or during processing.

### Table 2. Effect of treatment of sperm with tert-butyl hydroperoxide (TBHP: 0, 150 and 300 μmol/L) on sperm motility, embryo cleavage and blastocyst formation following insemination of matured oocytes

<table>
<thead>
<tr>
<th>TBHP (μmol/L)</th>
<th>n*</th>
<th>Motility</th>
<th>Cleavage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Blastocysts/oocyte (%)</td>
</tr>
<tr>
<td>0</td>
<td>5 (323)</td>
<td>61.0 ± 3.3</td>
<td>66.6 ± 5.3</td>
</tr>
<tr>
<td>150</td>
<td>5 (464)</td>
<td>52.0 ± 3.3†</td>
<td>60.6 ± 5.3</td>
</tr>
<tr>
<td>300</td>
<td>5 (315)</td>
<td>41.0 ± 3.3**</td>
<td>47.4 ± 5.3*</td>
</tr>
</tbody>
</table>

Data are least-squares means ± SEM. Means that differ from 0 μmol/L are indicated by †P < 0.05, **P < 0.01, ***P < 0.001.

*Number of replicates (total number of embryos evaluated per treatment).

### Acknowledgments

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### References


This is the ‘first scholarly book in Australasia devoted entirely to the legal relationship between humans and non-human animals’. Its editors are academic lawyers, 12 of the 15 authors are lawyers and the non-lawyers include a politician (Andrew Bartlett) and a political scientist. So why review it in a veterinary journal? Why should veterinarians be interested in a book of essays exploring animal welfare law?

Many veterinarians sit on animal ethics committees, animal welfare committees, or other working groups or committees where they endeavour to interpret animal welfare law or seek to shape animal welfare law and regulation, or they are employed by government, universities and other institutions that are bound by such laws. Even practitioners will confront aspects of welfare law, such as abused animals or neglected livestock. And if we compare the standard of care of animals in Australia with that in other countries, we think our legislation stands up well.

This book sets out to challenge the notion that animal welfare law actually protects animals. The authors mostly endorse an ‘animal rights’ viewpoint and highlight that, with the possible exception of companion animals, there are so many ‘exemptions’ in existing animal welfare legislation it makes a mockery of the term. As members of a profession that respects and values the benefits of enhanced animal health, welfare and production it behoves veterinarians to understand the ethical and philosophical basis of this viewpoint. Where do we, as individuals, stand on the continuum from a Kantian position (humans have no direct duties to animals) to that of Regan (and later) thinkers, who interpret the rights of animals as barring all human exploitation and affording animals equal rights with humans?

I don’t want to give the impression that this book is a diatribe against animal use or those with a more ‘traditional’ perspective on animal welfare. It is not. The authors discuss farm animals, companion animals, scientific experimentation, live export, hunting and animals in the wild in a logical and reasoned way. There are useful chapters on current Australian and New Zealand animal welfare legislation, animal cruelty sentencing, European and International legislation, and how our Federal politicians regard animal welfare.

Steven White (Griffith Law School, Brisbane) provides an overview of the various ethical perspectives influencing animal welfare over the past few centuries and relates these to legislation both current and (by some) desired. This chapter is a primer in animal ethics for undergraduate courses and is most useful for veterinarians who graduated without the benefit any teaching of ethics or welfare at university.

The chapter on companion animals in law by Leslie Anne Petrie (Flinders University, SA) acknowledges the importance and value of companion animals to individuals and to communities, but only considers the effect of animal welfare law on the animals. It ignores state-based legislation that controls the keeping of companion animals, and the effects on pets and their owners of poor residential and town planning.

Anrja Dale (Applied Animal Behaviour and Welfare, Unitec, Auckland) provides a valuable chapter on Codes of Practice, which should be required reading for all who are involved in the development of the new, national, enforceable Standards and Guidelines (which will progressively replace the Australian Model Codes and are designed to be incorporated into State and Territory legislation).

Animal law in Australasia is (mostly) easy to read for someone not trained in law. It may just equip the veterinary profession to hold its own as the animal welfare legislation changes in Australia in the years to come.

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