Melatonin in maturation media fails to improve oocyte maturation, embryo development rates and DNA damage of bovine embryos

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ABSTRACT: Melatonin (MEL) acts as a powerful scavenger of free radicals and direct gonadal responses to melatonin have been reported in the literature. Few studies, however, have evaluated the effect of MEL during in vitro maturation (IVM) on bovine embryos. This study tested the addition of MEL to maturation medium (MM) with no gonadotropins on nuclear maturation and embryo development rates and the incidence of DNA damage in resulting embryos. Cumulus-oocyte complexes were aspirated from abattoir ovaries and cultured in MM (TCM–199 medium supplemented with 10% fetal calf serum - FCS) at 39°C and 5% CO_2 in air. After 24 hours of culture in MM with 0.5 µg mL^{-1} FSH and 5.0 µg mL^{-1} LH; 10^{-9} M MEL) or 10^{-9} M MEL, 0.5 µg mL^{-1} FSH and 5.0 µg mL^{-1} LH, the oocytes were stained with Hoechst 33342 to evaluate nuclear maturation rate. After in vitro fertilization and embryo culture, development rates were evaluated and the blastocysts were assessed for DNA damage by Comet assay. There was no effect of melatonin added to the MM, alone or in combination with gonadotropins, on nuclear maturation, cleavage and blastocyst rates. These rates ranged between 88% to 90%, 85% to 88% and 42% to 46%, respectively. The extent of DNA damage in embryos was also not affected by MEL supplementation during IVM. The addition of 10^{-9} M MEL to the MM failed to improve nuclear maturation and embryo development rates and the incidence of DNA damage in resulting embryos, but was able to properly substitute for gonadotropins during IVM.
Key words: comet assay, embryos, gonadotropins, in vitro fertilization, melatonin

Melatonina no meio de maturação não melhorou as taxas de maturação dos ovócitos, de desenvolvimento embrionário e a fragmentação do DNA dos embriões bovinos

RESUMO: Melatonin (MEL) atua como um potente redutor de radicais livres. Efeito direto da MEL na função gonadal também foi observado. Existem poucos estudos relacionados ao efeito da MEL durante a maturação no desenvolvimento embrionário in vitro. Avaliou-se a adição de MEL no meio de maturação (sem gonadotrofinas) nas taxas de maturação nuclear e de desenvolvimento embrionário e na incidência de fragmentação do DNA nos embriões produzidos in vitro. Complexos cumulus-ovócitos foram aspirados de ovários provenientes de matadouros e cultivados em meio de maturação (Meio TCM–199 suplementado com 10% de soro fetal bovino) a 39°C e 5% CO_2 em ar. Após 24 horas de cultivo em meio de maturação com 0,5 µg mL^{-1} de FSH e 5,0 µg mL^{-1} de LH; 10^{-9} M de MEL ou 10^{-9} M de MEL, 0,5 µg mL^{-1} de FSH e 5,0 µg mL^{-1} de LH, os ovócitos foram corados com Hoechst 33342 para avaliação da taxa de maturação nuclear. A fragmentação do DNA dos blastocistos foi avaliada pela técnica do Cometa. Não houve efeito da MEL adicionada ao meio de maturação, com ou sem gonadotrofinas, nas taxas de maturação nuclear, clivagem e blastocistos. Os percentuais variaram entre 88% a 90%, 85% a 88% e 42% a 46%, respectivamente. O grau de fragmentação do DNA também não foi afetado pela adição de MEL ao meio de maturação. A adição de 10^{-9} M de MEL ao meio de maturação não exerce influência nas taxas de maturação nuclear e de desenvolvimento embrionário e nem da incidência de fragmentação do DNA dos embriões produzidos in vitro, mas pode substituir as gonadotropinas durante a maturação in vitro.
Palavras-chave: ensaio cometa, embriões, gonadotrofinas, fertilização in vitro, melatonina

Introduction

Oocyte maturation is one of the most important steps for successful in vitro production of bovine embryos (Dieleman et al., 2002). However, the in vitro maturation system is not as efficient as in vivo maturation (Hendriksen et al., 2000). One important factor known to influence the in vitro maturation process in mammals is the culture medium used for oocyte maturation. The composition of in vitro maturation medium affects oo-

cyte and embryo developmental competence, e.g., the stage of nuclear maturation, cleavage and blastocyst rates (Knijn et al., 2003).

The basic maturation media used in bovine embryo culture systems are generally composed of TCM–199 medium supplemented with a combination of FSH, LH and fetal calf serum (FCS) (Sargirkaya et al., 2007; Adona et al., 2008; Corrêa et al., 2008). It has been reported that FSH and LH added to maturation medium have a beneficial effect on the competence of oocytes to develop in vitro to the blastocyst stage (Papis et al., 2007; Adona et al., 2008; Corrêa et al., 2008). Although producing controversial results (Holm et al., 2002), FCS is successfully used as protein supplement in oocyte maturation medium (Sargirkaya et al., 2007; Adona et al., 2008; Corrêa et al., 2008).

The protective role of free radical scavengers in maturation culture medium has been documented (Tsantarioutou et al., 2007; Kang et al., 2009; Manjunatha et al., 2009). Melatonin acts as a potent scavenger of free radicals (Tan et al., 2007) and a direct gonadal response to melatonin has been also reported, including stimulation of progesterone (Adriaens et al., 2006) and estradiol synthesis (Sirotkin and Schaeffer, 1997), and improvement of oocyte quality and maturation (Chattorag et al., 2005; Kang et al., 2009). Although there are some studies describing the beneficial effect of melatonin on in vitro embryo development in different species (Ishizuka et al., 2000; Papis et al., 2007; Rodriguez-Osorio et al., 2007), most of them report the addition of melatonin in embryo culture medium and in those which study the effect of melatonin during in vitro maturation, the maturation media were also supplemented with gonadotropins (Chattoraj et al., 2005; Kang et al., 2009; Manjunatha et al., 2009).

The present work investigated whether melatonin added to a gonadotropin-free maturation medium affects nuclear maturation and embryo development rates as well as the incidence of DNA damage in the resulting embryos.

Material and Methods

Chemicals and reagents

Media components were cell-culture tested or Molecular Biology grade. Water was obtained from a water purification system. In all experiments, the same batch of fetal calf serum (FCS) was used.

In vitro maturation, fertilization and embryo culture procedures

The in vitro culture procedures were based on methods described previously (Adona et al., 2008; Corrêa et al., 2008). Ovaries obtained from slaughterhouse cows were transported to the laboratory in saline solution (NaCl 0.9%) supplemented with 100 UI mL⁻¹ penicillin G and 100 μg mL⁻¹ streptomycin sulfate at 35°C within 2 h. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 6-mm follicles with an 18 gauge needle and a 10 mL syringe and the aspirated follicular fluid was pooled in a 15 mL conical tube. After sedimentation, recovered COCs with homogeneous cytoplasm and at least three layers of cumulus cells were selected and rinsed three times in Hepes buffered TCM–199 medium + 10 μg mL⁻¹ gentamycin sulfate. The maturation procedure was performed in TCM–199 medium supplemented with 0.3 mol L⁻¹ sodium pyruvate, 10% fetal calf serum (FCS) and 10 μg mL⁻¹ gentamycin sulfate (maturation medium) and COCs were incubated for 24 h at 39°C under 5% CO_2 in air.

For in vitro fertilization (IVF), frozen-thawed semen from the same bull was prepared according to the discontinuous Percoll gradient technique (Parrish et al., 1994). The separated sperm was rinsed twice in TALP medium and then resuspended to a final concentration of 1×10^6 sperm cells mL⁻¹ in IVF medium. The IVF medium was TALP medium supplemented with 2 mol L⁻¹ penicillamine, 1 mol L⁻¹ hipotaurine, 250 mol L⁻¹ epinephrine and 10 μg mL⁻¹ heparin. Fertilization was performed in 100 μL IVF medium droplets (20-25 COCs/droplet) under mineral oil and under the same culture conditions as described for oocyte maturation. The day of in vitro insemination was considered as day zero (D0).

After 18-22 h, presumptive zygotes were rinsed in embryo culture medium and pipetted to remove remaining cumulus cells and adhering sperm cells. The presumptive zygotes were cultured in 90 μL droplets of SOF medium (Holm et al., 1999) supplemented with 2.77 mol L⁻¹ myo-inositol and 5 mg mL⁻¹ BSA under mineral oil, at 39°C under 5% CO_2 in air. After 48 h of in vitro fertilization (D2), cleavage rates were recorded and on Day 8 (D8) post-insemination the blastocyst development rates were recorded. Blastocyst yield for each IVM group was calculated in relation to the number of oocytes originally placed in the maturation medium.

Evaluation of meiotic stage of oocytes

After maturation culture, the oocytes were mechanically denuded from their cumulus cells in 0.5 mL phosphate buffered solution supplemented with 0.1% polyvinyl alcohol (PVA-PBS) and 0.1% hyaluronidase by gently pippeting using a small glass pipette. Denuded oocytes were incubated in PVA-PBS supplemented with 3.7% (w/v) formaldehyde and 10 μg mL⁻¹ Hoechst 33342 at room temperature for 30 min. The fixed oocytes were rinsed in PVA-PBS to remove the Hoechst 33342 and then mounted on slides to evaluate the meiotic stage. Oocytes were observed under fluorescent microscope and classified as: germinal vesicle stage (GV, immature oocytes) or metaphase II (MII, mature oocytes).

Evaluation of DNA damage

After embryo culture the DNA damage of the blastocysts was evaluated by the comet assay (Singh et al., 1988 modified by Visvardis et al., 1997) with slight modifications. After centrifugation (200 x *g*) for 10 min in PVA-PBS, embryos were suspended in 0.75% low melting point agarose in PBS at 37°C; the agarose drop was

placed on a microscope glass slide that was maintained on ice for 10 min and coated with agar gel. The slides were then placed on ice for 30 min to harden the agar and then immersed in lysis solution (2.5 M NaCl, 100 mol L^{-1} Na$_2$EDTA, 10 mol L^{-1} Tris, 1% Triton X–100 and 5 μg mL^{-1} proteinase K) at 50°C for 2 h. The slides were removed from the lysis solution and placed in a horizontal electrophoresis unit filled with fresh electrophoresis buffer (1 mol L^{-1} Na$_2$EDTA and 300 mol L^{-1} NaOH) for 20 min before electrophoresis at 25 V for 20 min at room temperature. The slides were then washed in 0.4 M Tris-HCl before staining with 10 μg mL^{-1} ethidium bromide for 20 min and then observed under an epifluorescence microscope. The embryos were evaluated using an adaptation of the scoring method previously reported by Visvardis et al. (1997). Comets were scored visually as belonging to one of four predefined classes according to tail length and given a value of 1, 2, 3 or 4. Class 1: no migrated DNA; class 2: slightly migrated DNA; class 3: partially migrated DNA; class 4: extensively migrated DNA.

Experimental design and statistical analysis

Pooled immature cumulus-oocyte complexes (COCs) were randomly distributed into three different in vitro maturation culture conditions. Twenty to twenty-five COCs were cultured in 100 μL droplets of maturation medium supplemented with: 1) 0.5 μg mL^{-1} FSH and 5.0 μg mL^{-1} LH; 2) 10^{-9} M melatonin or 3) 0.5 μg mL^{-1} FSH, 5.0 μg mL^{-1} LH and 10^{-9} M melatonin. Nine replicates of each treatment were used.

Differences in nuclear maturation and embryo development rates and frequency of embryo DNA damage were assessed using Chi-square test. All data were pooled from nine replicates. Differences with probability of 0.05 or less were considered significant.

Results and Discussion

Supplementation of a physiological concentration of melatonin (10^{-9} M) into the maturation culture medium, alone or in combination with gonadotropins, had no effect on nuclear maturation (Table 1) or cleavage and blastocyst rates (Table 2). Theses rates ranged between 88% to 90%, 85% to 88% and 42% to 46%, respectively. In contrast with our findings, positive effect of melatonin on oocyte nuclear maturation and on in vitro embryo development has been reported by Kang et al. (2009) and

Manjunatha et al. (2009). Melatonin during IVM increases the nuclear maturation and blastocyst rates in porcine (Kang et al., 2009) and in buffalo (Manjunatha et al., 2009). On the other hand, in agreement with our results, Tsantarioutou et al. (2007) observed no differences on bovine cleavage and blastocyst rates when melatonin was added to IVM medium. Those authors did not study the nuclear maturation rate. It seems that the effect of melatonin added only to IVM medium produced species-dependent results.

In bovine, the embryo culture conditions are a well documented process (Parrish et al., 1994; Holm et al., 1999; Papis et al., 2007; Sargirkaya et al., 2007; Adona et al., 2008; Corrêa et al., 2008). Oocyte in-vitro maturation is a viable phenomenon as oocyte matured, fertilized and cultured in vitro can generate embryos with full developmental potential after embryo transfer. In spite of the lack of knowledge about how the nutrient requirements of the cumulus-oocyte complex (COC) impacts subsequent embryo development, there are some studies that directly correlate the metabolic needs of the COC with developmental outcomes (Sutton et al., 2003).

Regarding the effects of melatonin, the concentrations of melatonin in human preovulatory follicular fluid are almost three-fold higher than serum levels (Ronnberg et al., 1990). There are some evidences that intrafollicular melatonin serves to protect both the oocytes and the steroid-secreting granulosa cells from toxic oxygen and nitrogen-based byproducts during the ovulatory process (Sugino, 2008). Melatonin has a strong positive impact on processes related to ovulation and early embryo de-

Table 1 – Nuclear maturation rates of cattle oocytes obtained from cumulus-oocyte complexes matured in the presence of FSH and LH (FSH-LH); or melatonin (MEL) alone or in combination with gonadotropins (FSH-LH-MEL).

Culture condition	No. of oocytes*	Nuclear maturation rates % (n)[NS]
FSH-LH (control)	171	88.9 (152)
MEL	158	90.5 (143)
FSH-LH-MEL	209	89.0 (198)

*Pooled data from nine replicates. Nuclear maturation rate: % of oocytes in metaphase II. NS = Non Significant by Chi-square test.

Table 2 – In vitro development rate (%) of bovine embryos (mean ± SEM) produced from fertilized oocytes matured in the presence of FSH and LH (FSH-LH); or melatonin (MEL) alone or in combination with gonadotropins (FSH-LH-MEL).

Culture condition	No. of oocytes*	Cleavage rate (n)[NS]	Blastocyst rate (n)[NS]
FSH-LH (control)	209	85.7 (178)	43.5 (91)
MEL	205	88.5 (181)	46.3 (95)
FSH-LH-MEL	208	85.7 (178)	42.8 (89)

*Pooled data from nine replicates. NS = Non Significant by Chi-square test.

velopment and that the levels of melatonin in the mature Graafian follicle are important for the optimal development of oocytes and their release from the ovaries. Melatonin accelerates the action of maturation-inducing hormone on carp oocyte maturation (Chattoraj et al., 2005; Maitra et al., 2005). Melatonin directly protects the oocyte from oxidative stress in human and mouse (Tamura et al. (2008). These beneficial actions likely relate in part to the direct free radical scavenging actions of melatonin, (Tan et al., 2007) processes that are receptor-independent, or they may in some cases also involve melatonin receptors which are known to be present in some ovarian cells including cumulus cells (Kang et al., 2009).

Our findings demonstrated that 10^{-9} M melatonin in gonadotropin-free maturation medium produced embryo development rates comparable to those treated with FSH + LH (Table 2). Because maturation media of in vitro bovine embryo systems are generally composed by TCM–199 medium supplemented with of 0.5 μg mL^{-1} FSH, 5 μg mL^{-1} LH and 10% FCS (Sargirkaya et al., 2007; Adona et al., 2008; Corrêa et al., 2008) the maturation medium of control group was supplemented with gonadotropins.

The maturation media were supplemented with gonadotropins and/or FCS in all studies where the beneficial effect of melatonin added to maturation and/or embryo culture media on in vitro embryo development was observed (Ishizuka et al., 2000; Rodriguez-Osorio et al., 2007; Papis et al., 2007; Kang et al., 2009; Manjunatha et al., 2009). In these studies, optimal blastocyst rates were achieved when physiological melatonin rates (Cos and Sanchez-Barceló, 2000), which varied from 10^{-9} M to 10^{-6} M, were added to culture media. In the present study, a concentration of melatonin close to physiological levels (10^{-9} M) was tested because elevated concentrations of melatonin negatively affect follicle survival in culture (Adriaens et al., 2006).

The presence of serum in the maturation medium can provide an explanation for the present observed results. Serum contains peptide growth factors several of which improve in vitro development to blastocyst stage (Kane et al., 1997) and some reports have demonstrated the ability of FCS added to gonadotropin-free maturation medium to support embryo development in vitro

(Lim et al., 1994). However, the direct effect of melatonin on the developmental competence of oocytes cannot be excluded since in the present study the same batch of FCS was used in all experiments to avoid differences in its biological activity observed in different commercial lots (Batt and Miller, 1988)

The extent of DNA damage in embryos was also not affected by melatonin supplementation during in vitro maturation (Table 3). The incidence of blastocysts with no migrated DNA fragment (Class 1) was 32.0%, 38.0% and 33.0% for FSH-LH, MEL and FSH-LH-MEL groups, respectively. There were no embryos with extensively migrated DNA fragment (Class 4) in any of the groups.

The comet assay has been used as a biomarker of oxidative stress-mediated DNA strand breaks in individual cells (Singh et al., 1988; Visvardis et al., 1997; Choucroun et al., 2001) and embryos (Takahashi et al., 2000). In fact, embryo development in vitro under ordinary culture systems is also compromised by the toxic effects of atmospheric oxygen concentration which is approximately 20% (Takahashi et al., 2000). This effect is probably due to oxidative stress since the addition of a free radical scavenger as melatonin to the culture medium improves the blastocyst rates (Papis et al., 2007). However, in the study performed by Papis and co-workers, melatonin was added only during the embryo culture but not in maturation medium as in the present study.

We did not find an effect of melatonin during in vitro maturation on DNA damage of resulting embryos. This can possibly be explained by the fact that melatonin is only acting locally in cumulus-oocyte complexes, probably at concentrations that are higher than physiological levels. Thus, additional research testing the local effects of different melatonin concentrations during vitro maturation culture is required.

Comet assay can also be used to detect fragments of DNA that occur in apoptosis (Choucroun et al., 2001) and it has been well documented that in vitro-produced embryos do undergo apoptosis (Knijn et al., 2003). The effect of melatonin on the regulation of cellular apoptosis processes has also been documented (Sainz et al., 2003). Rodriguez-Osorio et al. (2007) found a positive effect of melatonin on the total cell numbers per blastocyst, and this effect was attributed to its anti-

Table 3 – Frequency of DNA damage (mean % ± SEM) measured by comet assay in cattle blastocysts obtained from cumulus-oocyte complexes matured in the presence of FSH and LH (FSH-LH); or melatonin (MEL) alone or in combination with gonadotropins (FSH-LH-MEL).

Culture condition	Embryos (n)	Extent of DNA damage %[NS]			
		Class 1 (n)	Class 2 (n)	Class 3 (n)	Class 4 (n)
FSH-LH (control)	89	31.5 ± 6.0 (29)	53.8 ± 4.5 (47)	14.8 ± 4.2 (13)	0.0 ± 0.4 (0)
MEL	85	38.4 ± 6.0 (32)	52.2 ± 4.5 (45)	9.5 ± 4.2 (8)	0.0 ± 0.4 (0)
FSH-LH-MEL	86	32.9 ± 6.0 (28)	46.1 ± 4.5 (41)	20.1 ± 4.2 (16)	0.8 ± 0.4 (1)

Class 1: no migrated DNA; class 2: slightly migrated DNA; class 3: partially migrated DNA; class 4: extensively migrated DNA. NS = Non Significant by Student's t-test.

apoptotic property. To our knowledge, there are no published evidences of the relationship between melatonin supplementation of maturation medium and the incidence of embryo DNA fragmentation, irrespective of the origin of DNA breaks.

In the present study, the evaluation of embryos resulted from in vitro procedures by comet assay aimed to verify apoptosis status. In spite of this method being frequently used to evaluate the genotoxicity of test substances there is evidence that comet pictures might be associated with apoptotic nuclei (Choucroun et al., 2001). Additionally, a significant relationship between supplementation of maturation medium and apoptotic index of resulting embryos has been reported (Warzych et al., 2007). This confirms the effect of the composition of maturation media on embryo developmental competence.

Acknowledgement

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