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Review

Melatonin Scavenger Properties against Oxidative and Nitrosative Stress: Impact on *In Vitro* Embryo Production in Mammals

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Abstract: Oxidative and nitrosative stress are a common problem when manipulating gametes *in vitro*. *In vitro* development in mammalian embryos is highly affected by culture conditions, especially by reactive oxygen species (ROS) and reactive nitrogen species (RNS), because its absence or over production causes embryo arrest and changes in gene expression. Melatonin in gamete co-incubation during IVF has deleterious or positive effects depending on the concentration used in culture medium, demonstrating the delicate balance that must exist between antioxidant and pro-oxidant activity. Further research is needed to better understand the possible impact of melatonin on the different IVP steps in domestic animals, especially in seasonal breeds where this neuro-hormone system highly regulates its reproduction physiology.

Keywords: melatonin; antioxidants; RNS; ROS; embryo development; DNA integrity; DNA oxidation; gene expression

1. Introduction

1.1. Free radicals on reproduction (ROS/NOS)

The protocols of *in vitro* maturation, fertilization and embryo culture in assisted reproductive techniques (ART) have been greatly improved during the last decade. However, only a few embryos produced by ARTs are capable to carry out development to full term. This is mainly due to the lack of optimal *in vitro* embryo production conditions that cannot mimic to the *in vivo* conditions which in turn leads to several differences between both types of embryos, increasing the levels of ROS or RNS [1], among others. Both free radicals are generated as sub-products in physiological processes where the oxygen consumption is produced in the electron transport chain in cellular respiration [2].

There is a duality in the role of ROS and RNS. Physiological levels are needed in multiples process: ROS are necessary in human follicles to establish pregnancy [3], are potential markers in patients for predicting success of *in vitro* fertilization (IVF) [4], during the *in vitro* maturation of oocytes [5], in the resumption from diplotene arrest in oocytes [6], stimulating the release of intracellular Ca^{+2} in oocytes [7] or stimulating mitogen-activated protein kinase (MAPK) [8]. In sperm physiology, ROS participate in hyperactivation [9], sperm capacitation [10-14], through tyrosine phosphorylation [15]; and acrosome reaction [16].

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On the other side, RNS are necessary for the development of large antral follicles [17, 18], stimulate meiotic maturation in oocytes [19, 20], in the ovulatory process [21], in early folliculogenesis up to maturation [22] and in preimplantation embryo development [23, 24]. Also, RNS participate in sperm capacitation [25-27] and acrosome reaction [28]. However, when there is an imbalance between pro-oxidants molecules due to the increase of ROS/RNS levels within cells and/or the reduction of the antioxidant defense mechanisms, the phenomena called oxidative or nitrosative stress is triggered [29, 30].

1.2. *Oxidative stress*

Oxygen (O_2) is an essential element for aerobic organisms for which oxidative metabolism represents the main energy source. Partial reduction of O_2 results in ROS formation; these are molecules that contains one oxygen atom in their structure and possess at least one highly reactive unpaired electron in an outer orbital [31]. These molecules include two major groups: free radicals such as superoxide anion ($^{\bullet}O_2^-$) and hydroxyl radical ($^{\bullet}OH$); and molecules such as hydrogen peroxide (H_2O_2) [32]. The generation of $^{\bullet}O_2^-$ is the initial step for the formation of ROS, which is generated by acceptance of an electron by O_2, catalyzed by NADPH oxidase or xanthine oxidase. This radical can be converted in H_2O_2, by the action of superoxide dismutase (SOD), and then degraded to H_2O and O_2 by catalase or glutathione peroxidase [33]. Haber-Weiss reaction is the mechanism by which $^{\bullet}OH$ is generated. This reaction can generate more toxic free radicals through the interaction between $^{\bullet}O_2^-$ and H_2O_2 [29]. Fenton reaction also generates $^{\bullet}OH$ and consists of two reactions using iron ions (Fe^{+3} and Fe^{+2}) to generate this radical [29].

In pathological events, ROS has been involved in patients with endometriosis [34], in culture medium is negatively related with embryo implantation potential [35] or pregnancy [36], high levels of ROS are correlated with poor oocyte quality [37] and is associated with cell meiotic arrest [6].

We had investigate the induction of stress tolerance in bovine cumulus oocyte complexes (COCs) to generate oxidative stress resistance by incubation with H_2O_2 during the embryo production [38]. We observed that exposing COCs to low H_2O_2 levels could induce stress tolerance in these embryos, determined by embryo development, quality and gene expression pattern [39].

1.3. *Nitrosative stress*

Like ROS, RNS such as nitric oxide (NO) act as signaling molecules and modulate various aspects of reproduction physiology [40]; they influence and mediate the gametes and crucial reproductive processes such as sperm–oocyte interaction, implantation, and early embryo development [41]. Nitric oxide (NO) is generated either by a group of enzymes such as neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) [40, 42], or by non-enzymatic pathway from nitrite at low-pH under reducing conditions involving hydrogen peroxide and D-or L-arginine [40]. On the other hand, sustained high levels of RNS result in nitrosative stress and negative consequences for cells [40], leading to different pathologies [43]. The chemical reactivity of NO is rather low, but it reacts with $^{\bullet}O_2^-$ yielding peroxynitrite ($ONOO^-$) which is a potent oxidant that induces protein, lipid and DNA damage [44].

We had investigated the stress tolerance induction in oocytes to generate nitrosative stress resistance by incubation with NO donors during *in vitro* embryo production in bovine. However, the incubation of the oocytes either with 3-morpholinosidnonimine (SIN-1) [45] or with sodium nitroprusside (SNP) [46] did not generate differences on embryo quality or resistance to nitrosative stress.

Nowadays, scientific literature supports the administration of antioxidants compounds for counteracting oxidative and nitrosative stress in cells. Accordingly, we propose melatonin for its application on reproductive biotechnologies as is described below.

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1.4. Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a multifunctional molecule secreted by the pineal gland in response to environmental changes in light levels [43, 47]. It plays an important role in circadian sleep regulation [48] and reproductive function in seasonally breeding animals [49-51]. The melatonin pattern influences endocrine effects of photoperiod that result in physiological alterations in reproduction [52], also regulating the complex embryo-fetal developmental processes [53]. In fact, the cold and dark winter periods in Norway, may suppress ovarian activity and estrus expression in the cow, showing higher reproductive performance in the summer months compared to the winter season [54], as we had observed a decrease on cleavage rates and *in vitro* blastocyst production during winter season in our geographic zone [55].

Melatonin is a potent free radical scavenger [48, 56], quenching ROS directly and preventing the depletion of endogenous antioxidant enzymes [57]. It up-regulates gene expression and activity of several antioxidant proteins [57-60], preserves optimal mitochondrial function and homeostasis against oxidative stress [61], and also melatonin metabolites exhibit powerful antioxidant capacity [44, 62-65]. Melatonin readily combines with superoxide and liberates NO avoiding peroxynitrite formation, a free radical even more harmful than NO [66], and also is described as a direct peroxynitrite scavenger [67]. Here we present a summary of the last 5 years of the effect of melatonin on gametes and the different steps of the *in vitro* production of mammalian embryos (Table 1, Figure 1).

Table 1. Effect of melatonin on different steps of assisted reproductive techniques

Specie	Tissue	Treatment	Results	Reference
Porcine	Oocytes	10^{-9} M during *in vitro* maturation	Increases cleavage and blastocyst rate; and the total cell number of blastocyst. Also, it promotes the lipid metabolism, providing energy for oocyte maturation and embryo development.	[68]
Rat	Animal	Intraperitoneal injection of 20 mg/Kg for 4 weeks	Increases testosterone hormone in blood serum and body weight.	[69]
Human	Patient	3 mg per day since the fifth day of one cycle in women with diminished ovarian reserve	Increases the mean number of M-II oocytes, top-quality embryos with grade 1 and 2	[70]
Mouse	Spermatogonial stem cells	10 mg/Kg for 2 weeks after busulfan treatment	Relieves the loss and apoptosis in mouse testes. Also, it upregulates *MnSOD*	[71]
Mouse	Oocytes	10^{-9} to 10^{-3} M after *in vitro* maturation	Increases *in vitro* fertilization rate, reduces ROS and inhibits apoptosis	[72]
Bovine	Zygotes	1 μM for 3 hours after insemination and at 40°C	Reduces ROS levels in embryos	[73]
Mouse	Oocyte M-II	10^{-9} mol/L during vitrification/warming and PA	Increases blastocyst rate after warming, compared with control group.	[74]
Bovine	Sperm	10^{-3} M for 3 hours before *in vitro* fertilization	Improves the plasma membrane and acrosome integrity, mitochondrial activity and it decreases intracellular ROS levels. Also, it increases the blastocyst rate and it decreases apoptosis rate.	[75]
Human	Blastocyst	10^{-7} M in culture system in 3D (Encapsulation)	Increases the survival time of encapsulated embryos.	[76]
Bovine	Oocytes	10^{-6} or 10^{-9} M for 24 hours during *in vitro* maturation	In cumulus cells, it up-regulates *MnSOD* and *Cu-ZnSOD*. Also, it decreases fragmentation. In oocytes, it decreases ROS levels.	[77]
Human	Patient	3 mg for 14 days in patients with polycystic ovarian syndrome	Enhances the oocyte and embryo quality	[78]
Bovine	Embryos	10^{-7} M melatonin for 24 hr prior to exposure to 250 μM Paraquat (herbicide)	Decreases the incidence of apoptotic nuclei induced by Paraquat	[79]
Porcine	Oocytes	0.1 μM for 22-44h after endoplasmic reticulum stress during *in vitro* maturation	Improves oocyte maturation and cumulus cells expansion induced by endoplasmic reticulum stress	[80]
Bovine	Oocytes	Melatonin-loaded lipid-core nanocapsules at 10^{-6} M, 10^{-9} M and 10^{-12} M during *in vitro* maturation	Enhances *in vitro* embryo production, decreases ROS levels and the apoptotic nuclei, upregulates *GPX1* and *SOD2* and downregulates *CASP3* and *BAX*	[81]
Bovine	Zygotes	Melatonin-loaded lipid-core nanocapsules at 10^{-9} M during *in vitro* culture	Increases the hatching rate and embryo cell number, decreases cell apoptosis and ROS levels. Also, it downregulates *BAX*, *CASP3*, and *SHC1* genes, and upregulates *CAT* and *SOD2*.	[82]
Mouse	Oocyte	10^{-7} M during *in vitro* maturation	Improves the blastocyst rate and cell number of blastocysts	[83]
Mouse	Sperm	10 mg/kg body weight for 7 days during cadmium exposure	Reduces oxidative stress and inflammation induced by cadmium in male reproductive system	[84]
Mouse	Sperm	0.125 mg/mL in freezing extender during cryopreservation	Increases the progressive motility, decreases ROS levels and upregulates BCL-XL	[85]
Buffalo	Oocytes	250 μM during *in vitro* maturation	Improves fertilization rate	[86]
Bovine	Oocytes	1 μM during *in vitro* maturation of aged oocytes	Decreased the aberrant spindle organization, increases ATP production, increases the development of bovine oocytes and reduces apoptotic rate. Also, it downregulates BAX and CASP3 and increases BCL2.	[87]
Rabbit	Morula	10^{-3} M prior *in vitro* culture, prior vitrification	Promotes the blastocyst rate, it increases SOD activity and decreases LPO and NO levels.	[88]
Mouse	Preantral follicles	10 pM after vitrification, during culture	Increases diameter of follicles and their survival.	[89]
Bovine	Embryos produced by SCNT	10^{-11} to 10^{-2} M during *in vitro* culture	It increases the total cell number, ICM and the development of bovine SCNT embryos. Also, it suppress the expression of *p53* and *Bax*, upregulates *SOD1*, *Gpx4*, *BCL2L1* and *SOX2*.	[90]

Human	Sperm	0.01 mM in freezing extender before cryopreservation of sperm from infertile men	Increases motility and viability, decreases ROS and MDA levels	[91]
Porcine	Oocyte	10-7 M during *in vitro* maturation under heat stress	Improve polar body and blastocyst rate impaired by heat stress. Also it preserves normal levels of steroid hormone, reduces ROS, enhances GSH production and inhibits apoptosis.	[92]
Porcine	Oocyte and embryos	25 ng/mL during *in vitro* maturation and culture.	Increases blastocyst rate and decrease apoptotic nuclei in embryos.	[93]
Bovine	Sperm	1000 nmol	Increases higher wobbler coefficient, decreases sperm with intact acrosome and viable spermatozoa with ROS	[94]
Rabbit	Embryos	10^{-9} to 10^{-3} M during *in vitro* culture	Increases *in vitro* development and improves the hatching rate	[95]
Bovine	Zygotes	10^{-7} M during *in vitro* culture	Promotes the cleavage and blastocyst rate, accelerates the development of *in vitro* embryos and improves the quality of blastocysts.	[96]
Bovine	Zygotes	10^{-7} M for 2 days at the beginning of the *in vitro* culture	Increases the blastocysts and hatched blastocysts rate	[97]
Bovine	Zygotes	10^{-9} M for after 2 days of pre-culture and for the remaining 6 days of culture	Increases the blastocysts and hatched blastocysts rate	[97]
Bovine	GV oocytes	10^{-9} or 10^{-7} M during *in vitro* maturation	Improves the embryo development and the total cell number after *in vitro* fertilization. Also, it upregulates genes associated during *in vitro* maturation: *GDF9*, *MARF1* and *DNMT1α*.	[98]
Human	Animal	6 mg for 45 days	Increases the antioxidant capacity in seminal plasma, reduces the oxidative damage caused in sperm DNA, Also, it increases the quality of embryos	[99]
Mouse	2-cell embryos	10 μM during *in vitro* culture	Improves the quality and developmental rate of embryos. Also, it can prevent the cell death.	[100]
Rat	Sperm	10mg/kg weekly for 8 weeks	Improves sperm motility	[101]
Mouse	Embryos	10^{-12} M during *in vitro* culture of embryos produced by SCNT	Increases the embryo development	[102]
Ovine	Blastocysts	10-9 M during thawing after cryopreservation	Improves the embryo development after postwarming culture	[103]
Human	Patient	3 mg/day for 2 weeks	Increases the fertilization rate I the second cycle and improves the fertilization and good quality embryos rate.	[104]
Deer	Animal	Subcutaneous implantation of 40 mg	Elevates the serum FSH and LH levels, increases number of corpora luteal and the number of embryos	[105]
Human	Oocytes	10 μmol/L during *in vitro* maturation	Increases the preimplantation and pregnancy rate	[106]
Sheep	Animal	Subcutaneous implantation of 40 or 80 mg	Increases corpus luteal, the number of recovered embryos, the pregnancy and birth rates, and the lambs born per embryo	[107]
Porcine	Donor cell and embryos	10^{-10} M in the medium for donor cell and 10^{-9} M during *in vitro* culture of embryos produced by SCNT	Increases the proliferation of fetal fibroblasts, blastocysts rate, reduces the apoptotic nuclei. Also, it upregulates BCL2L1 and downregulates *BAX* and *p53*.	[108]
Mouse	Oocytes	10 to 100m nM during *in vitro* maturation	Increases the expansion, maturation, fertilization and blastocyst rate in a dose dependent manner.	[109]
Bovine	Oocytes	10^{-12} to 10^{-3} M during *in vitro* maturation under heat stress	Increases blastocyst rate of embryos submitted to heat stress	[110]
Murine	Pronuclear embryos	10^{-7} M during *in vitro* culture	Promotes embryo development, blastocyst rate, hatching rate and blastocyst cell number. Also, it improves the pregnancy rate. Even more, upregulates *SOD* and *BCL2* and downregulates *CAS3* and *p53*.	[111]
Ovine	Animal	Subcutaneous implant of 18 mg	Increases viability and pregnancy rate of undernourished ewes	[112]

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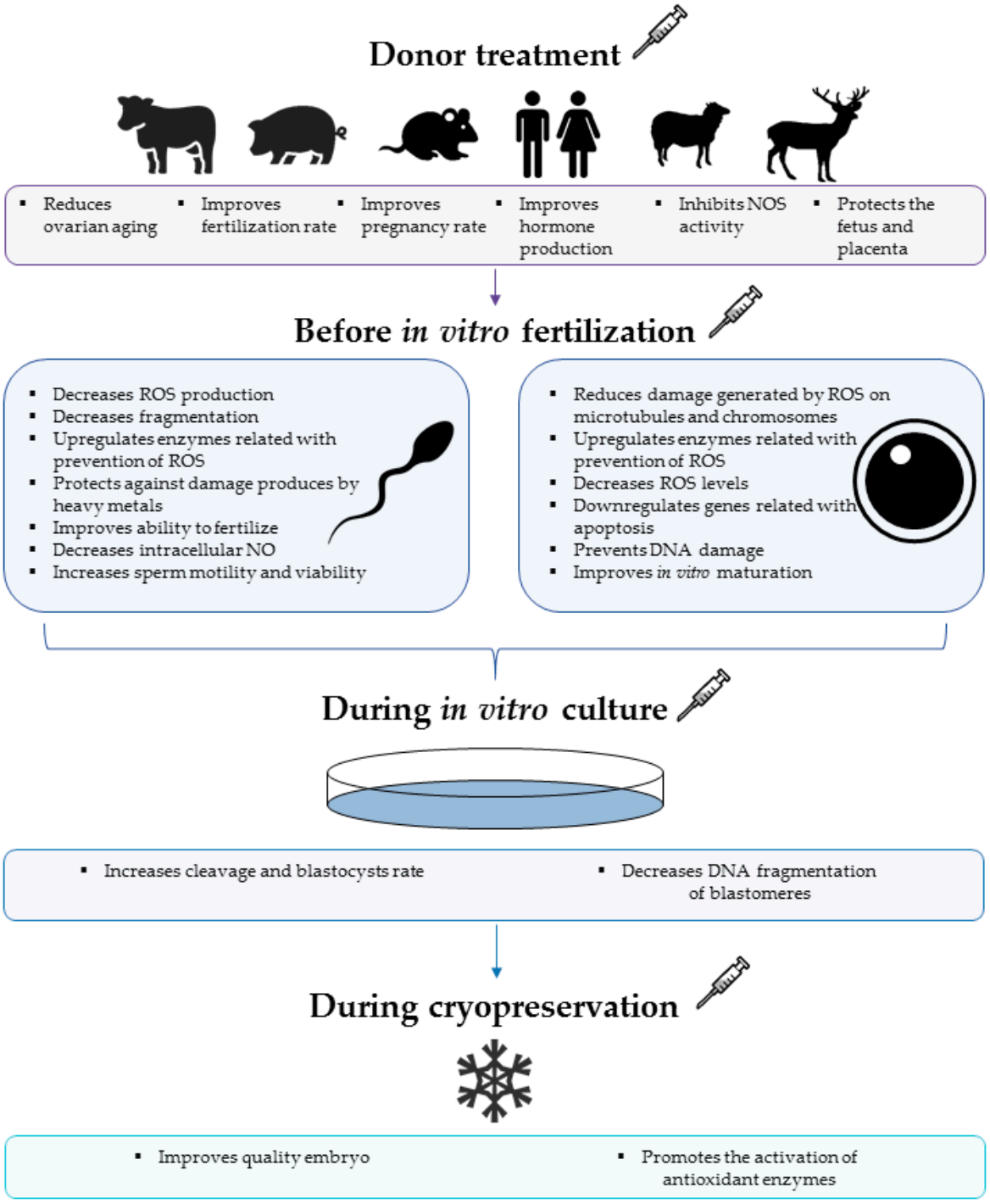

Figure 1. Melatonin application in different steps on gametes and *in vitro* embryo production and its effects.

2. Melatonin modulates oxidative stress on gametes and *in vitro* embryo production (IVP).

The ability of melatonin to pass the biological barriers due to its amphiphilic nature makes it an effective antioxidant for protecting macromolecules against ROS [65, 113]. In mammalian oocytes, melatonin can prevents the damage generated by hypochlorous acid (HOCl) on spindle microtubule and chromosome alteration in metaphase-II mouse oocytes [114], it can upregulates *MnSOD* [77, 115] and *Cu-ZnSOD* transcripts in cumulus cells [77], it decreases ROS levels in oocytes [77], it can

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suppress Bax protein expression and decreases Bax/Bcl-2 ratio in ovaries [115], prevents DNA damage [116] and decreases nuclear fragmentation in cumulus cells [77]. In human, long term treatments with melatonin reduce ovarian aging, increasing litter size, pool of follicles and telomere length [115]. Melatonin can protect the oocyte against the inhibitory effect of oxidative stress generated by H_2O_2 [116], leading to an increase on *in vitro* maturation rate [117], reducing the oxidative damage in oocytes during *in vitro* maturation and decreasing mitochondrial activity [117]. The mechanism by which melatonin promotes oocyte maturation is not clear, but it is believed to be mediated via melatonin membrane receptors, as the melatonin receptor agonist (IIK7) [98].

In follicular fluid, patients have shown an improvement in the fertilization and pregnancy rates after melatonin treatment [116] and melatonin levels are associated with oocyte quantity and quality [115, 118]. Also, melatonin improves progesterone production by corpus luteum in infertile women with luteal phase defect [119].

Spermatozoa are sensitive to oxidative stress, leading to apoptosis like process. Melatonin can decrease mitochondrial ROS production when sperm are exposed to oxidative stress [120], being a powerful antioxidant and anti-apoptotic agent in ejaculated human spermatozoa by inhibition of caspase-3 and caspase-9 activities [91, 120, 121]. Even more, melatonin can prevent mitochondrial ROS formation under basal conditions and at early time point upon oxidative stress induced by H_2O_2 exposure [122], increasing *MnSOD* expression [71], preventing DNA fragmentation [121] and therefore improving sperm quality [122]. As well as, melatonin supplementation of semen extenders increases sperm motility, viability, decreases ROS levels and lipid peroxidation [91], increasing sperm quality after the freezing-thawing processes [123]. This antioxidant compound can protect from testicular injury induced by oxidative stress after cadmium (Cd) exposure [84]. Also, melatonin helps to protect sperm from ROS induced by cell sorting, a widely used technique for *in vitro* fertilization or artificial insemination [123].

Our experience during supplementation of IVF medium with melatonin shows that this antioxidant has a dual effect over sperm function and embryo development in bovine [94]: lower concentrations (10 nM) modulates sperm quality by inducing changes on sperm motility increasing Wobbler coefficient. On the other hand, high melatonin concentration during sperm incubation (1000 nM) induced a decrease on viable sperm with intact acrosomes, induced high DNA fragmentation and high DNA oxidation than control, as a pro-oxidant. Accordingly, high melatonin concentrations in IVF (1000 nM) generated a decrease on blastocyst production but without affecting the embryo quality. During embryo culture, cells are exposed to higher oxygen concentrations, resulting in increased ROS production. Melatonin supplementation has a beneficial effect on *in vitro* fertilization in human patients [104, 116], improving blastocyst formation rate and decreasing DNA fragmentation of blastomeres [93].

Cryopreservation is a highly stressful process that reduces significantly the embryo developmental potential. Melatonin added to culture medium increases the cleavage and blastocyst rates [88, 124], increases hatching rate [103], increases the total cell number (TCN) [103] and improves trophectoderm (TE) and inner cell mass (ICM) ratio in vitrified embryos [124]. Also, melatonin reduces the apoptotic index [103, 124], promotes the activation of antioxidant enzymes like GST and SOD [88], decreases the level of oxidative substrates [88] and ameliorates the reduction of expression of important genes related in early embryo development, like NANOG and POU5F1 [88].

The evidence is clear that melatonin is involved in the protection against oxidative stress by scavenging free radicals, inducing the activity of antioxidant enzymes and preventing the induction of the mitochondrial pathway of apoptosis, improving gamete and embryo quality both in human and domestic animals during ART.

3. Potential use of melatonin against nitrosative stress during ART

Melatonin has been described to reduce nitrosative/oxidative stress in many different tissues and organelles [65], supporting its protective effect against drugs, toxins, metals and herbicides [125]. Melatonin acts on the NO/NOS system by reducing peroxynitrite formation in the brain in the

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first steps of the ischemic cascade, influencing the NO/NOS pathway and reducing oxidative and nitrosative stress [42]. During acute renal failure high levels of NO are produced by iNOS due to ROS/RNS activation, but it can be counteracted with melatonin as strong antioxidant and iNOS inhibitor agent as well as a scavenger of peroxynitrite, attenuating lipid peroxidation and protein oxidation in the kidneys [126]. Similarly, melatonin administration counteracted iNOS activation and mitochondrial damage in the liver during sepsis [127]. Melatonin preserves fetal growth in rats by protecting against ischemia/reperfusion-induced oxidative/nitrosative stress by preventing the oxidative damage in placental DNA and mitochondria [61]. Also, neuroprotective effect of melatonin is described by counteracting i-mtNOS induction, oxidative stress, and mitochondrial dysfunction [128].

Melatonin has shown to be protective for gamete handling *in vitro.* Proposed function of melatonin in the Graafian follicle are inhibiting the activity of the pro-oxidative enzyme nitric oxide synthase (NOS) [51]. Melatonin delays ovarian aging by multiple mechanisms including antioxidant action, reducing declines in oocyte quantity and quality in mice [129]. Accordingly, melatonin could be useful against nitrosative stress due to *in vitro* maturation of the oocytes.

A beneficial effect on male fertility is described for human and domestic animals: this compound induces a significant decrease on intracellular NO in human sperm, increasing sperm motility and viability [43]. Also, NO is one of the factors that changes during the annual reproductive cycle of the male adult buffalo: NO is mainly present in the caput epididymis during short photoperiods coinciding with maximum gonadal activity [130]. According to this, and considering the influence of melatonin on seasonal reproduction in these animals we can suggest the potential use of melatonin to modulate NO levels to increase buffalo fertility or in other seasonal breeders, both during semen storage or IVF.

On the other hand, ART can induce vascular dysfunction and arterial hypertension related to epigenetic alterations of the regulation of the eNOS gene; however this can be prevented by addition of melatonin during *in vitro* culture of embryos which doubled the success rate of IVF, prevented eNOS demethylation and normalized NO plasma concentration [131].

Melatonin has been found to protect the fetus and placenta from oxidative stress due to ROS and RNS [51]. On this way, melatonin could be a useful clinical treatment to increase or maintain umbilical blood flow by NO-dependent mechanisms in complicated pregnancy [66], as after embryo transfer of *in vitro* produced embryos (ET/IVP) in domestic animals.

Despite that more specific researches about melatonin effect against nitrosative stress in reproductive biotechnologies are scarce; melatonin has demonstrated direct and indirect beneficial effect against ROS. Therefore, considering that ROS can generate RNS, we can deduct that melatonin could have a protective action over nitrosative stress during gamete and embryo handling in the laboratory as it had been demonstrated in other tissues.

There is a long list of studies that support the use of melatonin against oxidative stress, however much remains to be investigated regarding the role that melatonin might have on nitrosative stress during *in vitro* manipulation and cryopreservation of gametes and embryos.

4. Conclusions

This review summarizes the experimental data published in literature about melatonin and its potential use against ROS/NOS as a powerful antioxidant for improving gamete and embryo quality in domestic animals.

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6. Author Contributions:

Raúl Sánchez conceived this paper; Pía Loren and Carolina Cheuquemán wrote this paper; María Elena Arias, Ricardo Felmer and Jennie Risopatrón revised the paper.

7. Conflict of Interest

None of the authors have any conflict of interest to declare.

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