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Full Length Research Paper

Effect of melatonin on *in vitro* maturation of bovine oocytes

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To investigate the effect of different concentrations of melatonin on bovine oocytes *in vitro* maturation, varying concentrations of melatonin (0, 0.01, 1, 100 µM), were included in the the maturation medium. Slaughterhouse derived oocytes were subjected to standard *in vitro* maturation procedures in high oxygen tension. After culture for 24 h, over 80% of COCs had full cumulus cells expansion. As melatonin concentration increased, the degree of cumulus cells expansion did not changed significantly. Oocytes incubated in 0.01 and 1 µM melatonin-containing media for 24 h, result in 73.11 and 70.68% maturation rate, respectively, which were no different from the control (72.24%). The maturation rate decreased (P < 0.05) significantly when melatonin concentrations was increased from 0.01 to 1 and 100 µM (73.11 vs 70.68% and 65.24% respectively). After culture in melatonin-containing media for 24 h, 100% oocytes achieved GVBD stage in all groups. There were no significant differences among groups at GVBD stage. But oocytes that remained at metaphase-I stage were significant (P < 0.05) different with 100 µM in compare to other groups (21.32 Vs 17.67, 15.68, 16.53). In conclusion in this experiment, melatonin cannot improve cumulus cell expansion and nuclear maturation of bovine oocytes. When concentrations is high, melatonin may affect bovine oocytes meiotic maturation at metaphase-1 stage, but it is improbable melatonin be toxic for bovine oocytes.

Key words: IVM, bovine oocytes, melatonin, meiotic maturation.

INTRODUCTION

In vitro handling and culture conditions cause oocytes and embryos to oxidative stress resulting from events such as exposure to light, elevated oxygen concentrations and unusual concentrations of metabolites and substrates (Agrawal et al., 2006). Reactive oxygen species, ROS, such as hydrogen peroxide (H2O2), superoxide (O2•) anions, or hydroxyl radical (•OH), damage cell membranes and DNA and play a role in apoptosis (Kitagawa et al., 2004; Agrawal et al., 2006). Therefore, it is important to protect oocytes from oxidative stress during *in vitro* maturation (IVM). One approach is to supplement the medium with antioxidant compounds. Many different free radical scavengers have been examined from the point of view of their potential protection against oxidative stress revealed on mammalian embryos subjected to *in vitro* culture systems. Starting with extra cellular enzymes, such as superoxide dismutase or catalase, other systems including taurine/hypotaurine, thioredoxin pyruvate (Agrawal et al., 2006) or low molecular weight thiols such as β-mercaptoethanol (Kitagawa et al., 2004), glutathione (Luvoni et al., 1996) and cysteine (Ali et al., 2003) were tested. Effects of culture media supplementation with tested antioxidants or free radical scavengers on oocyte and/or embryo quality and consequent development are not consistent and lead sometimes to contradictory conclusions (Agrawal et al., 2006).

Melatonin (N-aceyl-5-methoxytryptamine), an indole derivative secreted rhythmically from the pineal gland, plays a major role in regulating the circadian clock in mammals (Reiter, 1991). Also, this molecule has major effects on the reproductive system in mammals (Reiter, 1998; Sirotkin and Schaeffer, 1997). More recent studies have demonstrated that, beside its multiple actions on different physiological processes, melatonin as well as its

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metabolites is indirect antioxidants and powerful direct scavengers of free radicals (Reiter, 1998). In contrast to the majority of other known radical scavengers, melatonin is multifunctional and universal (Hardeland, 2005; Leon, 2005; Tomas-Zapico, 2005). It is soluble both in water and in lipids and hence, acts as a hydrophilic and hydrophobic antioxidant. Melatonin has been successfully tested for promoting mouse embryo development *in vitro* (Ishizuka et al., 2000). It has been also reported as having no detrimental effects on mouse or rat embryo development during toxicity tests (Melhinny et al., 1996) performed either *in vitro* or *in vivo* (Jahnke et al., 1999). Recently, an increased ratio of vitrified sheep blastocysts developed with melatonin application for 24 and 48 h *in vitro* was reported (Abecia et al., 2002). Melatonin is likely to become the drugs of choice for improving oocytes quality for woman who can not get pregnant because of poor oocytes quality (Takazuki et al., 2003). The beneficial effect of supplementing culture medium with melatonin had been reported in mouse (Ishizuki, 2000) and bovine (Poleszczuk et al., 2007) *in vitro* embryo development. It has been reported that exogenous melatonin has beneficial effects on nuclear and cytoplasmic maturation during porcine IVM (Kang et al., 2008). Melatonin might improve the efficiency of porcine cloning by producing somatic cell nuclear transfer (SCNT) embryos with decreased apoptosis (Chio et al., 2008).

The specific objective of this study was to evaluate effects of melatonin supplementation during bovine IVM on cumulus cells expansion and nuclear maturation in commercial tissue culture medium 199 in high oxygen concentrations (20% O2).

MATERIALS AND METHODS

All chemicals were purchased from Sigma, unless otherwise indicated.

Collection of COCs

Bovine ovaries were collected at a local slaughterhouse in a termoflask and were transported to the laboratory in normal saline solution (30 - 35°C) containing 100 IU/ml penicillin and 100 µg/ml streptomycin (15140-122; Gibco/ Invitrogen) within 2 h after slaughter. Ovaries were washed twice in freshly prepared saline solution with antibiotic. Visible follicles 2 to 8 mm in size were aspirated by using an 18 -gauge needle connected to a 10 cc syringe containing 0.5 - 1 ml of aspiration medium, (Oocyte Collection Medium + 50 IU/ml heparin from drug store) and the contents recovered into a 50 ml conical tube and allowed to settle for 10 min in water bath at 38°C. after preliminary evaluation COCs were rinsed with oocyte collection medium which consisted of HEPES-TCM 199 supplemented with 2% FBS (10270-106; Gibco/Invitrogen), 0.2 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin.

In vitro maturation

Only oocytes enclosed in a compact cumulus with evenly granulated cytoplasm for maturation were selected. The COCs were washed 4 times in oocyte Collection Medium and one time in oocyte maturation medium (OMM), OMM was bicarbonate-buffered TCM199 (31100-027; Gibco/ Invitrogen) supplemented with 10% FBS, 0.2 mM sodium pyruvate, 5 µg/ml bovine follicle stimulating hormone (bFSH) (Sioux Biochemical, Sioux Center, IA), 0.01 IU/ml bovine luteinizing hormone (bLH) (Sioux Biochemical, Sioux Center, IA) and 100 IU/ml penicillin and 100 µg/ml streptomycin. Oocyte maturation was performed under mineral oil by culturing approximately 8 - 10 COCs in 50 µl of maturation medium in 60 × 15 dishes at 38.5°C in 5% CO2, 20% O2 (high oxygen concentrations), in air and high humidity for 24 h.

Melatonin stock solution was prepared with an ethanol/TCM199 system. 23.23 mg melatonin was first dissolved in 1.0 ml absolute ethanol and then was diluted with TCM199 by serial dilution. In this way 0.01 µM, 1 µM and 100 µM melatonin stock solution (100x) was Prepared. The stock solutions were stored in refrigerator at 0 – 4°C for no longer than 2 weeks.

Amount of ethanol concentrations in our experiment was 0.1% in the maturation medium and we used it as vehicle group.

Assessment of the cumulus expansion and nuclear status

COCs showing fully expanded cumulus cells after 24 h maturation period, were assessed by phase contrast invert microscope (Olympus, Japan) and those which COCs not expanded or showed incomplete expansion did not account.

Oocytes were then placed in TCM199 medium + hyaluronidase at room temperature, followed by pipetting to remove the cumulus cells. Denuded oocytes first assessed for pervitilline space and first polar body excursion by phase contrast invert microscope, oocytes that had polar body take account as matured and metaphase-II oocytes. Oocytes that had not polar body were recovered and transferred to glass slides for other stages evaluating. Vaseline and paraffin wax were used to keep the cover slip in contact with the oocytes. The slides were immersed in ethanol: acetic acid (3:1) for at least 24 h to fix them (Sun et al., 2001). Oocytes were then stained with 1% (w/v) aceto-orcein. in order to decolorized, aceto-giycerol (20, 20, 60%; glycerol, acetic acid, distillated water) (Hewitt et al., 1998) was used then examined under a phase contrast invert microscope at 400× magnification. Oocytes were classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) stage of the maturation process.

Experimental design and statistical analysis

In current study different concentrations of melatonin (0, 0.01, 1 and 100 µM) and 0.1% ethanol (vehicle) were included in the maturation medium. One-way ANOVA was used to determine significant differences in data levels. Turkey test was followed to determine statistical differences between groups. Statistical analyses were preformed using PROC GLM of SAS software (Version 9.1). The significant differences among the treatments were determined when $p < 0.05$. Data are expressed as mean ± S.E.

RESULTS

Expansion of cumulus and pervitilline space formation

After culture in 0, 0.01, 1 and 100 µM melatonin-containing media for 24 h, over 80% of Cumulus-oocyte-complexes had full cumulus cell expansion. As melatonin

Table 1. Effect of melatonin on cumulus expansion of bovine oocytes *in vitro*.

Melatonin concentrations (µM)	Ethanol concentrations (%)	Number of COC_s	Cumulus expansion (%)	S.E
0 (Control)	0	185	152 (82.94)[a]	1.034
0 (Vehicle)	0.1	152	127 (83.69)[a]	1.034
0.01	0.1	159	132 (83.11)[a]	1.034
1	0.1	188	154 (81.99)[a]	1.034
100	0.1	177	146 (82.72)[a]	1.034

Percentages are based on the number of oocytes examined. a Within the same column, values with different letters were significantly different (P < 0.05).

Table 2. Effect of melatonin on nuclear statues of bovine oocyte maturation *in vitro*.

Melatonin concentrations (µM)	Ethanol concentrations (%)	Number of COCs	Nuclear statues after 24 h (Mean ± S.E)			
			GV, n (%)	GVBD, n (%)	M-I, n (%)	M-II, n (%)
0 (Control)	0	135	0	14 (11.21 ± 1.12) [a]	23 (16.53 ± 1.22) [b]	98 (72.24 ± 1.17) [ab]
0 (Vehicle)	0.1	133	0	13 (11.01 ± 1.12) [a]	24 (17.98 ± 1.22) [ab]	96 (71.61 ±1.17) [ab]
0.01	0.1	127	0	14 (11.90 ± 1.12) [a]	20 (15.68 ± 1.22) [b]	93 (73.11 ± 1.17) [a]
1	0.1	116	0	14 (12.03 ± 1.12) [a]	19 (17.67 ± 1.22) [b]	83 (70.68 ± 1.17) [b]
100	0.1	127	0	15 (12.59 ± 1.12) [a]	28 (21.32 ± 1.22) [a]	83 (65.24 ± 1.17) [c]

Percentages are based on the number of oocytes examined.
[a,b,c]Within the same column, values with different letters were significantly different (P < 0.05).

concentration increased, the degree of cumulus expansion did not increase or decreased significantly (Table 1). After removal of cumulus cells, matured oocytes in control and other groups were clearly visible with presence of first polar body in pervitilline space (we count them as metaphase-II stage oocytes). We did not observed any non-pervitilline-space oocytes in this study and majority of oocytes developing visible space.

Evaluation of meiotic maturation

Oocytes incubated in 0.01 and 1 µM melatonin-containing media for 24 h result in 73.11 and 70.68% maturation rate, respectively, which were no different from the control (72.24%). The maturation rate significantly decreases (P < 0.05 to the control) to 65.24% when melatonin concentrations were increased to 100 µM. We observed a dose dependent response to melatonin treatment in this experiment.

After culture in 0, 0.01, 1 and 100 µM melatonin-containing media for 24 h, 100% Oocytes achieved GVBD stage and we did not observed any GV-Stage oocytes. There was no significant difference among groups at GVBD stage. But oocytes that remained at metaphase-I stage, were significant (P < 0.05) different with 100 µM in compare to other groups (21.32 Vs 17.67%, 15.68, 16.53%).

Absolute ethanol which we used for diluting melatonin (0.1% as vehicle group), had no detrimental effect on neither cumulus cell expansion nor nuclear status (P < 0.05) (Table 2).

DISCUSSION

It is generally accepted that melatonin exerts its primary reproductive action at the level of the brain and pituitary (Vanecek, 1998) however presence of high melatonin levels in follicular fluid (Brzezinski et al., 1987) and the presence of receptors in granulosa cells (Soares et al., 2003) suggest a potential beneficial property of melatonin reproductive processes not only through the hypothalamo–hypophysial system but also by direct effects on reproductive organs. This is confirmed by the presence of melatonin and serotonin and its receptors in the reproductive organs, by action on the production of hormones, growth factors and cyclic nucleotides by ovarian cells, by effects on oocyte maturation and by effects on the secretory functions of the testis and oviduct. Thus the available data suggest a dual control of reproduction by melatonin and serotonin.

Cumulus cells are known to play a crucial role during oocyte maturation. Cumulus cells during maturation are essential for acquiring developmental competence by oocyte *in vitro* (Gordon, 2003). It is reported that 1 µM

melatonin reduce cumulus cells apoptosis by activating its receptors on cumulus cells (Na et al., 2005). However, in our study cumulus-cell expansion did not show significant difference in compare to control. It has been reported that type of medium and supplements which is used for IVM, may influence rate of cumulus cell expansion (Gordon, 2003). It has been showed that mouse COC_s modification did not influenced by 1 to 2 mM melatonin (Adrians et al., 2006). Therefore type of medium and supplements which was used for IVM may cover melatonin effect on cumulus cell expansion in our experiment. Also melatonin may influence apoptotic indexes in bovine cumulus cells that remain to be clarified.

The formation of the perivitelline space is one of the major events associated with normal oocyte maturation. The space starts to form in bovine cumulus cell enclosed oocytes around 2 h in IVM. An intricate network of gap junctions exists between the innermost layer of cumulus cells and the oolemma via cytoplasmic processes that traverse the zona pellucida in the growing oocyte (Kidder and Mhawi, 2002). There are many factors secreted by cumulus cells such as glycosaminoglycans and steroid hormones that assist cytoplasmic maturation (Dode and Graves, 2002). As the oocyte matures, these intricate cytoplasmic contacts are gradually withdraw back to their mother cells and an obviously perivitelline space forms. It has been reported that some toxic factors such asnicotine (Liu et al., 2006) in bovine oocytes maturation medium, prevent formation of the perivitelline space. In the present study concentrations of melatonin did not on ovarian function. Melatonin and serotonin regulate affect the formation of the perivitelline space. General melatonin toxicity is reported to be extremely low (Jahnke et al., 1999). Therefore it is improbable that melatonin be toxic for oocytes in this study.

Manipulation of gametes and embryo in an *in vitro* environment, increase risk of exposure of these cells to high levels of reactive oxygen species (ROS) (Agawal et al., 2006). Increased oxidative stress damage mitochondria and consequently will impair ATP production and hamper meiotic and mitotic spindles formation in growing oocyte. The adverse effect will compromise fertility potential (Peter and Adashi, 2004). When porcine cumulus-free oocytes exposure to ROS, meiotic maturation arrest, increase degenerated oocytes and occur apoptosis (Wiener-Megnazi et al., 2004) and antioxidants reduce chromosome abnormality (Tarin et al., 1998). Melatonin can decrease H_2O_2 level in mitochondria, elevates 0000glutathione homeostasis and improves mitochondria performance and activates repairing mechanism of mitochondria genome (Leona et al., 2004). Also melatonin activate nuclear factor kappa-B (NF_k-B), that is a crucial factor for developing mouse embryo from one cell to 4 cell stage (Ali et al., 2003). Incubation of mouse GV oocyte with 300 µM H_2O_2, significantly decrease first polar body excursion, but when H_2O_2 and melatonin added to maturation medium together, inhibitory effect of H_2O_2 on oocyte meiotic maturation blocked (Tamura et al., 2008). Role of ROS in IVM media is still controversial. In a study, bovine COC_s were matured in TCM199 + 10% steer serum and addition of vitamin E to IVM medium fail to vary meiotic maturation (Cetica et al., 2001). In addition physiological concentrations of ROS have a role in meiotic resumption, in fact high concentrations of antioxidants inhibit spontaneous resumption of meiosis (Takami et al., 1999). Lipid content of oocytes is variable in animal species and may be a factor to determine oocyte sensitivity to oxidative stress and oocyte requirement to antioxidant (Manjunatha et al., 2007). It is reported that 10 µM to 50 µM melatonin, increase nuclear maturation and transformable embryo of buffalo oocyte *in vitro* (Manjunatha et al., 2007). But 1 µM melatonin increase nuclear and cytoplasmic maturation of porcine oocytes (Kang et al., 2008). It is clear that lipid content of buffalo oocytes is higher than porcine and bovine oocytes. Therefore buffalo oocytes are more sensitive to oxidative stress and its requirement to antioxidant is high. In our study TCM199 + 10% FBS and supplements such as pyruvate may be sufficient for bovine oocytes during IVM. Therefore 0.01 µM melatonin fails to increase nuclear maturation. However melatonin effect on glutathione content after maturation (which is a good biomarker of ooctyte developmental competence), fertilization rate and embryo development, remain to be clarified. Melatonin is probably able to influence not only secretory but also generative ovarian functions. Melatonin injecttions increased oocyte metabolism and inhibited ovulation in Bufo arenarium. It stimulated the reinitiation but not the completion of meiosis or the cleavage of bovine oocytes matured and fertilized *in vitro* (Sirotkin and Schaeffer, 1997). Therefore as we observed in our study, oocytes that remained at metaphase-I stage, were significant ($P < 0.05$) different with 100 µM in compare to other groups (21.32 Vs 17.67%, 15.68, 16.53%). Results of our experiment are according to Sirotkin and Schaeffer (1997).

In conclusion in this study, melatonin cannot improve cumulus expansion, but had no detrimental effects on cumulus cells expansion and privetilline space formation. Melatonin may affect oocyte meiotic maturation at metaphase-1 stage when melatonin concentration is high, and it is improbable that melatonin may be toxic for meiotic maturation of bovine oocytes.

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REFERENCES

Abecia JA, Forcada F, Zuniga O. (2002). The effect of melatonin on the

secretion of progesterone in sheep and on the development of ovine embryos *in vitro*. Vet. Res. Commun. 26: 151-158.
Adrians I, Jacquet P, Contrrindt R, Janssen K, Smiz J (2006). Melatonin has dos-depended effect on folliculogenesis, oocyte maturation capacity and steroidogenesis. Toxicol. 228: 333-343.
Agawal A, Tamer MS, Mohamed AB, Jashoman B, Juan GA (2006). Oxidative stressing assisted reproductive techniques. Steril. Fertil. 86: 503-512.
Brzezinski A, Seibel M, Lynch H, Deng M, Wurtman R (1987). Melatonin in human preovulatory follicular fluid. J. Clin. Endocrin. Metabol. 64: 865-867.
Cetica PD, Pintos LN, Dalvit GC, Beconi MT (2001). Antioxidant enzyme activity and oxidative stress in bovine oocyte *in vitro* maturation. IUBMB Life. 51:57- 64.
Chio J, Park S, Lee E, Kim J, Joeng Y, Lee J, Park S, Hyun S, Hossein MS, Jeong Y, Kim S, Hawang W (2008). Anti-apoptotic effect of melatonin on preimplantation development of porcine parthenogenetic embryos. Mol. Reprod. Dev. 75:127-1135.
Dode MA, Graves C (2002). Involvement of steroid hormones on *in vitro* maturation of pig oocytes. Theriogenol. 57: 811-821.
Gordon I (2003). Laboratory production of cattle embryos. Second edition CABI International publishing, Wallingford. p. 157.
Hardeland R (2005). Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. Endocr. 27:119-130.
Hewitt DA, Watson PF, England GCW (1998). Nuclear staining and culture requirements for in vitro maturation of domestic bitch oocytes. Theriogenology. 49: 1083-1101.
Ishizuka B, Kuribayashi Y, Murai K, Amemiya A, Itoh M (2000). The effect of melatonin on in vitro fertilization and embryo development in mice. J. Pineal. Res. 28:48-51.
Jahnke G, Marr M, Myers C, Wilson R, Travlos G, Price C (1999). Maternal and developmental toxicity evaluation of melatonin administered orally to pregnant Sprague-Dawley rats. Toxicol Sci. 50: 271–279.
Kang Jk, Koo OJ, Kwon DK, Park HJ, Jang G, Kang SK, Lee BC (2008). Effects of melatonin on *in vitro* maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. J. Pineal Res. In press.
Kidder GM, Mhawi AA (2002). Gap junctions and ovarian folliculogenesis. Reprod. 123: 613-620.
León J, Acuña-Castroviejo D, Escames G, Tan DX, Reiter RJ (2005). Melatonin mitigates mitochondrial malfunction. J. Pineal Res. 38: 1-9.
Leona J, Acun˜a-Castroviejob D, Sainza RM, Mayoa JC, Tana DX, Reiter RJ (2004). Melatonin and mitochondrial function. Life Sci. 75:765-790.
Luvoni GC, Keskintepe L, Brackett BG (1996). Improvement in bovine embryo production *in vitro* by glutathione-containing culture media. Mol. Reprod. Dev. 43: 437-443.
Manjunatha BM, Devaraj M, Gupta PSP, Ravindra, JP. Nandi S (2007). Effect of taurine and melatonin in the culture medium on buffalo in vitro embryo development. Reprod Dom Anim. In press.
Melhinny AS, Davis FC, Warner CM (1996). The effect of melatonin on cleavage rate of C57BL/6 and CBA/Ca preimplantation embryos cultured in vitro. J. Pineal Res. 21: 44-48.
Na k, Kim J, Lee J, Yoon T, Cha k, Lee D (2005). Effect of melatonin on the maturation of mouse GV oocytes and apoptosis of cumulus cells *in vitro*. Fertil. Steril. 84: S 103, Abst.
Peter CKL, Adashi EY (2004).The Ovary. Second Edition Elsevier Inc. pp. 113-130.
Poleszczuk O, Papis K, Wanta-Mnchalska E (2007). An effect of melatonin on development of bovine embryos cultured in vitro under optimal or enhanced oxygen tension. J. Pineal Res. 43: 231-236
Reiter RJ (1998). Melatonin and human reproduction. Ann Med. 30: 103-108.
Reiter RJ, Tan DX, Osuna C, Gitto E (2000). Actions of melatonin in the reduction of oxidative stress: a review. J. Biomed Sci. 7: 444-458.
Reiter RJ (1991). Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr. Rev. 12: 151-180.
Sirotkin AV, Schaeffer HJ (1997). Direct regulation of mammalian reproductive organs by serotonin and melatonin. J. Endocr. 154: 1-5.
Soares JM, Masana MI, Ersahin C, Dubocovich ML (2003). Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. J. Pharma. Exp. 306: 694-702.
Takami M, Preston SL, Toyloy VA, Behrman H, Angell R (1999). Antioxidants reversibly inhibit the spontaneous resumption of meiosis. Am. J. Physiol. 276: E684-E688.
Takazuki A, Nakamura A, Tamura A, Shiamura K, Morioka H (2003). Melatonin as a new drug for improving oocyte quality. Reprod Medic Biol. 2: 139-144.
Tamura H, Takasaki A, Mivwa I, Taniguchi K, Maekawa RH (2008). Oxidative stress impairs oocyte quality and melatonin protects oocyte from free radical damage and improves fertilization rate. J. Pineal Res. 44: 270-287.
Tarin JJ, Vendrell FJ, Ten J, Cano A (1998). Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. Mol. Hum. Reprod. 4:281-284.
Tomas-Zapico C, Coto-Montes A (2005). A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J. Pineal Res. 39: 99-104.
Vanecek J (1998). Cellular mechanims of melatonin action. Physiol. Rev. 78: 687-721.
Wiener-Megnazi Z, Vardi L, Lissak A, Shnizer S, Zeev Reznick A, Ishai D (2004). Oxidative stress indices in follicular fluid as measured by the thermochemiluminescence assay correlate with outcome parameters in *in vitro* fertilization. Fertil. Steril. 82(Suppl 3):11171-11176.