

Insulin-like growth factor-1 protects preimplantation embryos from anti-developmental actions of menadione

James I. Moss · Eduardo Pontes · Peter James Hansen

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Abstract Menadione is a naphthoquinone used as a vitamin K source in animal feed that can generate reactive oxygen species (ROS) and cause apoptosis. Here, we examined whether menadione reduces development of preimplantation bovine embryos in a ROS-dependent process and tested the hypothesis that actions of menadione would be reduced by insulin-like growth factor-1 (IGF-1). Menadione caused a concentration-dependent decrease in the proportion of embryos that became blastocysts. All concentrations tested (1, 2.5, and 5.0 μM) inhibited development. Treatment with 100 ng/ml IGF-1 reduced the magnitude of the anti-developmental effects of the two lowest menadione concentrations. Menadione also caused a concentration-dependent increase in the percent of cells positive for the TUNEL reaction. The response was lower for IGF-1-treated embryos. The effects of menadione were mediated by ROS because (1) the anti-developmental effect of menadione was blocked by the antioxidants dithiothreitol and Trolox and (2) menadione caused an increase in ROS generation. Treatment with IGF-1 did not reduce ROS formation in menadione-treated embryos. In conclusion, concentrations of menadione as low as 1.0 μM can compromise development of bovine preimplantation embryos to the blastocyst stage of development in a ROS-dependent mechanism. Anti-developmental actions of

menadione can be blocked by IGF-1 through effects downstream of ROS generation.

Keywords Preimplantation embryo · Menadione · Reactive oxygen species · Insulin-like growth factor-1 · Apoptosis

Introduction

Menadione (2-methylnaphthalene-1,4-dione or vitamin K₃) is a naphthoquinone used as a vitamin K source in animal feed and was once studied as an anticarcinogen (Lamson and Plaza 2003). In the presence of molecular oxygen, menadione generates superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and other reactive oxygen species (ROS) (Monks et al. 1992). One result of menadione exposure is apoptosis (Sata et al. 1997; Criddle et al. 2006; Wochna et al. 2007). Given actions of free radicals on spermatozoa and preimplantation embryos (Guérin et al. 2001; Sikka 2004), menadione exposure could possibly lead to fertilization failure or embryonic death. Consequences of exposure to menadione for the developing embryo are not known but treatment of boar sperm with menadione reduced motility, mitochondrial transmembrane potential and ATP content (Guthrie et al. 2008).

Here, we examined whether menadione reduces development of preimplantation bovine embryos. In addition, we tested the hypothesis that actions of menadione would be reduced by insulin-like growth factor-1 (IGF-1). This multifunctional growth factor acts as a survival factor in the preimplantation embryo to reduce the anti-developmental effects of several stresses including radiation (Spanos et al. 2000), tumor necrosis factor- α (Byrne et al. 2002), hydrogen peroxide (Kurzawa et al. 2004), and heat

J. I. Moss · E. Pontes · P. J. Hansen (✉)
Department of Animal Sciences, University of Florida,
PO Box 110910, Gainesville, FL 32611-0910, USA
e-mail: Hansen@animal.ufl.edu

E. Pontes
Faculdade de Medicina Veterinária e Zootecnia,
Universidade de São Paulo, São Paulo, Brazil

shock (Jousan and Hansen 2004, 2007). One rationale for testing effects of IGF-1 was to evaluate the possible use of IGF-1 as a therapeutic strategy for mitigating infertility due to menadione exposure. The molecular and biochemical basis for the cytoprotective effect of IGF-1 in the preimplantation embryo is largely unknown. Here, it was tested whether cytoprotective actions of IGF-1 involve reduction in ROS production or apoptosis.

Materials and methods

Materials

Materials for production of HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP were as described previously (Soto et al. 2003; Jousan and Hansen 2007). Oocyte collection medium (OCM) consisted of Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (HyClone, Logan, UT) supplemented with 2% (v/v) bovine steer serum containing 2 U/ml heparin (Pel-Freez, Rogers, AR), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) consisted of TCM-199 (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/ml estradiol 17-β, 20 µg/ml bovine follicle stimulating hormone (Folltropin-V; Bioniche Life Sciences Inc., London, ON, Canada), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, and 1 mM glutamine. KSOM-BE2 was prepared as described by Soto et al. (2003). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL). Percoll was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Menadione was purchased from Sigma-Aldrich (St. Louis, MO) and was maintained in 10 mM stocks dissolved in ethanol, stored at 4°C in the dark and used within 2 weeks. A modified recombinant human insulin-like growth factor-1 (Arg³-IGF-1) was purchased from Sigma-Aldrich or Upstate Biotechnology (Lake Placid, NY) and prepared in acetic acid as described by Block et al. (2003). Dithiothreitol and Trolox were from Sigma-Aldrich. The In Situ Cell Death Detection Kit (TMR red) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). SYTOX[®] Green nucleic acid stain was purchased from Invitrogen (Carlsbad, CA). Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY) and RQ1 RNase-free DNase was from Promega (Madison, WI). All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

Embryo production

Procedures for production of embryos *in vitro* were performed as previously described (Soto et al. 2003) except

where mentioned. Briefly, cumulus oocyte complexes (COCs) were obtained by slashing 2–10-mm follicles on the surface of ovaries obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with at least one complete layer of compact cumulus cells were washed three times in OCM. Groups of 10 COCs were placed in 50-µl drops of OMM overlaid with mineral oil and matured for 20–22 h at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air. Matured COCs were then washed once in HEPES-TALP and transferred in groups of 50 to 4-well plates containing 450 µl of IVF-TALP and 20 µl of PHE [0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 mM epinephrine in 0.9% (w/v) NaCl] per well. These were fertilized with 30 µl Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls that was diluted to give a final concentration of 1 × 10⁶ sperm/ml. After 20–22 h at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1,000 U/ml hyaluronidase in HEPES-TALP, and placed in groups of 30 in 50-µl drops of KSOM-BE2 overlaid with mineral oil. Embryos were cultured at 38.5°C in a humidified atmosphere of 5% (v/v) CO₂ and 5% (v/v) O₂ with the balance N₂ until treatment.

Interactions between menadione and IGF-1 affecting developmental capacity of embryos

Embryos ≥16 cells were harvested from culture drops at Day 5 after insemination and randomly placed in groups of 7–15 in wells of a 24-well polystyrene plate (Nalge Nunc International, Rochester, NY). For each replicate, the number of embryos per well was similar for all treatments. Each well, which was not covered with mineral oil to avoid movement of menadione into the oil, contained 150 µl of either control medium [KSOM-BE2 containing vehicle for IGF-1 (40 µM acetic acid)] or KSOM-BE2 containing 100 ng/ml IGF-1. After 1 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO₂ and 5% (v/v) O₂ with the balance N₂, menadione freshly diluted in KSOM-BE2 or KSOM-BE2 containing IGF-1 was added in a volume of 150 µl to achieve a final concentration of 0 [vehicle control; KSOM-BE2 containing 40 µM acetic acid and 0.10% (v/v) ethanol], 1.0, 2.5, or 5.0 µM menadione. Embryos were returned to the incubator for 24 h. Embryos were then removed from the treatment dishes and rinsed serially in 50 µl microdrops of KSOM-BE2 and then placed in 50 µl microdrops of KSOM-BE2 overlaid with mineral oil. Embryos were returned to the incubator and development to the blastocyst stage was assessed on Day 8 after insemination. The experiment was replicated 14 times using 120–204 embryos per treatment.

Induction of apoptosis by menadione as influenced by IGF-1

Embryos ≥ 16 cells were harvested from culture drops at Day 6 after insemination and placed in 24-well plates of KSOM-BE2 containing vehicle or 100 ng/ml IGF-1. After 1 h of incubation, menadione was added to a final concentration of 0, 1, 2.5, or 5.0 μM and embryos were incubated for 24 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2 and 5% (v/v) O_2 with the balance N_2 . Embryos were then fixed in 4% (w/v) paraformaldehyde and analyzed for apoptosis using the TUNEL assay as previously described (Jousan and Hansen 2007) using TMR red conjugated to dUTP and SYTOX[®] Green. Each embryo was analyzed for total cell number (green nuclei) and TUNEL positive blastomeres (red nuclei) with FITC or rhodamine filters, respectively, using a 40 \times objective. Digital images were acquired using AxioVision software and a high-resolution black and white Zeiss AxioCam MRm digital camera. Total cell number and the percent of blastomeres that were TUNEL positive were determined for each embryo. The experiment was replicated 5 times using 21–33 embryos per treatment.

Protection of embryos from anti-developmental effects of menadione by antioxidants

Embryos ≥ 16 cells were harvested from culture drops at Day 6 after insemination and placed in 24-well plates in groups of 5–12. Wells contained either vehicle (KSOM-BE2 + 40 μM acetic acid), 100 ng/ml IGF-1, 100 μM dithiothreitol, or 100 μM Trolox (a cell-permeable, water-soluble derivative of vitamin E). After 1 h at 38.5°C, an additional 150 μl of KSOM-BE2 contained the same treatment as previously (vehicle, IGF-1, dithiothreitol, or Trolox) and either vehicle or menadione to yield a final concentration of 5 μM . Embryos were returned to the incubator for 24 h, removed from treatment dishes, rinsed serially in KSOM-BE2 and then placed in fresh 50- μl drops of KSOM-BE2 overlaid with mineral oil. Development was allowed to proceed at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2 and 5% (v/v) O_2 with the balance N_2 . Development was assessed on Day 8 post-insemination. The experiment was replicated 6 times using 50–60 embryos per treatment.

Effect of IGF-1 on induction of ROS by menadione

Embryos ≥ 16 cells were harvested from culture drops at Day 6 after insemination and placed in 24-well plates in groups of 5–8 in either vehicle (40 μM acetic acid) or 100 ng/ml IGF-1. After 1 h at 38.5°C, an additional 150 μl of KSOM-BE2 containing vehicle [final concentration of 0.1% (v/v)

ethanol] or KSOM-BE2 with menadione was added to yield a final concentration of 5 μM . In addition, all wells contained 10 μM of the ROS detection reagent, 5-(and-6)-carboxy-2',7'-dihydrofluorescein diacetate (H2DFFDA). At 1 h after addition of menadione and H2DFFDA, embryos were washed four times in 10 mM PO_4 buffer, pH 7.4 containing 0.9% (w/v) NaCl and 1 mg/ml polyvinylpyrrolidone (PBS-PVP). The embryos were then immediately placed in wells of 72-well Terasaki plates (Nunc 136528) in 15 μl PBS-PVP and examined using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) and a FITC filter for fluorescence. Images were acquired using AxioVision software and an AxioCam MRm digital camera. Using the computer mouse, a circular draw function was manually performed around the internal side of the zona pellucida and intensity per unit area was determined using AxioVision software. The experiment was replicated 4 times using 22–25 embryos per treatment.

Statistical analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS for Windows, version 9.2, SAS Institute Inc., Cary, NC). Percent development was calculated for each replicate and transformed by arcsin transformation before analysis. Percent TUNEL positive cells was calculated on an embryo basis and then transformed using an arcsin transformation. Independent variables included menadione concentration, cytoprotectant, and replicate. The mathematical model included main effects and all interactions. Replicate was considered random and other main effects were considered fixed. *F* tests were calculated using error terms calculated from expected mean squares. All values reported are least-squares means \pm SEM. Probability values for percent data are based on the analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

Results

Interactions between menadione and IGF-1 affecting developmental capacity of embryos

As shown in Fig. 1, development of embryos to the blastocyst stage was affected by an interaction between menadione concentration and IGF-1 ($P < 0.001$). In the absence of IGF-1, menadione caused a concentration-dependent decrease in the proportion of embryos that became blastocysts. All concentrations tested reduced development; no embryos became blastocysts at the highest concentration (5.0 μM). Treatment with IGF-1 reduced

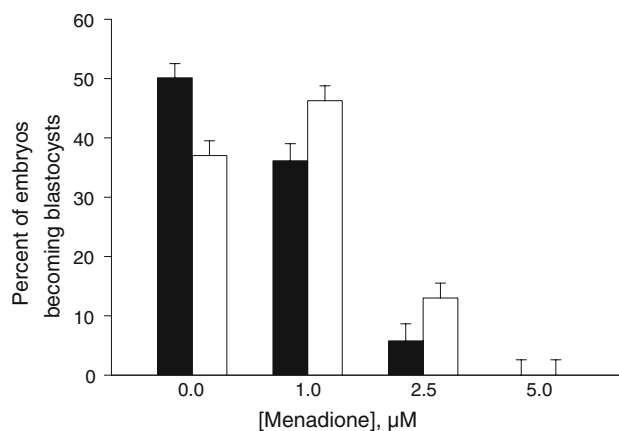


Fig. 1 Interactions between menadione and insulin-like growth factor-1 (IGF-1) affecting developmental capacity of embryos. Embryos ≥ 16 cells were treated for 24 h at Day 5 after insemination and the percent of embryos becoming blastocysts determined at Day 8 after insemination. Data are least-squares means \pm SEM for embryos cultured in the absence (*black bars*) and presence (*white bars*) of 100 ng/ml IGF-1. The experiment was replicated 14 times using 120–204 embryos per treatment. Development was affected by menadione concentration ($P < 0.001$) and the interaction between menadione concentration and IGF-1 ($P < 0.001$)

the magnitude of the anti-developmental effects of menadione. This action of IGF-1 was apparent at 1.0 μM , where IGF-1 prevented the reduction in development caused by menadione, and at 2.5 μM , where IGF-1 partially blocked the effects of menadione, but not at 5.0 μM , where no embryos became blastocysts regardless of the presence of IGF-1.

Induction of apoptosis by menadione as influenced by IGF-1

Since one effect of menadione is to increase apoptosis, an experiment was performed to determine whether menadione increases apoptosis in embryos and, if so, whether this effect is reduced by IGF-1 (Fig. 2). The percent of cells positive for the TUNEL reaction was affected by an interaction between menadione concentration \times IGF-1 ($P < 0.05$). Menadione caused a concentration-dependent increase in the percent of cells positive for the TUNEL reaction in control- and IGF-1-treated embryos. At the highest concentration, 5 μM , 73% of blastomeres were apoptotic as indicated by TUNEL labeling. The effect of menadione was reduced by the presence of IGF-1 at 1.0 and 2.5 μM but not at 5 μM .

Protection of embryos from anti-developmental effects of menadione by antioxidants

An experiment was conducted to verify that the anti-developmental actions of menadione were caused by ROS.

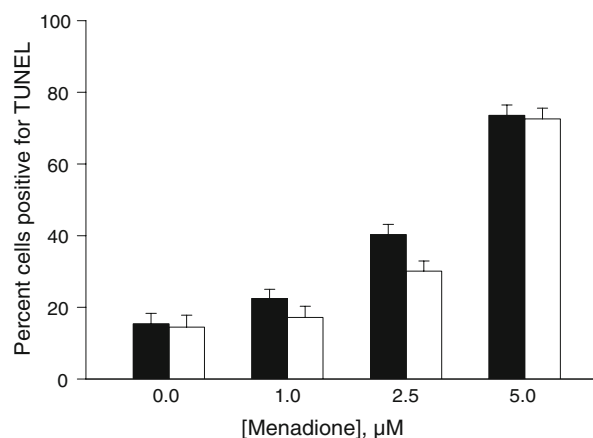
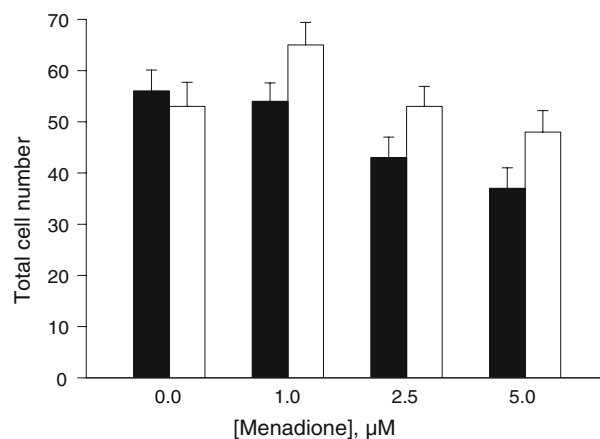


Fig. 2 Induction of apoptosis by menadione as influenced by insulin-like growth factor-1 (IGF-1). Embryos ≥ 16 cells were treated at Day 6 after insemination and total cell number (*top panel*) and the percent of cells positive for the TUNEL reaction (*bottom panel*) 24 h later were determined. Data are least-squares means \pm SEM for embryos cultured in the absence (*black bars*) and presence (*white bars*) of 100 ng/ml IGF-1. The experiment was replicated 5 times using 21–33 embryos per treatment. Total cell number was affected by menadione concentration ($P < 0.025$) and IGF-1 ($P = 0.05$). The percent of cells positive for the TUNEL reaction was affected by menadione concentration ($P < 0.01$), IGF-1 ($P < 0.06$), and the interaction between menadione concentration and IGF-1 ($P < 0.05$)

The approach was to treat embryos with 5.0 μM menadione in the presence of the antioxidants dithiothreitol and Trolox. In addition, the protective effects of IGF-1 were evaluated. As shown in Fig. 3, all three treatments reduced the effect of menadione on development to the blastocyst stage (menadione \times cytoprotectant type, $P < 0.05$) with dithiothreitol completely blocking actions of menadione and IGF-1 and Trolox reducing effects of menadione on development.

Effect of IGF-1 on induction of ROS by menadione

An experiment was done to determine whether IGF-1 protects embryos from the actions of menadione by

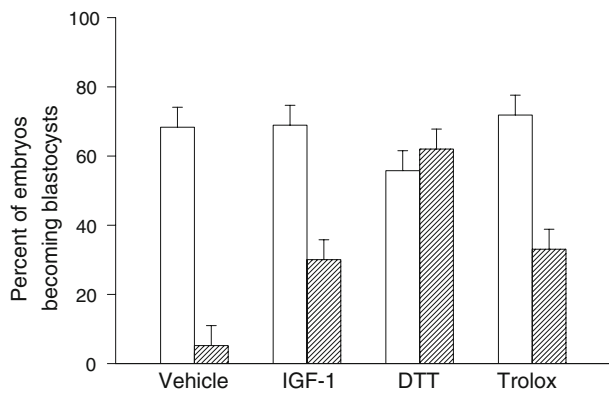


Fig. 3 Protection of embryos from anti-developmental effects of menadione by antioxidants. Embryos ≥ 16 cells were treated for 24 h at Day 6 after insemination and the percent of embryos becoming blastocysts determined at Day 8 after insemination. Embryos were treated with either vehicle, insulin-like growth factor-1 (IGF-1), dithiothreitol (DTT), or Trolox in the absence (*white bars*) or presence (*hatched bars*) of 5.0 μM menadione. Data are least-squares means \pm SEM. The experiment was replicated 6 times using 50–60 embryos per treatment. Development was affected by menadione ($P < 0.05$) and the interaction between cytoprotectant type and menadione ($P < 0.01$)

reducing ROS production (Fig. 4). There was a concentration-dependent increase in production of ROS caused by menadione ($P < 0.01$). However, this increase was not affected by IGF-1 as indicated by the lack of significant effect of IGF-1 and the interaction between menadione concentration and IGF-1.

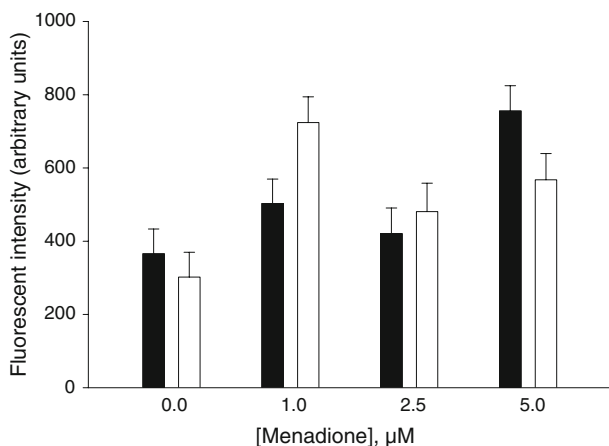


Fig. 4 Effect of IGF-1 on induction of reactive oxygen species by menadione. Amount of reactive oxygen species in embryos ≥ 16 cells at Day 6 after insemination generated at 1 h after addition of menadione was determined using the fluorescent probe H₂DFFDA. Data are least-squares means \pm SEM for embryos cultured in the absence (*black bars*) and presence (*white bars*) of 100 ng/ml IGF-1. The experiment was replicated 4 times using 22–25 embryos per treatment. Fluorescent intensity was affected by menadione concentration ($P < 0.01$) but not by IGF-1 or the interaction between menadione concentration and IGF-1

Discussion

The preimplantation embryo is susceptible to disruption by ROS. Among the oxidizing agents and prooxidants shown to block development are tertiary butyl hydroxide (Gardiner and Reed 1994), hydrogen peroxide (Kurzawa et al. 2004), a high concentration of oxygen in the gaseous environment (Orsi and Leese 2001; de Castro e Paula and Hansen 2008), heat shock (Sakatani et al. 2008), and sodium nitroprusside (Soto et al. 2003). Data presented in this paper indicate that menadione is another embryotoxic molecule that acts through generation of ROS. That the effects of menadione are due to ROS was indicated by the increase in ROS production caused by menadione and the inhibition of the anti-developmental actions of menadione by administration of the antioxidants dithiothreitol or Trolox. The finding that IGF-1 reduced actions of menadione illustrates the ability of this pleiotropic growth factor to protect embryos from oxidative stress. In particular, IGF-1 acts to protect embryos from menadione by blocking downstream effects of ROS production such as apoptosis and not by blocking ROS generation itself.

The bovine preimplantation embryo seems to be more sensitive to menadione than several other cells. Embryonic development was reduced by concentrations of menadione as low as 1.0 μM and, depending on the experiment, completely blocked or greatly reduced by 5.0 μM menadione. In contrast, the IC_{50} for inhibition of proliferation of various tumor cells lines was 6–47 μM (Oztopçu et al. 2004; Sasaki et al. 2008; Osada et al. 2008). Proliferation of endothelial cells was stimulated by 1.0 μM menadione and 5 μM had only slight inhibitory effects (Łuczak et al. 2004). Among the explanations for the increased sensitivity of the embryo to menadione is the fact that successful development requires differentiation as well as proliferation. Moreover, the intracellular antioxidant glutathione is deficient in the preimplantation embryo (Gardiner and Reed 1994, 1995; Aréchiga et al. 1995; Lim et al. 1996). Deficiencies in glutathione concentrations and biosynthesis are likely to make the embryos very sensitive to damaging actions of ROS.

A clinical implication to our findings is that feeding menadione may have more deleterious effects on female fertility than anticipated. To our knowledge, there are no reports regarding circulating concentrations of menadione in females fed diets containing menadione. Research to determine such concentrations are warranted.

The result that IGF-1 protects embryos from the anti-developmental actions of menadione is consistent with protective effects of this growth factor against a variety of insults including radiation (Spanos et al. 2000), tumor necrosis factor- α (Byrne et al. 2002), and heat shock (Jousan and Hansen 2004, 2007). The mechanism by which

IGF-1 protects embryos from menadione involves inhibition of events downstream from ROS generation. There was no effect of IGF-1 on ROS concentrations as determined using the fluorescent probe H2DFFDA. However, IGF-1 did reduce the increase in apoptotic cells caused by menadione. Consistent with the conclusion that IGF-1 blocks deleterious effects of ROS is the earlier finding that IGF-1 reduces effects of hydrogen peroxide on development (Kurzawa et al. 2004).

The anti-apoptotic properties of IGF-1 are an important mechanism for cytoprotection in a wide variety of cells (Kurmasheva and Houghton 2006). It is possible, however, that inhibition of apoptosis is not the only mechanism engaged by IGF-1 to protect embryos from menadione. For bovine embryos exposed to heat shock, IGF-1 reduces the increase in apoptotic cells caused by culture at elevated temperature through activation of the phosphatidyl 3-kinase/Akt pathway (Jousan and Hansen 2004, 2007; Jousan et al. 2008). Inhibition of phosphatidyl 3-kinase with LY 294002 does not eliminate the thermoprotective effects of IGF-1 on development, however, even though it blocks anti-apoptotic actions of IGF-1 (Jousan and Hansen 2007).

One possibility is that IGF-1 counteracts actions of menadione on DNA polymerase γ . The IC_{50} value for inhibition of this enzyme by menadione is 6.0–6.8 μ M (Sasaki et al. 2008). Given its role in mitochondrial replication, inhibition of DNA polymerase γ could lead to reduced capacity for oxidative respiration. One possible mechanism by which IGF-1 could reduce consequences of inhibition of DNA polymerase γ by menadione would be through increased synthesis of p53. This protein can translocate to mitochondria in response to ROS and other insults and interact with mitochondrial DNA polymerase γ to increase mitochondrial replication (Achanta et al. 2005). IGF-1 has been shown, under some circumstances, to increase the amount of p53 in human lymphocytes (Cianfarani et al. 1998), cultured human cardiac muscle cells (Wang et al. 1998), and MCF-7 malignant breast cancer cells (Clark et al. 2005).

In conclusion, concentrations of menadione as low as 1.0 μ M can compromise development of bovine preimplantation embryos to the blastocyst stage of development in a ROS-dependent mechanism. Anti-developmental actions of menadione can be blocked by IGF-1 through effects downstream of ROS generation. Consequences of supplementing animal feed with menadione for fertility should be examined.

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