Asian Pacific Journal of Reproduction

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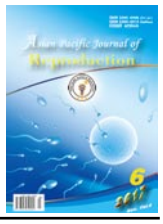

doi: 10.4103/2305-0500.217317

Melatonin as an antioxidant preserving sperm from domestic animals

Alfredo Medrano[1]✉, Carlos F BSc Contreras[1], Fernando MSc Herrera[2], Alicia MSc Alcantar-Rodriguez[1]

[1]*Department of Animal Sciences, Faculty of Superior Studies Cuautitlan, National Autonomus University of Mexico*

[2]*Posgraduate Master Programme on Animal Production and Health Sciences, National Autonomus University of Mexico*

ARTICLE INFO

Article history:
Received 30 August 2017
Revision 12 September 2017
Accepted 30 October 2017
Available online 1 November 2017

Keywords:
Semen
Sperm viability
Freeze-thawing
ROS

ABSTRACT

The role of melatonin on sperm function as well as its use as antioxidant for sperm conservation is analysed in this review. Melatonin has been included in the cooling/freezing media for the conservation of spermatozoa. Depending on the animal species, the best dose to improve sperm quality and fertile capacity is in the range from 0.01 mM to 3.00 mM. Since the work started on the use of melatonin as antioxidant for the conservation of spermatozoa (2011), a search for references was done on the subject using internet and our university libraries: journals, proceedings, thesis, *etc*. The search focused on animal spermatozoa, but a collection of papers on human spermatozoa was also carried out.

1. Introduction

Sperm preservation and artificial insemination (AI) are important tools of animal reproductive biotechnologies. In most of domestic animal species, AI is a fundamental instrument for genetic progress[1], but it also provides a mean to solve mating problems for other species (such as dogs)[2]. Sperm cryopreservation is mainly used to carry out AI programs, and to keep and transport genetic material for the conservation of several animal species[3].

Different protocols for the cryopreservation of spermatozoa have been developed. However, fertility obtained by AI employing frozen-thawed spermatozoa is usually lower than that by natural mating or AI using either fresh or cooled spermatozoa[4]. This low fertility is mainly attributed to a series of morphological and physiological changes sperm suffer during cryopreservation[3,5]. These changes are produced by drastic variations, thermal and osmotic, as well as the excessive production of the so-called reactive oxygen species (ROS), which produce oxidative stress during the cryopreservation process. Damaging effects of ROS on sperm include cryocapacitation, lipid peroxidation, acrosome injuries, metabolic alterations, DNA denaturation, and loss of fertilizing capacity[3-7].

For this, the inclusion of antioxidants in the cooling/freezing media for sperm preservation may significantly improve sperm survival. Antioxidants are substances capable of retarding or preventing the oxidation of any oxidizing substrate. Different compounds have been used to accomplish that role. One of them is melatonin natural hormone that participates in several steps of sperm physiology, and has improved sperm viability and fertility when it is added to sperm extenders in many cases.

✉First and corresponding author: Alfredo Medrano, FES Cuautitlán - Universidad Nacional Autonoma de Mexico, Carretera Cuautitlán-Teoloyucan s/n Cuautitlán Izcalli, Edo México 54714, Mexico.
E-mail: amedrano@unam.mx

How to cite this article: Alfredo Medrano, Carlos F BSc Contreras, Fernando MSc Herrera, Alicia MSc Alcantar-Rodriguez. Melatonin as an antioxidant preserving sperm from domestic animals. Asian Pac J Reprod 2017; 6(6): 241-246.

2. Oxidative stress in the spermatozoa

Oxidative stress in the spermatozoa refers to the damage these cells may suffer on their morphology and functionality caused by the formation of high amounts of free radicals molecules, such as the so-called ROS present during semen manipulation, processing and cryopreservation[8]. Free radicals molecules include ROS, transition metals, and reactive nitrogen species, but ROS are the most damaging for cells[9,10].

Free radicals are generated *in vivo* as by-products of normal cell metabolism. This production is associated with physiological processes or accidental production, especially in animal cells[11]. However, under controlled conditions, ROS play important functions such as intra- or inter-cell signalling[12].

In the sperm, normal production of ROS is involved in the activation of motility by increasing synthesis of cyclic adenosine monophosphate and protein phosphorylation[13]. Also, ROS participate in sperm maturation, capacitation, hyperactivation, acrosome reaction, and the interaction with the oocyte. All of these roles mediated by cyclic adenosine monophosphate and tyrosine phosphorylation[8,9]. However, ROS must be continuously inactivated to keep cell physiological balance.

Other generating sources of ROS are the presence of leucocytes in the semen as well as immature, immotile, or abnormal spermatozoa[14]. Oxidative stress by increasing amounts of ROS may directly damage sperm plasma membrane, which will become disorganized and in consequence associated receptors and enzymes are inactivated. These changes will modify membrane fluidity and permeability, and finally may cause cell death[15]. Other side effects of ROS oxidative stress are loss of motility, acrosome integrity, and mitochondrial membrane potential[16].

Damage to sperm DNA is another important negative effect of oxidative stress since it may cause different degrees of infertility. DNA fragmentation refers to both denaturation and damage that may not be repaired[17]. Mature spermatozoa are unable to repair any damage to DNA. However, small but not extended damage to DNA may be repaired by the fertilized oocyte or embryo[18].

3. Oxidative stress and sperm cryopreservation

Increased production of ROS during cryopreservation has been widely documented. ROS produced before, during or after cryopreservation are responsible for sperm oxidative stress. However, loss of sperm antioxidant activity caused by structural damage to sperm cytoskeleton as well as the alteration of antioxidant enzymes may also contribute[15].

Sperm oxidative stress is promoted by some processes of cryopreservation such as centrifugation and dilution since they either remove or dilute antioxidant compounds present in seminal plasma[19,20].

For this, the inclusion of antioxidants in the cooling/freezing media for sperm may significantly improve sperm survival. However, it should be considered oxidative stress is one part of the complex stress sperm, which is subjected during cryopreservation[21]. Antioxidants are substances capable of retarding or preventing the oxidation of any oxidizing substrate such as lipids, proteins, and DNA[22]. The basic mechanism of action is to avoid oxygen to bind to other molecules in a specific microenvironment[22].

The natural antioxidant protection for the sperm begins to work since early spermatogenesis, and continues during maturation in the epididymis. Due to cytoplasm reduction during spermatogenesis, the amount of antioxidant enzymes is limited in the ejaculated sperm. However, it is compensated by antioxidants present in seminal plasma[23].

To minimize damage to the sperm caused by ROS during cryopreservation, many compounds and different protocols of adding them to cooling/freezing media have been tested. Antioxidants, catalase, superoxide dismutase, α-tocopherol, ascorbic acid, carotenoids, and melatonin are some examples[24–26]. In most of the cases, the addition of antioxidants to sperm extenders has improved sperm cryosurvival.

4. Melatonin

Melatonin is a lipophilic molecule derived from tryptophan, present in vertebrates, invertebrates, bacteria, unicellular organisms, and even in plants[27]. It is produced in the pineal gland (principal site), cerebellum, retina, skin, gastrointestinal cells, Harder's gland, thymus, mononuclear peripheral cells, placenta, ovary, testicle, bone marrow, liver, hippocampus, and platelets[27,28].

Melatonin produced in the pineal gland is not stored but directly secreted to both cerebrospinal fluid and cardiovascular system[28]. It may move freely through morph-physiological barriers such as the blood-brain and placenta, thus it may be distributed along the whole body[29,30]. In mammals, melatonin plays a series of biological functions such as regulation of circadian cycles, signalling for

seasonal reproduction, antioxidant, and immunomodulation[31–37].

Extra-pineal melatonin apparently does not pass to blood in relevant quantities but it serves locally as antioxidant, and as local intra- or inter-cell signalling[31,38]. Tissue melatonin levels are higher than those in blood[34].

4.1. Antioxidant properties of melatonin

As mentioned, at cellular level melatonin plays a role as antioxidant based on its capacity to sequester and neutralize free radicals; the direct mechanism is by electron donation[31]. Therefore, melatonin should be in the vicinity of the free radicals at the moment when they are formed; due to its lipophilic structure, melatonin may work efficiently in several cellular compartments[39].

Some metabolites produced by the interaction of melatonin with free radicals keep a high antioxidant capacity themselves. The relationship between the antioxidant capacity of melatonin and that of its metabolites is known as antioxidant cascade[31,34,40].

Melatonin may also play indirect actions though the stimulation of several antioxidant enzymes such as glutathione peroxidase/reductase, catalase, and superoxide dismutase[37,41].

4.2. Effects of melatonin on the spermatozoa

In the sperm, melatonin reduces oxidative damage in the mitochondria, DNA fragmentation, peroxidation of plasma membrane lipids, and apoptotic markers by improving antioxidant activity of enzymatic systems, and reducing ROS levels[42–45]. In addition to the presence of melatonin in seminal plasma[44,46], it has been demonstrated a direct action of melatonin as a modulator of sperm capacitation[47–49]. Melatonin effect may be due to its antioxidant properties. In this context, melatonin has a high capacity to eliminate ROS, while certain levels of ROS are necessary for sperm capacitation[8].

Thus, high levels of melatonin as those present at physiological levels in seminal plasma may protect sperm from oxidative damage, and prevent capacitation at the same time[44]. Nevertheless, when sperm are in the female reproductive tract seminal plasma is removed, and they are exposed to lower concentrations of melatonin; also, melatonin normally present in follicular fluid is diluted when it passes throughout the oviduct, and thus capacitation takes place[50]. It has been proposed that melatonin may regulate capacitation by means of binding to calmodulin into sperm cytoplasm; calmodulin participates in several sperm physiological processes such as hyperactivation, capacitation, and acrosome reaction[51].

Melatonin may also exert its actions by direct binding to specific receptors on sperm plasma membrane, and two of them from mammals have been reported: MT1 and MT2[48]. These receptors, in particular MT2, seem to be involved in sperm capacitation[48,52]. Since melatonin regulates bicarbonate secretion and mobilization of intracellular calcium in some somatic cells, it is reported that melatonin may regulate sperm capacitation in a similar way[28].

4.3. Effects of melatonin on sperm preservation

The role of melatonin on sperm preservation is based on its antioxidant and antiapoptotic properties that may either improve or maintain sperm quality[53,54]. There are several reports on the use of melatonin as antioxidant added to the sperm media in different species. Jang *et al*[37] added 100 nM melatonin to boar spermatozoa then diluted in a commercial extender, and sperm were kept at 37 ℃ for 12 h; values of motility, plasma membrane integrity, and mitochondrial activity were higher in the melatonin-treated spermatozoa than in those without melatonin. Also, the rate of development of embryos produced by *in vitro* fertilization using melatonin-treated spermatozoa increased significantly.

Succu *et al*[55] added respectively 0.001, 0.010, 0.100, 1.000, and 10.000 mM melatonin to a freezing extender for ram spermatozoa; values of sperm motility, DNA integrity, intracellular concentration of ATP, and fertilization rate after freeze-thawing were higher in the sperm supplemented with 1.000 mM melatonin than in the sperm from the other groups. In contrast, Souza *et al*[56] added 100 pM, 100 nM, and 100 μM melatonin to a freezing extender for ram spermatozoa; values of sperm motility, plasma membrane integrity, acrosome integrity, and mitochondrial activity after freeze-thawing were higher in the sperm supplemented with 100 pM melatonin than in the sperm from the control group (0 mM melatonin). This beneficial effect on sperm motility may be attributed to protection of the mitochondrial function by melatonin[57,58].

Ashrafi *et al*[59] added respectively 0.0, 0.1, 1.0, 2.0, 3.0, and 4.0 mM melatonin to a freezing extender for bull spermatozoa; values of sperm motility, viability, plasma membrane integrity, normal sperm, and enzymes activity (superoxide dismutase and catalase) improved significantly as melatonin concentration increased. These authors concluded that the addition of melatonin at 2.0 and 3.0 mM to bull semen freezing extender may improve sperm cryosurvival. This beneficial effect of melatonin may be explained by the reduction of lipid peroxidation which is due to an increase in the total antioxidant capacity of the spermatozoa. El-Raey *et al*[60] added respectively 0.10 and 0.25 mM melatonin to a freezing extender for buffalo spermatozoa; values of sperm motility, plasma membrane integrity, acrosome integrity, and conception rate after freeze-thawing were

higher in the sperm supplemented with 0.10 and 0.25 mM melatonin than in the sperm from the control group (0 mM melatonin).

Lanconi *et al*[61] added 1 mM melatonin to a freezing extender for horse spermatozoa; values of sperm plasma membrane integrity, acrosome integrity, and high mitochondrial activity improved after freeze-thawing in the sperm supplemented with 1 mM melatonin but no in the control group (0 mM melatonin). Affonso *et al*[62] added 1 μM melatonin to a cooling extender (skim milk medium) for horse spermatozoa; after 8 h of cooling at 5 ℃, percentages of spermatozoa showed intact plasma and acrosomal membranes, and high mitochondrial potential were higher than those from the control group (0 mM melatonin).

Karimfar *et al*[63] added respectively 0.001, 0.005, 0.010, 0.050, 0.100, and 1.000 mM melatonin to a freezing extender for human spermatozoa; values of sperm motility, viability, and reduction of intracellular concentration of ROS after freeze-thawing were higher in the sperm supplemented with 0.010 mM melatonin than in the sperm from the other groups.

All these reports indicate that protective effect of melatonin on sperm quality, cryosurvival, and fertile capacity is dependent on melatonin inclusion level, both between and within animal species.

In dogs, the effect of melatonin on sperm cryosurvival is not as clear as in other species. Varesi *et al*[64] added 1 mM melatonin to a freezing extender for dog sperm collected from the epididymis; values of motility and acrosome integrity were similar after freeze-thawing for sperm diluted in 0 mM or 1 mM melatonin. The authors have cryopreserved spermatozoa from both German and Belgian Shepherd dogs, adding 1 and 2 mM melatonin to the freezing extender (egg yolk, Tris, 5% glycerol); values of motility, plasma membrane integrity, acrosome integrity, capacitation status, and membrane fluidity after freeze-thawing in supplemented groups were similar to 0 mM melatonin group.

The authors have also tested the effect of melatonin on sperm quality during cooling and storage at 5 ℃ for 48 h. Melatonin 1 mM and 2 mM were added respectively to the cooling extender for ram[25], and goat buck[65] spermatozoa. Ram sperm quality was better in those treatments supplemented with melatonin; in contrast, sperm quality was similar between goat buck with or without melatonin, throughout storage at 5 ℃. In the case of goat buck, a negative effect of DMSO (employed to dissolve melatonin) on sperm quality was detected; in that work, control treatments were the freezing extender without or with DMSO/PBS (1:9 v/v) added at 50 μL per mL of diluted spermatozoa. Similar observation was reported by Gwayi and Bernard[66] in rat spermatozoa, where ethanol (0.5% final concentration) was employed to dissolve melatonin. In contrast, Martín-Hidalgo *et al*[67] found no negative effects of ethanol (0.01%) on boar spermatozoa.

On the other hand, the effect of melatonin on sperm quality during refrigeration has also been tested. Martín-Hidalgo *et al*[67] added 1 μM melatonin to a commercial extender for boar spermatozoa, and kept them at 17 ℃ for up to 7 d. It was concluded that melatonin did not improve boar sperm function.

Inclusion of high levels of melatonin on semen extenders may reduce sperm fertilizing potential after freeze-thawing; since ROS production is excessively neutralized, oxidative phosphorylation may be inhibited, and in consequence sperm motility and viability are reduced[56]. For this, it is important to identify the optimum dose of melatonin for the conservation of sperm from the different animal species.

Melatonin is a universal antioxidant presented in animal and vegetal cells. It is involved in several sperm functions such as the activation of motility, sperm maturation, capacitation, hyperactivation, and the acrosome reaction. It has been tested as an antioxidant for the conservation of spermatozoa from different animal species, and the effectivity seems to depend on the dose at which is employed. Usually, when an excessive dose of melatonin is added to the sperm cooling/freezing extender, sperm fertility may be reduced.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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