PAPP-A ENHANCES THE ANTIOXIDATIVE EFFECTS OF IGF-1 DURING BOVINE IN VITRO EMBRYO PRODUCTION

Fernanda Fagali Franchi[1,4*], Priscila Helena dos Santos[1], Patricia Kubo Fontes[1], Ana Elisa Valencise Quaglio[1], Sarah Gomes Nunes[1], Gisele Zoccal Mingoti[2], Anthony César de Souza Castilho[3*]

[1] São Paulo State University (Unesp), Department of Pharmacology, Institute of Biosciences, Botucatu- São Paulo, Brazil.; fernanda.franchi@unimi.it, santos.priscilah@gmail.com, pkfontes@gmail.com, anaequaglio@hotmail.com, sarah.nunes@unesp.br.

[2] São Paulo State University (Unesp), School of Veterinary Medicine, Laboratory of Reproductive Physiology, Araçatuba, São Paulo, Brazil; gisele.mingoti@unesp.br

[3] University of Western São Paulo (Unoeste), [3]Department of Animal Science, Presidente Prudente-, São Paulo, Brazil.; castilho.anthony@gmail.com

[4] Reproductive and Developmental Biology Laboratory, Department of Veterinary Medicine and Animal Science, University of Milan, Milan, Italy.

*Correspondence: castilho.anthony@gmail.com; fernanda.franchi@unimi.it.

ABSTRACT

We investigated whether exogenous pregnancy-associated plasma protein-A (PAPP-A) enhances the antioxidant role of insulin-like growth factor-1 (IGF-1) in bovine *in vitro* embryo production (IVP). We performed standard *in vitro* maturation (IVM) and *in vitro* culture (IVC) or added menadione to promote an oxidative stressed microenvironment and evaluated the antioxidant effect of IGF-1 alone or in combination with PAPP-A (IGF-1/PAPP-A). In IVM, the treatments did not affect oocyte nuclear development, total GSH content, *cumulus* cell gene expression, and blastocyst yield. Nevertheless, IGF-1/PAPP-A treatment prevented an increase in reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) levels. In IVC, the treatments did not affect the total GSH content on blastocysts and IVC media, but IGF-1 and IGF-1/PAPP-A treatments increased blastocyst yield compared to the menadione group. In addition, IGF-1/PAPP-A treatment

had lower ROS levels and regulated genes related to embryonic quality compared to the control and menadione groups. Overall, we showed that PAPP-A could enhance the antioxidant role of IGF-1 during IVP in cattle by avoiding higher ROS levels in oocytes and blastocysts and modulating the transcriptional abundance of genes involved in oxidative protection and embryonic quality.

Keywords: Pappalysin, oxidative stress, ROS, cattle, insulin-like growth factor-1, gene expression.

1. INTRODUCTION

Reactive oxygen species (ROS) are produced as natural products of aerobic metabolism, even when cells are under basal conditions. Most ROS production is a natural result of the oxidative phosphorylation process that occurs in the mitochondrial electron transport chain to generate adenosine triphosphate (ATP)(Reviewed by Guerin et al., 2001). However, excessive ROS levels have been associated with oxidative stress, which can cause lipid peroxidation, mitochondrial function damage, and DNA structure alterations. The latter has been demonstrated by events of base pair loss or fragmentation of the DNA core (Birben et al., 2012; Nasr-Esfahani et al., 1990; Noda et al., 1991; Richter et al., 1995; Roth, 2018). Some specific cells and/or conditions require higher ATP production, such as the oocytes and embryos during the early development stages (Chappel, 2013). Under physiological conditions, these cells rely on an antioxidant system that assures reduced and/or absence of any cell damage caused by increased ROS levels production. However, high ROS levels have been described in bovine oocytes submitted to *in vitro* maturation (IVM) and bovine blastocysts produced by *in vitro* embryo production (IVEP) (Dalvi et al., 2005; Morado et al., 2009), such high ROS levels

in *in vitro* conditions even have led to embryo development arrest at the 2-cell stage in mice (Nasr-Esfahani et al., 1990).

Supporting the fact that an increase in ROS levels has harmful effects, two studies demonstrated that bovine embryos when cultured with menadione, a pro-oxidant stressor inducer of ROS production when under oxygen presence (Monks et al., 1992; Nutter et al., 1992), indeed resulted in decreased the blastocyst yield production and increased apoptotic cells number (de Assis et al., 2015; Moss et al., 2009). On the contrary, the insulin-like growth factor-1 (IGF-1) has shown a systemic antioxidant effect. (Higashi et al., 2010; Sukhanov et al., 2007). Corroborating with that, it has been demonstrated that IGF-1 media supplementation prevented menadione deleterious effects (Monks et al., 1992; Nutter et al., 1992), although such effect seems to be independent of ROS concentrations in bovine embryo culture (Moss et al., 2009).

The IGF-1 bioavailability system is regulated by IGF-binding proteins (IGFBPs), which decrease IGF-1 activity. Oppositely, the pregnancy-associated plasma protein-A (PAPP-A) increases IGF-1 availability and facilitates IGF-1 binding to its receptors (Froesch et al., 1985; Jones & Clemmons, 1995; Lawrence et al., 1999; LeRoith et al., 1992). PAPP-A is mainly secreted by granulosa cells (Conover et al., 1999). It acts by cleaving IGFBP-2, IGFBP-4, and IGFBP-5 in many species' tissues, including cattle (Laursen et al., 2007; Laursen et al., 2001; Mazerbourg et al., 2003; Mazerbourg & Monget, 2018; Monget et al., 2003; Rivera & Fortune, 2003). The relevance of its role in reproductive tissues has been demonstrated by producing knockout mice for PAPP-A presence, which resulted in reduced animal fertility, lower *cumulus*-oocyte complexes (COCs) quantification, and lower progesterone and estradiol levels during ovarian superovulation cycles (Laursen et al., 2001; Nyegaard et al., 2010). Because of that, PAPP-A has been used as a dominant follicle selection marker. Moreover, its

contributions to the reproductive field have not been limited to *in vivo* conditions. Under *in vitro* conditions, the presence of PAPP-A increased the IGF-1 availability in the COCs *in vitro* maturation media, which later in the blastocyst production was associated with modulation on genes involved with lipid metabolism, mitochondrial functionality, and blastocyst competence (Dellaqua et al., 2023; Giroto et al., 2019).

Collectively, due to its physiological relevance, many studies have evaluated the effect of IGF-1 supplementation on ROS production during *in vitro* embryo production (IVEP), however, the results of these *in vitro* studies are controversial. Taking into consideration the IGF-1 bioavailability system, we hypothesize that the effect of IGF-1 supplementation in the IVEP system might be enhanced by its association with PAPP-A. Therefore, our objective was to evaluate the antioxidant effect of IGF-1 associated with PAPP-A on the bovine embryo production system, regarding the oocyte and blastocyst quality. To the best of our knowledge, the synergist of PAPP-A and IGF-1 has not yet been investigated.

2. MATERIALS AND METHODS

Unless otherwise mentioned, all products were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) with high purity.

2.1. Experimental design

To expand our understanding of the IGF-1/PAPP-A system involvement with oxidative stress, ROS production was induced in oocytes and embryos samples withmenadione treatment (5 µM), as previously described (Cavallari et al., 2019; Chen et al., 2017; de Assis et al., 2015; Moss et al., 2009). In a pilot experiment, the effect of PAPP-A alone on ROS concentration was investigated, however, no modulation was

observed in this parameter (data not shown) Therefore, this present study followed with PAPP-A associated with IGF-1 (Figure 1).

FIGURE 1

2.2. *Cumulus*-oocyte complex collection

Ovaries from crossbred female cattle (*Bos taurus indicus*) were obtained in a slaughterhouse and transported to the laboratory in sodium chloride 0.9% at 36 °C. Follicles with a diameter of 2-8 mm were aspirated with an 18-gage needle and collected in a 15 mL conical tube. After sedimentation, COCs were collected and selected using a stereomicroscope. Only COCs with homogeneous cytoplasm and a compact multilayer of grade 1 and 2 *cumulus* were used for the experiments, as described by Stojkovic et al. (Stojkovic et al., 2001).

2.3. In vitro maturation

The selected COCs were washed in buffered wash medium and randomly transferred to four-well dishes containing 10 µL/COC basic IVM medium, consisting of TCM199 with bicarbonate and Earle's salts, recombinant follicular stimulating hormone (0.1 IU/mL solution; Gonal-f; Merck Serono, Bari, Italy), sodium pyruvate (22 µg/mL), amikacin (75 µg/mL), and fatty acid-free bovine serum albumin (BSA; 4 mg/mL). The basic IVM medium was used from the control group (C group). In addition, three other groups consisted of IVM medium supplemented with menadione (5 µM): only menadione (M group), menadione with IGF-1 (100 ng/mL, IGF-1 recombinant human; Life Technologies, Carlsbad, CA, USA; M/I group), or menadione with IGF-1 and PAPP-A (100 ng/mL, recombinant human Pappalysin-1; R&D Systems Inc., Minneapolis, MN, USA; M/I-P group) according to the experimental design (Figure 1a). After 24 hours at

129 38.5 °C in humidified air with 5.5% CO_2, the COCs were submitted to *cumulus* cell
130 removal, and oocyte and cumulus cell samples were collected separately for analysis.
131
132 **2.4. In vitro fertilization**
133 The presumptive matured COCs (not treated, basic IVM media) were washed
134 three times with in vitro fertilization (IVF) medium and placed in plates with 90 µL of
135 IVF medium (25 COCs/droplet) and covered with silicone oil (Quimesp Química,
136 Guarulhos, São Paulo, Brazil). The IVF medium contained thyroid albumin-lactate-
137 pyruvate supplemented with BSA (6 mg/mL), pyruvate (22 µg/mL), amikacin (75
138 µg/mL), heparin (30 µg/mL), and penicillamine–hypotaurine–epinephrine solution (20
139 µM penicillamine, 10 µM hypotaurine, and 1 µM epinephrine). Nelore bull semen from
140 a single ejaculation (CRV Lagoa, Sertãozinho, São Paulo, Brazil) was thawed at 37 °C
141 for 30 s and spermatozoa were washed in a discontinuous gradient with BotuFIV Select
142 SPERM® (Botupharma, Botucatu, Brazil) prepared by adding 45% to 90% BotuFIV
143 Select SPERM® in a 1.5 mL centrifuge tube. The sperm samples were added to the top of
144 the gradient and centrifuged at 3000 × g for 5 minutes (MiniSpin®; Eppendorf, Hamburg,
145 Germany). The supernatant was then removed, and the sperm pellet obtained was counted
146 in a Neubauer chamber. Sperm were resuspended in IVF medium to reach a final
147 concentration of 1×10^6 cells/mL, and 7 µL were added to each IVF droplet and incubated
148 for 18 hours at 38.5 °C in a saturated humidity atmosphere containing 5.5% CO_2.
149 Subsequently, the presumptive zygotes (PZ) were denuded of the remaining surrounding
150 *cumulus* cells by vortexing and washed three times in Synthetic Oviduct Fluid (SOF)
151 medium (Holm et al., 1999).
152
153 **2.5. In vitro culture**

Embryo culture was performed in four-well dishes with 10 µL/PZ of IVC medium (SOF medium, sodium pyruvate (0.2 mM), BSA (5 mg/mL), and 2.5%; v/v fetal bovine serum). On day 4 post-IVF, all structures were transferred to a new culture medium as previously performed by Moss et al. (2009). All structures were placed in a new four dish containing IVC medium without supplementation (control, C group) or in wells supplemented with menadione (5 µM; M group), menadione with IGF-1 (100 ng/mL; M/I group) or menadione with IGF-1 and PAPP-A (100 ng/mL; M/I+P group), as shown in the experimental design (Figure 1b). On day 5, the structures were washed three times in IVC medium and transferred to a new four-well dish with IVC medium (without treatments) until day 7. All cultures were performed in a humidified and controlled atmosphere (5% CO_2, 5% O_2, and 90% N_2) at 38.5 °C. Blastocyst yield was determined on day 7 after IVF and embryos were collected for analysis (Figure 1b).

2.6. Transcript profile of *cumulus* cells and blastocysts

Three out of six replicates were randomly selected for gene expression analysis of *cumulus* cell samples (n=3 samples/group). For blastocyst analysis, a sample was defined as a pool of three blastocysts (n=5 samples/group).

Total RNA was extracted using the PicoPure™ RNA Isolation Kit (Applied Biosystem®, Foster City, CA, USA) according to the manufacturer's protocol. After purification, the RNA samples were eluted in 12 µL of RNAse-free water. Total RNA (100 ng/reaction for *cumulus* cell samples and total RNA samples for embryos) was incubated with DNAse I (1 IU/µg; Invitrogen, São Paulo, Brazil) and then reverse transcribed with random primers according to the protocol of the High-Capacity Kit (Applied Biosystem®, Foster City, CA, USA).

Subsequently, the mRNA abundance of 96 genes (see Supplementary Table S1) was assessed in the *cumulus* cells and blastocysts samples. All analyzes were performed using the Biomark HD assay (Fluidigm, South San Francisco, CA, USA) as previously described (Dellaqua et al., 2023; Fontes et al., 2020; Franchi et al., 2019; Giroto et al., 2019). The relative expression values for each gene were calculated with the ΔCt method and the data were normalized using the geometric mean values of the most stable reference genes for each cell type (NormFinder software tested). The geometric mean of the mRNA abundance of *ACTB, GAPDH, PPIA,* and *RPL30* was used to normalize the blastocyst samples data, while *ACTB, B2M, PPIA,* and *RPL30* were used for the *cumulus* cells data.

2.7. Assessment of nuclear progression, mitochondrial activity, and intracellular reactive oxygen species in oocytes and embryos

A combined fluorescent staining technique using Hoechst 33342, MitoTracker® Red CMXRos, and CellROX™ Green Reagent (Invitrogen) was used to determine the nuclear maturation stage, indirect mitochondrial inner membrane potential (MMP), and ROS concentration in oocytes, respectively. In the blastocyst samples, only the ROS concentrations of the non-expanded blastocysts were analyzed.

After IVM, oocytes were denuded by vortexing and washed in phosphate-buffered saline (PBS; pH 7.2) containing 1 mg/mL polyvinylpyrrolidone (PBS-PVP). Next, oocytes were incubated with Hoechst (1 µg/mL), CellROX™ Green (5 µM), and MitoTracker® Red (0.5 µM). For blastocyst samples, CellROX™ Green (5 µM) and Hoechst (1 µg/mL) were used. In both sample types, the incubations were done for 30 minutes at 5.5% CO_2 and 38.5 °C. After, samples were washed three times in PBS-PVP and fixed with 4% paraformaldehyde for 15 minutes. Last, samples were washed three

times in PBS-PVP, placed in glycerol microdroplets on a microscope slide, covered with a coverslip, and analyzed with a Leica DM4500 epifluorescence microscope (Leica Camera AG, Wetzlar, Germany) to determine DNA nuclear progression and ROS and MMP fluorescence intensities. Images were acquired with LAS software using a camera attached to the microscope. Quantification of average pixel intensity was performed using Image J software (NIH, Bethesda, MD, USA) for ROS and MMP analysis.

2.8. Glutathione total content determination

Total GSH content was quantified using a recycling assay previously as described (Anderson, 1985). Two sample types were analyzed, cells and culture media samples. The cell samples were embryos (n=5 pools of 5 embryos/pool/group), denuded oocytes (n=5 pools of 10 oocytes/pool/group), and *cumulus* cells (n=5 pools of *cumulus* cells from 10 oocytes/pool/group) were stored in trichloroacetic acid (TCA) at -80 °C. The culture medium samples were IVM media, IVC media (Day 5), and IVC media (Day 7), all samples consisted of 20 µL volume (n=5 samples/group), which were stored at -80 °C and TCA was added immediately before the GSH assay. Each sample (except the medium samples) was vortexed and centrifuged at 10000 ×g for 20 s. Standards containing 1.56 to 100 nmol/mL reduced GSH were prepared simultaneously with the samples in TCA. The samples and standards were stored on ice until they were loaded onto the microtiter plate. Then, 20 µL of each sample or standard was added to a 96-well microtiter plate, followed by 140 µL of NADPH (0.3 mM), 5 µL of sodium phosphate buffer (143 mM) with EDTA (6.3 mM), and 20 µL of DTNB (6 mM). All reagents were diluted in sodium phosphate buffer (143 mM) with EDTA (6.3 mM). The plate was incubated at 30 °C for 5 minutes and then 15 µL of GSH reductase (4.0 IU/mL) was added. Immediately, the

plate was analyzed at 412 nm in a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) with 10 replicate measurements.

2.9. Statistical analysis

Meiosis progression and blastocyst rates were calculated as percentages and arcsine-transformed. All data were tested for normality using the Shapiro-Wilk test. The data of gene expression, total GSH content, MMP, and ROS fluorescent intensity, which showed a non-normal distribution, were log-transformed to achieve a normal distribution. For parametric data, analysis of variance (ANOVA) followed by the Tukey test was performed to compare means, and for non-parametric data, the Kruskal-Wallis test followed by the Wilcoxon test was performed to compare means. All data are expressed as mean ± standard error of the mean, and differences were considered significant when $P \leq 0.05$.

3. RESULTS

3.1. Effects of IGF-1/PAPP-A treatment during bovine IVM

When the treatments were performed during the IVM, no difference was observed regarding the percentage of oocytes that reached metaphase II and the blastocyst yield (Table 1). The total GSH content of the COCs, embryos, and IVM medium was also unaffected (Table 2). On the other hand, when analyzing oocyte samples, similar MMP levels were detected in the IGF-1/PAPP-A group (M/I+P) and the control group (C), which was lower compared to the menadione (M) and IGF-1 (M/I) groups (Figure 2b). In addition, the ROS levels in the oocyte samples from IGF-1/PAPP-A (M/I+P) were lower than menadione (M) and IGF-1 (M/I) groups, although, higher than the control group (C, Figure 2b). Concerning the transcripts abundance analysis, in the *cumulus* cells samples,

the *VNN1* gene, a stress marker, was remarkably lower in the IGF-1 alone (M/I group) and it combined with PAPP-A (M/I+P group) when compared to the menadione group (M) (Figure 3). While, in the blastocyst samples, no difference was observed among the groups, although *CDX2* appears to be more abundant in the control (C) and IGF-1/PAPP-A (M/I+P) groups compared to the M and M/I groups (P=0.0847) and *FOXO2* tended to be more abundant in the M/I+P group (P=0.0647; Figure S1).

TABLE 1

TABLE 2

FIGURE 2

FIGURE 3

3.2. Effects of IGF-1/PAPP-A treatment during bovine IVC

When the treatments were performed during the IVC, the blastocyst yield was higher in the IGF-1 alone (M/I) and it combined with PAPP-A (M/I+P) groups compared to the menadione (M) group (Figure 4b). Moreover, lower ROS levels were detected in the IGF-1/PAPP-A (M/I+P) group compared to control (C) and menadione (M) groups (Figure 4). Furthermore, the transcripts abundance of *ACSL3, EGFR, HMOX1, IMPDH2,* and *SLC2A5* were higher in the IGF-1/PAPP-A group (M/I+P) compared to the menadione group (M; Figure 5). While no alteration was observed in the total GSH quantification in the IVC (D5 and D7) medium or the blastocysts samples (Table 3).

FIGURE 4

FIGURE 5

TABLE 3

4. DISCUSSION

In this study, we proposed a new strategy for the use of recombinant IGF-1 in the *in vitro* embryo production technology. For the first time, the association of recombinant PAPP-A with IGF-1 enhanced the antioxidant role of the latter when under the *in vitro* stressed microenvironment. The results show that exogenous IGF-1/PAPP-A, when added to the IVM media, prevents ROS and MMP levels increase in the oocyte after maturation. In addition, blastocysts derived from these oocytes had lower ROS levels. Moreover, when IGF-1/PAPP-A was added to the IVC media, it demonstrated an impressive modulation in the blastocyst transcriptional profile.

Previous studies have presented conflicting results regarding the effects of ROS or IGF-1 on the oocyte nuclear stage (Ascari et al., 2017; Chen et al., 2017; Gomez et al., 1993; LaRosa & Downs, 2006; Lorenzo et al., 1994; Reed et al., 1993; Rieger et al., 1998; Takami et al., 1999; Tamura et al., 2008; Yang et al., 2022). A study in rodents has shown a reduced progression in the oocyte nuclear maturation when they are under a stressful environment induced by menadione. In the same study, the authors demonstrated that the oocyte maturation progress until the MII stage could be restored by using an antioxidant agent,, such as melatonin (Chen et al., 2017). In disagreement with this data, in our study, the menadione treatment did not impair the oocyte nuclear maturation (Table 1). This same absence of menadione effect on meiosis progression was observed in murine COCs, which was demonstrated to be due to the protection effect of the *cumulus* cells (LaRosa & Downs, 2006). Therefore, the presence of *cumulus* cells surrounding the oocytes might be an explanation for the lack of effect of the menadione treatment in our study, although higher ROS levels were observed in these oocytes.

Corroborating with this latter result, other studies also observed higher ROS levels in oocytes and embryos exposed to menadione treatment. And again, treatments with antioxidants, such as melatonin, were able to prevent it (Ascari et al., 2017; Cavallari et

306 al., 2019; Chen et al., 2017; de Assis et al., 2015; Moss et al., 2009). Although IGF-1 is
307 classified as an antioxidant, a previous study demonstrated no effect of IGF-1 in
308 modulating ROS levels (Yang et al., 2022). In the same way, our study showed no
309 modulation of ROS levels by the IGF-1 group (M/I), however, when IGF-1 was
310 associated with PAPP-A (M/I+P), the ROS levels in oocytes were significantly lower than
311 in the menadione group.

312 Interestingly, the quantification of ROS levels in blastocysts showed a similar
313 pattern in the menadione-treated and control group, which might be due to the *in vitro*
314 condition already being a stressful environment. Under these conditions, the IGF-
315 1/PAPP-A combination (M/I+P) resulted in lower ROS levels compared to the control
316 and menadione groups. IGF-1 has been shown to lower systemic and vascular ROS
317 concentrations while increasing total serum antioxidant activities and capacity, thereby
318 reducing oxidative damage in the brain and liver of rodents (Garcia-Fernandez et al.,
319 2008; Higashi et al., 2010; Sukhanov et al., 2007). The discrepancy in the outcomes of
320 using IGF-1 alone or combined with PAPP-A might be the key factor. As mentioned
321 before, the IGFBPs decrease IGF-1 activity, and their expression is known to occur in
322 oocytes and embryos during their development (Armstrong et al., 2002; Satrapa et al.,
323 2013; Sawai et al., 2005; Winger et al., 1997). Moreover, BSA and/or fetal bovine serum
324 are often added to IVM and IVC media, and both supplements contain IGFBPs (Slaaby
325 et al., 2008; Zheng et al., 2006). As proof of that, it has been shown that BSA presence in
326 culture media reduces IGF-1/IGF-1R binding. In contrast, PAPP-A addition in bovine
327 IVM media increases IGF-1-free concentrations, even with BSA supplementation (Giroto
328 et al., 2019). Therefore, we suggest that PAPP-A presence is required to increase IGF-1
329 activity to reduce ROS levels in bovine IVP systems.

Moreover, the increase in ROS levels has also been correlated to mitochondrial damage, which typically leads to lower MMP levels (Hao et al., 2011; Loor et al., 2010). On the other hand, MMP is generated by protons moving during ATP synthesis, which means that higher MMP has been associated with greater energy production (Cottet-Rousselle et al., 2011). However, under unbalanced energy demand, the exponential increase in MMP levels leads to high ROS production (Lambert & Brand, 2009; Murphy, 2009; Starkov, 2008), which even impairs ATP synthesis and results in detrimental effects in the cells (Berruyer et al., 2004; Miwa & Brand, 2003; Ramzan et al., 2010; Rottenberg et al., 2009; Starkov & Fiskum, 2003). Therefore, it is expected from normal cell function maintenance of MMP levels (Kaim & Dimroth, 1999; Korshunov et al., 1997; Ramzan et al., 2010; Rottenberg et al., 2009; Starkov & Fiskum, 2003). Regarding this correlation, it has been proposed that controlling the MMP might be a mechanism for mitochondrial ROS regulation. For instance, a 10 mV reduction in MMP was sufficient to eliminate approximately 70% of the ROS production by complex I (Miwa & Brand, 2003). It was observed in the present study high ROS levels in oocytes from M and M/I groups, which were associated with high MMP levels. This pattern might indicate a disbalance in the cells from these groups. However, higher ROS levels were detected in the M/P+I group compared to the control group, while the MMP level was similar in the M/P+I and C groups. Based on this, we suggest that the decrease of MMP levels by the IGF-1/PAPP-A association (M/I+P) could be a mechanism to reduce ROS levels in oocytes.

Regarding the relative transcripts abundance analyzis, it appears that neither IGF-1 (M/I) nor PAPP-A (M/I+P) affected *cumulus* cells compared to control or menadione. Except by *VNN1*, whose relative mRNA abundance was downregulated by IGF-1 (M/I) alone and in combination with PAPP-A (M/I+P) compared to the menadione group (M). *VNN1* is a stress marker that modulates the release of glutathione (GSH) when cells are

exposed to oxidative stress (Berruyer et al., 2004; Nivet et al., 2013). GSH appears to be the most important protection against oxidative stress in oocytes and embryos (Feuerstein et al., 2007; Gardiner & Reed, 1994; Takahashi et al., 1993), and indeed studies have observed that the use of antioxidant substances during IVM or IVC leads to an inverse correlation between GSH and ROS levels (Kere et al., 2013; Kwak et al., 2012; Wang et al., 2014). However, the GSH levels analyzed in the present study were unaffected by the experimental conditions when quantified in the COCs, embryos, IVM, and IVC media. Interestingly, Sovernigo, et al. (2017) found that only some antioxidants in cattle show this inverse correlation between ROS and GSH levels, which might explain why our results showed that the IGF-1 is not directly related to the GSH pathway.

Concerning the relative transcripts abundance in blastocysts, few genes were upregulated in the M/I+P compared to the M group. The modulated genes were *IMPDH2* and *EGFR*, which are related to cell replication, proliferation, and differentiation (Adamson & Wiley, 1997; Jackson et al., 1975; Kozhevnikova et al., 2012; Zimmermann et al., 1998); *ACSL3,* which plays a key role in lipid biosynthesis and has been shown to restore survival rates after vitrification of low-quality embryos (Mashek et al., 2007; Soupene & Kuypers, 2008; Valente et al., 2019); *HMOX1* is reported to activate the transcriptional machinery that drives the induction of antioxidant genes (Lin et al., 2007), and *SLC2A5* that is a membrane transporter which has a high specificity for fructose (Burant et al., 1992; Mueckler & Thorens, 2013). It is also known that *SLC2A5* transcription began at the time of bovine embryonic genome activation, suggesting that the early embryo is capable of transporting this energy substrate and that it may be associated, for example, with the production of ribose 5-phosphate, an essential precursor for nucleotide synthesis (Augustin et al., 2001).

GRAPHICAL ABSTRACT

5. CONCLUSION

Taking it all together, we suggest that PAPP-A might be a crucial factor for the antioxidant role of IGF-1 in bovine *in vitro* production. Only associated with PAPP-A that IGF-1 prevented an increase in the ROS levels in oocytes and embryos under menadione-induced stress. Furthermore, the combination of these proteins appears to activate pathways associated with repair processes, which we demonstrated to result in higher embryo quantity and quality. We conclude that IGF-1 needs PAPP-A association to act as an antioxidant factor in bovine oocytes and embryos produced *in vitro*.

Author Contributions: Conceptualization and design of the study, F.F.F, and A.C.S.C; methodology, F.F.F, and A.C.S.C.; software, F.F.F, and G.Z.M.; formal analysis, F.F.F; acquisition of data, F.F.F, P.H.S, P.K.F, A.E.V.Q, S.G.N; resources, A.C.S.C.; writing—original draft preparation, F.F.F.; writing—review and editing, F.F.F and A.C.S.C.; final approval of the version F.F.F.; supervision, project administration and funding acquisition, A.C.S.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES), Finance Code 001”, “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant number: 403063/2016-7”, and by “São Paulo Research Foundation (FAPESP), grant numbers: 2013/11480-3; 2015/04505-5; 2018/06674-7”.

Acknowledgments: We would like to thank the Laboratory of Ovarian Molecular Physiology and Professor Dr. Jose Buratini Junior, the Multi-user Laboratory of

Phytomedicines, Pharmacology, and Biotechnology (FitoFarmaTec), Progest Biotechnology in Animal Reproduction and Dr. Raquel Zaneti Puelker.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

Adamson, E. D., & Wiley, L. M. (1997). The EGFR gene family in embryonic cell activities. *Curr Top Dev Biol*, *35*, 71-120. https://doi.org/10.1016/s0070-2153(08)60257-4

Anderson, M. E. (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol*, *113*, 548-555. https://doi.org/10.1016/s0076-6879(85)13073-9

Armstrong, D. G., Baxter, G., Hogg, C. O., & Woad, K. J. (2002). Insulin-like growth factor (IGF) system in the oocyte and somatic cells of bovine preantral follicles. *Reproduction*, *123*(6), 789-797. http://www.ncbi.nlm.nih.gov/pubmed/12052233

Ascari, I. J., Alves, N. G., Jasmin, J., Lima, R. R., Quintao, C. C. R., Oberlender, G., . . . Camargo, L. S. A. (2017). Addition of insulin-like growth factor I to the maturation medium of bovine oocytes subjected to heat shock: effects on the production of reactive oxygen species, mitochondrial activity and oocyte competence. *Domest Anim Endocrinol*, *60*, 50-60. https://doi.org/10.1016/j.domaniend.2017.03.003

Augustin, R., Pocar, P., Navarrete-Santos, A., Wrenzycki, C., Gandolfi, F., Niemann, H., & Fischer, B. (2001). Glucose transporter expression is developmentally regulated in in vitro derived bovine preimplantation embryos. *Mol Reprod Dev*, *60*(3), 370-376. https://doi.org/10.1002/mrd.1099

Berruyer, C., Martin, F. M., Castellano, R., Macone, A., Malergue, F., Garrido-Urbani, S., . . . Galland, F. (2004). Vanin-1-/- mice exhibit a glutathione-mediated tissue resistance to oxidative stress. *Mol Cell Biol*, *24*(16), 7214-7224. https://doi.org/10.1128/MCB.24.16.7214-7224.2004

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organ J*, *5*(1), 9-19. https://doi.org/10.1097/WOX.0b013e3182439613

Burant, C. F., Takeda, J., Brot-Laroche, E., Bell, G. I., & Davidson, N. O. (1992). Fructose transporter in human spermatozoa and small intestine is GLUT5. *J Biol Chem*, *267*(21), 14523-14526. http://www.ncbi.nlm.nih.gov/pubmed/1634504

Cavallari, F. C., Leal, C. L. V., Zvi, R., & Hansen, P. J. (2019). Effects of melatonin on production of reactive oxygen species and developmental competence of bovine oocytes exposed to heat shock and oxidative stress during in vitro maturation. *Zygote*, *27*(3), 180-186. https://doi.org/10.1017/S0967199419000236

Chappel, S. (2013). The role of mitochondria from mature oocyte to viable blastocyst. *Obstet Gynecol Int*, *2013*, 183024. https://doi.org/10.1155/2013/183024

Chen, D., Li, X., Liu, X., Liu, X., Jiang, X., Du, J., . . . Ma, W. (2017). NQO2 inhibition relieves reactive oxygen species effects on mouse oocyte meiotic maturation and embryo development. *Biol Reprod*, *97*(4), 598-611. https://doi.org/10.1093/biolre/iox098

Conover, C. A., Oxvig, C., Overgaard, M. T., Christiansen, M., & Giudice, L. C. (1999). Evidence that the insulin-like growth factor binding protein-4 protease in human ovarian follicular fluid is pregnancy associated plasma protein-A. *J Clin Endocrinol Metab*, *84*(12), 4742-4745. https://doi.org/10.1210/jcem.84.12.6342

Cottet-Rousselle, C., Ronot, X., Leverve, X., & Mayol, J. F. (2011). Cytometric assessment of mitochondria using fluorescent probes. *Cytometry A*, *79*(6), 405-425. https://doi.org/10.1002/cyto.a.21061

Dalvit, G. C., Cetica, P. D., Pintos, L. N., & Beconi, M. T. (2005). Reactive oxygen species in bovine embryo in vitro production. *Biocell*, *29*(2), 209-212. http://www.ncbi.nlm.nih.gov/pubmed/16187501

de Assis, P. M., Castro, L. S., Siqueira, A. F., Delgado Jde, C., Hamilton, T. R., Goissis, M. D., . . . Assumpcao, M. E. (2015). System for evaluation of oxidative stress on in-vitro-produced bovine embryos. *Reprod Biomed Online*, *31*(4), 577-580. https://doi.org/10.1016/j.rbmo.2015.06.014

Dellaqua, T. T., Franchi, F. F., dos Santos, P. H., Giroto, A. B., Nunes, S. G., de Lima, V. A. V., . . . de Souza were no effects Castilho, A. C. (2023). Molecular phenotypes of bovine blastocyst derived from in vitro-matured oocyte supplemented with PAPP-A. *Veterinary Research Communications*. https://doi.org/10.1007/s11259-023-10072-7

Feuerstein, P., Cadoret, V., Dalbies-Tran, R., Guerif, F., Bidault, R., & Royere, D. (2007). Gene expression in human cumulus cells: one approach to oocyte competence. *Hum Reprod*, *22*(12), 3069-3077. https://doi.org/10.1093/humrep/dem336

Fontes, P. K., Castilho, A. C. S., Razza, E. M., & Nogueira, M. F. G. (2020). Bona fide gene expression analysis of samples from the bovine reproductive system by microfluidic platform. *Anal Biochem*, *596*, 113641. https://doi.org/10.1016/j.ab.2020.113641

Franchi, F. F., Satrapa, R. A., Fontes, P. K., Santos, P. H., Razza, E. M., Emanuelli, I. P., . . . de Souza Castilho, A. C. (2019). Equine chorionic gonadotropin drives the transcriptional profile of immature cumulus-oocyte complexes and in vitro-produced blastocysts of superstimulated Nelore cows. *Mol Reprod Dev*, *86*(11), 1639-1651. https://doi.org/10.1002/mrd.23251

Froesch, E. R., Schmid, C., Schwander, J., & Zapf, J. (1985). Actions of insulin-like growth factors. *Annu Rev Physiol*, *47*, 443-467. https://doi.org/10.1146/annurev.ph.47.030185.002303

Garcia-Fernandez, M., Delgado, G., Puche, J. E., Gonzalez-Baron, S., & Castilla Cortazar, I. (2008). Low doses of insulin-like growth factor I improve insulin resistance, lipid metabolism, and oxidative damage in aging rats. *Endocrinology*, *149*(5), 2433-2442. https://doi.org/10.1210/en.2007-1190

Gardiner, C. S., & Reed, D. J. (1994). Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. *Biol Reprod*, *51*(6), 1307-1314. https://doi.org/10.1095/biolreprod51.6.1307

Giroto, A. B., Fontes, P. K., Franchi, F. F., Dos Santos, P. H., Razza, E. M., Nogueira, M. F. G., . . . Castilho, A. C. S. (2019). Use of pregnancy-associated plasma protein-A during oocyte in vitro maturation increases IGF-1 and affects the transcriptional profile of cumulus cells and embryos from Nelore cows. *Mol Reprod Dev*. https://doi.org/10.1002/mrd.23259

Gomez, E., Tarin, J. J., & Pellicer, A. (1993). Oocyte maturation in humans: the role of gonadotropins and growth factors. *Fertil Steril*, *60*(1), 40-46. http://www.ncbi.nlm.nih.gov/pubmed/8513957

Guerin, P., El Mouatassim, S., & Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update*, *7*(2), 175-189. http://www.ncbi.nlm.nih.gov/pubmed/11284661

Hao, C. N., Geng, Y. J., Li, F., Yang, T., Su, D. F., Duan, J. L., & Li, Y. (2011). Insulin-like growth factor-1 receptor activation prevents hydrogen peroxide-induced oxidative stress, mitochondrial dysfunction and apoptosis. *Apoptosis*, *16*(11), 1118-1127. https://doi.org/10.1007/s10495-011-0634-9

Higashi, Y., Sukhanov, S., Anwar, A., Shai, S. Y., & Delafontaine, P. (2010). IGF-1, oxidative stress and atheroprotection. *Trends Endocrinol Metab*, *21*(4), 245-254. https://doi.org/10.1016/j.tem.2009.12.005

Holm, P., Booth, P. J., Schmidt, M. H., Greve, T., & Callesen, H. (1999). High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology*, *52*(4), 683-700. https://doi.org/10.1016/S0093-691X(99)00162-4

Jackson, R. C., Weber, G., & Morris, H. P. (1975). IMP dehydrogenase, an enzyme linked with proliferation and malignancy. *Nature*, *256*(5515), 331-333. https://doi.org/10.1038/256331a0

Jones, J. I., & Clemmons, D. R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev*, *16*(1), 3-34. https://doi.org/10.1210/edrv-16-1-3

Kaim, G., & Dimroth, P. (1999). ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage. *EMBO J*, *18*(15), 4118-4127. https://doi.org/10.1093/emboj/18.15.4118

Kere, M., Siriboon, C., Lo, N. W., Nguyen, N. T., & Ju, J. C. (2013). Ascorbic acid improves the developmental competence of porcine oocytes after parthenogenetic activation and somatic cell nuclear transplantation. *J Reprod Dev*, *59*(1), 78-84. https://doi.org/10.1262/jrd.2012-114

Korshunov, S. S., Skulachev, V. P., & Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett*, *416*(1), 15-18. https://doi.org/10.1016/s0014-5793(97)01159-9

Kozhevnikova, E. N., van der Knaap, J. A., Pindyurin, A. V., Ozgur, Z., van Ijcken, W. F., Moshkin, Y. M., & Verrijzer, C. P. (2012). Metabolic enzyme IMPDH is also a transcription factor regulated by cellular state. *Mol Cell*, *47*(1), 133-139. https://doi.org/10.1016/j.molcel.2012.04.030

Kwak, S. S., Cheong, S. A., Jeon, Y., Lee, E., Choi, K. C., Jeung, E. B., & Hyun, S. H. (2012). The effects of resveratrol on porcine oocyte in vitro maturation and subsequent embryonic development after parthenogenetic activation and in vitro fertilization. *Theriogenology*, *78*(1), 86-101. https://doi.org/10.1016/j.theriogenology.2012.01.024

Lambert, A. J., & Brand, M. D. (2009). Reactive oxygen species production by mitochondria. *Methods Mol Biol*, *554*, 165-181. https://doi.org/10.1007/978-1-59745-521-3_11

LaRosa, C., & Downs, S. M. (2006). Stress stimulates AMP-activated protein kinase and meiotic resumption in mouse oocytes. *Biol Reprod*, *74*(3), 585-592. https://doi.org/10.1095/biolreprod.105.046524

Laursen, L. S., Kjaer-Sorensen, K., Andersen, M. H., & Oxvig, C. (2007). Regulation of insulin-like growth factor (IGF) bioactivity by sequential proteolytic cleavage of IGF binding protein-4 and -5. *Mol Endocrinol*, *21*(5), 1246-1257. https://doi.org/10.1210/me.2006-0522

Laursen, L. S., Overgaard, M. T., Soe, R., Boldt, H. B., Sottrup-Jensen, L., Giudice, L. C., . . . Oxvig, C. (2001). Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. *FEBS Lett*, *504*(1-2), 36-40. http://www.ncbi.nlm.nih.gov/pubmed/11522292

Lawrence, J. B., Oxvig, C., Overgaard, M. T., Sottrup-Jensen, L., Gleich, G. J., Hays, L. G., . . . Conover, C. A. (1999). The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A. *Proc Natl Acad Sci U S A*, *96*(6), 3149-3153. https://doi.org/10.1073/pnas.96.6.3149

LeRoith, D., McGuinness, M., Shemer, J., Stannard, B., Lanau, F., Faria, T. N., . . . Roberts, C. T., Jr. (1992). Insulin-like growth factors. *Biol Signals*, *1*(4), 173-181. https://doi.org/10.1159/000109323

Lin, Q., Weis, S., Yang, G., Weng, Y. H., Helston, R., Rish, K., . . . Dennery, P. A. (2007). Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *J Biol Chem*, *282*(28), 20621-20633. https://doi.org/10.1074/jbc.M607954200

Loor, G., Kondapalli, J., Schriewer, J. M., Chandel, N. S., Vanden Hoek, T. L., & Schumacker, P. T. (2010). Menadione triggers cell death through ROS-dependent mechanisms involving PARP activation without requiring apoptosis. *Free Radic Biol Med*, *49*(12), 1925-1936. https://doi.org/10.1016/j.freeradbiomed.2010.09.021

Lorenzo, P. L., Illera, M. J., Illera, J. C., & Illera, M. (1994). Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation in vitro by the addition of epidermal growth factor and insulin-like growth factor I. *J Reprod Fertil*, *101*(3), 697-701. https://doi.org/10.1530/jrf.0.1010697

Mashek, D. G., Li, L. O., & Coleman, R. A. (2007). Long-chain acyl-CoA synthetases and fatty acid channeling. *Future Lipidol*, *2*(4), 465-476. https://doi.org/10.2217/17460875.2.4.465

Mazerbourg, S., Bondy, C. A., Zhou, J., & Monget, P. (2003). The insulin-like growth factor system: a key determinant role in the growth and selection of ovarian follicles? a comparative species study. *Reprod Domest Anim*, *38*(4), 247-258. http://www.ncbi.nlm.nih.gov/pubmed/12887564

Mazerbourg, S., & Monget, P. (2018). Insulin-Like Growth Factor Binding Proteins and IGFBP Proteases: A Dynamic System Regulating the Ovarian Folliculogenesis. *Front Endocrinol (Lausanne)*, *9*, 134. https://doi.org/10.3389/fendo.2018.00134

Miwa, S., & Brand, M. D. (2003). Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochem Soc Trans*, *31*(Pt 6), 1300-1301. https://doi.org/10.1042/bst0311300

Monget, P., Mazerbourg, S., Delpuech, T., Maurel, M. C., Maniere, S., Zapf, J., . . . Overgaard, M. T. (2003). Pregnancy-associated plasma protein-A is involved in insulin-like growth factor binding protein-2 (IGFBP-2) proteolytic degradation in bovine and porcine preovulatory follicles: identification of cleavage site and characterization of IGFBP-2 degradation. *Biol Reprod*, *68*(1), 77-86. https://doi.org/10.1095/biolreprod.102.007609

Monks, T. J., Hanzlik, R. P., Cohen, G. M., Ross, D., & Graham, D. G. (1992). Quinone chemistry and toxicity. *Toxicol Appl Pharmacol*, *112*(1), 2-16. https://doi.org/10.1016/0041-008x(92)90273-u

Morado, S. A., Cetica, P. D., Beconi, M. T., & Dalvit, G. C. (2009). Reactive oxygen species in bovine oocyte maturation in vitro. *Reprod Fertil Dev*, *21*(4), 608-614. https://doi.org/10.1071/RD08198

565 Moss, J. I., Pontes, E., & Hansen, P. J. (2009). Insulin-like growth factor-1 protects preimplantation
566 embryos from anti-developmental actions of menadione. *Arch Toxicol*, *83*(11), 1001-1007.
567 https://doi.org/10.1007/s00204-009-0458-3
568 Mueckler, M., & Thorens, B. (2013). The SLC2 (GLUT) family of membrane transporters. *Mol Aspects Med*,
569 *34*(2-3), 121-138. https://doi.org/10.1016/j.mam.2012.07.001
570 Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem J*, *417*(1), 1-13.
571 https://doi.org/10.1042/BJ20081386
572 Nasr-Esfahani, M. H., Aitken, J. R., & Johnson, M. H. (1990). Hydrogen peroxide levels in mouse oocytes
573 and early cleavage stage embryos developed in vitro or in vivo. *Development*, *109*(2), 501-507.
574 http://www.ncbi.nlm.nih.gov/pubmed/2401209
575 Nivet, A. L., Vigneault, C., Blondin, P., & Sirard, M. A. (2013). Changes in granulosa cells' gene expression
576 associated with increased oocyte competence in bovine. *Reproduction*, *145*(6), 555-565.
577 https://doi.org/10.1530/REP-13-0032
578 Noda, Y., Matsumoto, H., Umaoka, Y., Tatsumi, K., Kishi, J., & Mori, T. (1991). Involvement of superoxide
579 radicals in the mouse two-cell block. *Mol Reprod Dev*, *28*(4), 356-360.
580 https://doi.org/10.1002/mrd.1080280408
581 Nutter, L. M., Ngo, E. O., Fisher, G. R., & Gutierrez, P. L. (1992). DNA strand scission and free radical
582 production in menadione-treated cells. Correlation with cytotoxicity and role of NADPH quinone
583 acceptor oxidoreductase. *J Biol Chem*, *267*(4), 2474-2479.
584 http://www.ncbi.nlm.nih.gov/pubmed/1370822
585 Nyegaard, M., Overgaard, M. T., Su, Y. Q., Hamilton, A. E., Kwintkiewicz, J., Hsieh, M., . . . Giudice, L. C.
586 (2010). Lack of functional pregnancy-associated plasma protein-A (PAPPA) compromises mouse
587 ovarian steroidogenesis and female fertility. *Biol Reprod*, *82*(6), 1129-1138.
588 https://doi.org/10.1095/biolreprod.109.079517
589 Ramzan, R., Staniek, K., Kadenbach, B., & Vogt, S. (2010). Mitochondrial respiration and membrane
590 potential are regulated by the allosteric ATP-inhibition of cytochrome c oxidase. *Biochim Biophys*
591 *Acta*, *1797*(9), 1672-1680. https://doi.org/10.1016/j.bbabio.2010.06.005
592 Reed, M. L., Estrada, J. L., Illera, M. J., & Petters, R. M. (1993). Effects of epidermal growth factor, insulin-
593 like growth factor-I, and dialyzed porcine follicular fluid on porcine oocyte maturation in vitro. *J*
594 *Exp Zool*, *266*(1), 74-78. https://doi.org/10.1002/jez.1402660111
595 Richter, C., Gogvadze, V., Laffranchi, R., Schlapbach, R., Schweizer, M., Suter, M., . . . Yaffee, M. (1995).
596 Oxidants in mitochondria: from physiology to diseases. *Biochim Biophys Acta*, *1271*(1), 67-74.
597 http://www.ncbi.nlm.nih.gov/pubmed/7599228
598 Rieger, D., Luciano, A. M., Modina, S., Pocar, P., Lauria, A., & Gandolfi, F. (1998). The effects of epidermal
599 growth factor and insulin-like growth factor I on the metabolic activity, nuclear maturation and
600 subsequent development of cattle oocytes in vitro. *J Reprod Fertil*, *112*(1), 123-130.
601 https://doi.org/10.1530/jrf.0.1120123
602 Rivera, G. M., & Fortune, J. E. (2003). Proteolysis of insulin-like growth factor binding proteins -4 and -5 in
603 bovine follicular fluid: implications for ovarian follicular selection and dominance. *Endocrinology*,
604 *144*(7), 2977-2987. https://doi.org/10.1210/en.2002-0077
605 Roth, Z. (2018). Symposium review: Reduction in oocyte developmental competence by stress is
606 associated with alterations in mitochondrial function. *J Dairy Sci*, *101*(4), 3642-3654.
607 https://doi.org/10.3168/jds.2017-13389
608 Rottenberg, H., Covian, R., & Trumpower, B. L. (2009). Membrane potential greatly enhances superoxide
609 generation by the cytochrome bc1 complex reconstituted into phospholipid vesicles. *J Biol Chem*,
610 *284*(29), 19203-19210. https://doi.org/10.1074/jbc.M109.017376
611 Satrapa, R. A., Castilho, A. S., Razza, E. M., Pegorer, M. F., Puelker, R., & Barros, C. M. (2013). Differential
612 expression of members of the IGF system in OPU-derived oocytes from Nelore (Bos indicus) and
613 Holstein (Bos taurus) cows. *Anim Reprod Sci*, *138*(3-4), 155-158.
614 https://doi.org/10.1016/j.anireprosci.2013.02.023
615 Sawai, K., Kageyama, S., Moriyasu, S., Hirayama, H., Minamihashi, A., & Onoe, S. (2005). Analysis of mRNA
616 transcripts for insulin-like growth factor receptors and binding proteins in bovine embryos
617 derived from somatic cell nuclear transfer. *Cloning Stem Cells*, *7*(3), 189-198.
618 https://doi.org/10.1089/clo.2005.7.189
619 Slaaby, R., Andersen, A. S., & Brandt, J. (2008). IGF-I binding to the IGF-I receptor is affected by
620 contaminants in commercial BSA: the contaminants are proteins with IGF-I binding properties.
621 *Growth Horm IGF Res*, *18*(4), 267-274. https://doi.org/10.1016/j.ghir.2007.09.003

Soupene, E., & Kuypers, F. A. (2008). Mammalian long-chain acyl-CoA synthetases. *Exp Biol Med (Maywood)*, *233*(5), 507-521. https://doi.org/10.3181/0710-MR-287

Sovernigo, T. C., Adona, P. R., Monzani, P. S., Guemra, S., Barros, F., Lopes, F. G., & Leal, C. (2017). Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. *Reprod Domest Anim*, *52*(4), 561-569. https://doi.org/10.1111/rda.12946

Starkov, A. A. (2008). The role of mitochondria in reactive oxygen species metabolism and signaling. *Ann N Y Acad Sci*, *1147*, 37-52. https://doi.org/10.1196/annals.1427.015

Starkov, A. A., & Fiskum, G. (2003). Regulation of brain mitochondrial H2O2 production by membrane potential and NAD(P)H redox state. *J Neurochem*, *86*(5), 1101-1107. https://doi.org/10.1046/j.1471-4159.2003.01908.x

Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P. B., & Wolf, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod*, *64*(3), 904-909. http://www.ncbi.nlm.nih.gov/pubmed/11207207

Sukhanov, S., Higashi, Y., Shai, S. Y., Vaughn, C., Mohler, J., Li, Y., . . . Delafontaine, P. (2007). IGF-1 reduces inflammatory responses, suppresses oxidative stress, and decreases atherosclerosis progression in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol*, *27*(12), 2684-2690. https://doi.org/10.1161/ATVBAHA.107.156257

Takahashi, M., Nagai, T., Hamano, S., Kuwayama, M., Okamura, N., & Okano, A. (1993). Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos. *Biol Reprod*, *49*(2), 228-232. https://doi.org/10.1095/biolreprod49.2.228

Takami, M., Preston, S. L., Toyloy, V. A., & Behrman, H. R. (1999). Antioxidants reversibly inhibit the spontaneous resumption of meiosis. *Am J Physiol*, *276*(4), E684-688. https://doi.org/10.1152/ajpendo.1999.276.4.E684

Tamura, H., Takasaki, A., Miwa, I., Taniguchi, K., Maekawa, R., Asada, H., . . . Sugino, N. (2008). Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res*, *44*(3), 280-287. https://doi.org/10.1111/j.1600-079X.2007.00524.x

Valente, R. S., Almeida, T. G., Alves, M. F., Camargo, J., Basso, A. C., Belaz, K. R. A., . . . Sudano, M. J. (2019). Modulation of long-chain Acyl-CoA synthetase on the development, lipid deposit and cryosurvival of in vitro produced bovine embryos. *PLoS One*, *14*(8), e0220731. https://doi.org/10.1371/journal.pone.0220731

Wang, F., Tian, X., Zhang, L., He, C., Ji, P., Li, Y., . . . Liu, G. (2014). Beneficial effect of resveratrol on bovine oocyte maturation and subsequent embryonic development after in vitro fertilization. *Fertil Steril*, *101*(2), 577-586. https://doi.org/10.1016/j.fertnstert.2013.10.041

Winger, Q. A., de los Rios, P., Han, V. K., Armstrong, D. T., Hill, D. J., & Watson, A. J. (1997). Bovine oviductal and embryonic insulin-like growth factor binding proteins: possible regulators of "embryotrophic" insulin-like growth factor circuits. *Biol Reprod*, *56*(6), 1415-1423. http://www.ncbi.nlm.nih.gov/pubmed/9166693

Yang, S., Yang, Y., Hao, H., Du, W., Pang, Y., Zhao, S., . . . Zhao, X. (2022). Supplementation of EGF, IGF-1, and Connexin 37 in IVM Medium Significantly Improved the Maturation of Bovine Oocytes and Vitrification of Their IVF Blastocysts. *Genes (Basel)*, *13*(5). https://doi.org/10.3390/genes13050805

Zheng, X., Baker, H., Hancock, W. S., Fawaz, F., McCaman, M., & Pungor, E., Jr. (2006). Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. *Biotechnol Prog*, *22*(5), 1294-1300. https://doi.org/10.1021/bp060121o

Zimmermann, A. G., Gu, J. J., Laliberte, J., & Mitchell, B. S. (1998). Inosine-5'-monophosphate dehydrogenase: regulation of expression and role in cellular proliferation and T lymphocyte activation. *Prog Nucleic Acid Res Mol Biol*, *61*, 181-209. https://doi.org/10.1016/s0079-6603(08)60827-2

FIGURE LEGEND

Figure 1. Experimental design. Experiment 1: (a) Insulin-like growth factor-1 (IGF-1) and pregnancy associated plasma protein-A (PAPP-A) addition in bovine in vitro maturation (IVM). Immature *cumulus*-oocyte complexes (COCs) (50 COCs/group) were selected and randomly distributed among the groups: C (IVM medium), M (IVM medium + menadione), M/I (IVM medium + menadione + IGF-1), and M/I+P (IVM medium + menadione + IGF-I + PAPP-A). The COCs were matured for 24 h, and then the oocytes were separated to *cumulus* cells mechanically or followed to in vitro fertilization (IVF) and culture (IVC) until day 7. The IVM medium was collected to assess the total glutathione (GSH) content. (b) IGF-1 and PAPP-A addition in bovine IVC. Experiment 2: immature COCs (50 COCs/well) were selected and matured for 24 h, followed by IVF and IVC for 3 days in IVC medium. On day 4, all the structures were distributed among the groups: C (IVC medium), M (IVM medium + menadione), M/I (IVM medium + menadione + IGF-1), and M/l+P (IVM medium + menadione + IGF-/ + PAPP-A) and incubated for 24 h. On day 5, embryos were washed thrice in IVC medium and replaced in a four-well dish with the control IVC medium (without any treatment) until day 7. The IVC medium was collected at days 5 and 7 to assess the total GSH content.

Figure 2. Effects of insulin-like growth factor-1 (IGF-1) with or without pregnancy-associated plasma protein A (PAPP-A) on bovine *cumulus*-oocyte complex in vitro maturated in a stressed environment induced by menadione presence. The symbols + and - indicate presence and absence, respectively. (a) Representative image of oocytes stained by MitoTracker® Red CMXRos dye (red) and CellROX™ Green dye (green). (b) Pixels intensity of mitochondrial membrane potential (MMP) of oocytes (n=77-88 oocytes/group; 5 replicates; p=0.0001) detected by MitoTracker® Red CMXRos dye and reactive oxygen species (ROS) (n=123-132 oocytes/group; 5 replicates; P= 0.0001) detected using CellROX™ Green dye. The symbols + and - indicate presence and absence, respectively. Data are presented as mean ± standard error of the mean. Differences were considered significant when $P \leq 0.05$. Different letters indicate statistical differences. C: Control; M: Menadione; M/I: Menadione + IGF-1; M/I+P: Menadione + IGF-1 + PAPP-A.

Figure 3. mRNA relative abundance of genes differently expressed in the *cumulus* cells. The symbols + and - indicate presence and absence, respectively. Target genes were normalized with the reference genes (*ACTB, B2M, PPIA,* and *RPL30*) geometric mean using the ΔCt method. Data are presented as fold change [2^(-ΔCt)] ± standard error of the mean. Differences were considered significant when $P \leq 0.05$. Different letters indicate statistical differences. C: Control; M: Menadione; M/I: Menadione + IGF-1; M/I+P: Menadione + IGF-1 + PAPP-A.

Figure 4. Effects of insulin-like growth factor-1 (IGF-1) with or without pregnancy associated plasma protein-A (PAPP-A) during bovine in vitro culture on embryo development of blastocysts submitted to the stressed environment induced by menadione presence on day 4 post-in vitro fertilization for 24 h. The symbols + and - indicate presence and absence, respectively. (A) Representative image of blastocycts stained by hoechst 33342 (blue) and CellROX™ Green dye (green) (B) Percentage rate of blastocysts produced at day 7 (4 replicates) and pixels intensity of reactive oxygen species (ROS) detected using CellROX™ Green dye in the blastocysts (n=20-30/blastocysts/group; 4 replicates). Data are presented as mean ± standard error of the mean, and differences were considered significant when $P \leq 0.05$. Different letters

indicate statistical differences. C: Control; M: Menadione; M/I: Menadione + IGF-1; M/I+P: Menadione + IGF-1 + PAPP-A.

Figure 5. mRNA relative abundance of genes differently expressed in the blastocysts. The symbols + and - indicate presence and absence, respectively. Target genes were normalized with the reference genes (*ACTB, GAPDH, PPIA,* and *RPL30*) geometric mean using the 2^(-ΔCt) method. Data are presented as mean ± standard error of the mean. Differences were considered significant when $P \leq 0.05$. Different letters indicate statistical differences. C: Control; M: Menadione; M/I: Menadione + IGF-1; M/I+P: Menadione + IGF-1 + PAPP-A.

Figure 6. The proposed system with PAPP-A, a metalloprotease that cleaves the IGFBPs, as an enhancer of IGF-1 antioxidative role in IVP. The addition of IGF-1/PAPP-A in bovine IVM of COCs exposed to the stressed environment induced by menadione presence enhances the IGF-1 antioxidative role due to avoid the increased ROS concentration and controlling MMP levels in oocytes besides the downregulation of VNN1, a stress markers in *cumulus* cells. The IGF-1/PAPP-A association also showed to enhance the IGF-1 antioxidative role when applied in IVC. The proteins association increased the blastocyst yield and avoided the high ROS concentration in blastocysts besides modulated transcripts encoding proteins involved with antioxidative protection, lipid metabolism, and embryonic quality showing the PAPP-A is a feasible enhancer to IGF-1 antioxidative role. Red arrows: downregulation/decrease ($P < 0.05$); blue arrow: upregulation/increase ($P > 0.05$); green arrow: upregulation ($0.1 > P > 0.05$).

TABLE LEGEND

Table 1. Oocytes' nuclear stage and embryo development from COCs exposed to the stressed environment during IVM for 24 h with no treatment (M), IGF-1 (M/I), IGF-1/PAPP-A (M/I+P), and control (C).

C: control; IVM medium.

M: IVM medium + menadione (5 μM);

M/I: IVM medium + menadione + IGF-1 (100 ng/mL);

M/I+P: IVM medium + menadione + IGF-1 + PAPP-A (100 ng/mL).

Blastocyst yield: total of blastocysts/total of oocytes in IVM.

Abbreviations: TII, telophase II; MII, metaphase II.

Table 2. Total GSH content (mean ± SEM) in IVM medium and COCs submitted for 24h to the stressed environment with no treatment (M), IGF-1 (M/I), IGF-1/PAPP-A (M/I+P), and control (C).

C: control; IVM medium.

M: IVM medium + menadione (5 μM);

M/I: IVM medium + menadione + IGF-1 (100 ng/mL);

M/I+P: IVM medium + menadione + IGF-1 + PAPP-A (100 ng/mL).

771 *Cumulus* cells pool: *cumulus* cells from 10 COCs; n = 5 pools/group.

772 Medium samples: n = 5 samples of 20 µL.

773 Oocytes pool: 10 oocytes; n = 5 pools/group.

774 **Table 3.** Total GSH content (mean ± SEM) in IVC medium on days 5 and 7 and blastocyst
775 submitted for 24h to the stressed environment with no treatment (M), IGF-1 (M/I), IGF-
776 1/PAPP-A (M/I+P) and control (C).
777
778 C: control; IVC medium.

779 M: IVC medium + menadione (5 µM);

780 M/I: IVC medium + menadione + IGF-1 (100 ng/mL);

781 M/I+P: IVC medium + menadione + IGF-1 + PAPP-A (100 ng/mL);

782 Blastocyst pool: 5 blastocysts; n = 5 pools/group.

783 Medium samples: n = 5 samples of 20 µL.

784