In Vitro Maturation Culture Temperature Alters the Enzymatic Antioxidant Activity of Bovine Oocytes and Embryos

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***Abstract*—** It is known that basic variable in the cellular environment is temperature and low temperature decrease cellular metabolism rate. Also, low cellular metabolism may reduce oxidative activity and intracellular antioxidant enzyme activity, resulting in low ROS production. The aim of this study was therefore to investigate the effect of 36.5 °C (low) and 38.5 °C (conventional) incubation temperatures during IVM on glutathione peroxidase activity of oocytes and blastocysts following fertilization. Bovine oocytes were maturated in medium–199 for 22 hours at either 36.5 °C or 38.5 °C and they were subjected to IVF. Putative zygotes were then cultured randomly into SOFaa embryo culture media with or without antioxidant (a mixture of 1mM GSH and 1500 IU SOD). Matured oocytes with first polar body and embryo development to the blastocyst stage were selected for determine antioxidant enzyme activity. Antioxidant enzyme activity was determined by glutathione peroxidase activity, which was recorded spectrophotometrically at 340 nm wavelength. Antioxidant enzyme activity were lower ($P<0.05$) in oocytes matured at low temperature than those of conventional temperature. Percentages of embryos developed to blastocyst stages were higher ($P<0.05$) in embryos cultured in SOFaa medium with antioxidant. Antioxidant enzyme activity were lower ($P<0.05$) of blastocysts from embryos obtained oocytes matured low temperature and cultured in antioxidants supplemented media. The results of present study show that decreasing the in vitro maturation temperature decrease antioxidant enzyme activity in both oocyte and blastocyst may be due to decrease oxidative metabolism and ROS production. Perhaps oocytes matured low temperature could be overcome the increase in ROS because of the presence of their own enzymatic antioxidant system without the need of an additional antioxidant system even after fertilization.

***Keywords*—** bovine, temperature, antioxidant activity, oxidative stress, oocytes, blastocysts

I. Introduction

Various approaches have been employed to improve maturation and developmental competence of bovine oocytes following in vitro fertilization [1]. Improve in vitro maturation (IVM) competence in bovine oocytes has been subjected in many investigations by supplementation such as follicular fluid, co-culture [2], growth factors or gonadotropic hormones into IVM media [1]. However, developmental competence of bovine oocytes not only depends on the composition of the culture medium but also incubation conditions such as gases tension, humidity and temperature [3]. IVM of bovine oocytes are performed at 38 °C to 39 °C, as this temperature is close to the rectal temperature in cattle [4]. However recent reports demonstrated that the temperature in preovulatory follicles is 1.5 to 2°C cooler than their adjacent stroma in cattle [5]. The existence of follicular cooling raises the question of whether oocytes develop advantageously at lower temperatures. Reduced temperature may be required for successful oogenesis or oocyte maturation, or for subsequent embryonic or fetal development [6].

The role of ROS in IVM and its influence on later embryonic development is controversial [7]. A physiological amount of ROS may be indicative of healthy developing oocytes [8], but the presence of excessive ROS can contribute to the meiotic arrest of the oocyte [7] and embryonic developmental arrest, cell death and apoptosis [3]. In vitro embryo production systems have developed mechanisms to control ROS levels, including enzymatic (superoxide dismutase, glutathione peroxidase, catalase) and nonenzymatic antioxidant agents (α-tocopherol, ascorbic acid, β-carotene, and glutathione, among others [9]. Superoxide anion and hydroxyl radical activity in embryonic cells is scavenged by endogenous superoxide dismutase to superoxide anion, and endogenous glutathione peroxidase and catalase transform hydrogen peroxide into water and oxygen in vitro [7, 10].

The extent of ROS damage to cell systems is dependent on the balance between their production and removal rates [11]. Generation of ROS is an inevitable consequence of oxidative reactions, but various protective scavengers for example, superoxide dismutase, catalase and glutathione reductase remove radical species before they can cause significant damage in in vitro embryo production systems. However, if metabolic activity of in vitro produced embryos can be reduced, oxidative metabolism may be decreased, resulting in low production of ROS. Thus in vitro produced embryos may not need to addition of an antioxidant system against to ROS in culture media. Also these embryos attempt to scavenge ROS with its antioxidant mechanisms. Probably, establishment of in vitro environmental conditions, which will not increase metabolism of embryo, may eliminate necessity of supplemental antioxidant defense systems against to ROS

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in culture media in vitro.

Leese [12] put forward the ‘Quiet Embryo Hypothesis’, which proposed that low (quiet) embryo metabolism would better serve the embryo viability than active metabolism. Additionally, this hypothesis indicates that oocyte and early embryo function at a lower temperature, which represent preovulatory follicle temperature in in vivo condition, could encourage the expression of a low (quiet) metabolism than core body temperature in in vitro [3]. Therefore, it was hypothesized that IVM at a temperature that mimics the thermal environment of the bovine preovulatory follicles in vivo may provide a better culture condition for optimum metabolic activity and antioxidant capacity of oocytes and may be from preimplantation embryos to the blastocyst stage. The aim of this study was therefore to investigate the effect of low (36.5 °C) and conventional (38.5 °C) incubation temperatures during IVM on antioxidant activity (glutathione peroxidase activity) of oocytes and embryos cultured SOFaa media with or without antioxidant and developed to blastocysts obtained from oocytes maturated at low and conventional temperature.

II. Material and Methods

Cumulus-oocyte complexes (COCs) were aspirated with an 18-gauge needle fixed to a 10 ml syringe from 2 to 8 mm in diameter follicles. COCs were collected in 3-4 ml hepes-buffered medium-199 (Sigma, M7528) containing earle's salts and supplemented with 1% v/v antibiotic-antimycotic solution, 100 µg/ml L–glutamine and 5% v/v heat-inactivated fetal calf serum (FCS). Maturation medium was sodium bicarbonate-buffered medium-199 (Sigma, M4530) containing Earle's salts and L-glutamine supplemented with 5.5 µg/ml sodium pyruvate, 1% v/v antibiotic-antimycotic solution, 10% v/v heat-inactivated FCS, 5.0 µg/ml LH, 0.5 µg/ml FSH and 10 ng/ml EGF. COCs were separately placed in 500 µl of maturation medium (approximately 25-35 COCs per well) covered with 300 µl mineral oil in four-well dishes (Nunc, Roskilde, Denmark) and matured for 22 hour filled with humidified 5% CO2 in air at 36.5 °C (low) or 38.5 °C (conventional) incubation temperatures. At the end of maturation period COCs were completely denuded of cumulus cells by vortexing and first polar body of oocytes was evaluated. In vitro matured denuded oocytes were washed 3 times in Ca2+ and Mg2+ -free PBS supplemented with 1 mg/ml polyvinyl alcohol and were stored in microtubes (approximately 25 / 10 µl) at -80 °C until enzyme activity analysis of glutathione peroxidase.

After the completion of maturation culture COCs were washed twice in hepes-buffered medium 199 and then twice in in vitro fertilizasyon (IVF) medium. The IVF medium was glucose-free modified TALP supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/ml fatty acid-free BSA, 10 mg/ml heparin-sodium salt and 0.5 µl/ml antibiotic-antimycotic solution (adjusted pH 7.4 and 280-300 mOsm/kg). COCs were transferred into 48 µl fertilization drops (approximately 15 COCs per drop) covered with mineral oil. Frozen–thawed semen from a single bull was used for the fertilization of oocytes. Percoll density gradient system was used for the separation of the motile fraction of the frozen–thawed semen (Parrish et al., 1995). The sperm concentration was counted by hemocytometer using a phasecontrast microscope at a magnification of 400×. Sperm was then diluted to 50×106/ml spermatozoa with fertilization medium. The sperm motility was visually checked for acceptable motility (at least 80% progressively motile). The oocytes were fertilized with 2 µl diluted semen per fertilization drops for 22 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

After in vitro fertilization, the putative zygotes were washed three times in hepes-buffered Medium 199 and they were vortexed 5 min to remove cumulus cells layer. The denuded zygotes were washed twice in hepes-buffered medium-199 and then twice in synthetic oviduct fluid (SOF) embryo culture media. The SOF embryo culture media was supplemented with 40 µg/ml sodium pyruvate, 8 mg/ml fatty acid-free BSA, 20 µl/ml MEM non-essential amino acids solution (100×), 10µl/ml BME Amino Acids Solution (50×) and 0.5 µl/ml antibiotic-antimycotic solution on the day of use. The zygotes were placed in 50 µl drops (approximately 15 zygotes per drop) of SOFaa embryo culture media with or without antioxidant (a mixture of 1mM GSH and 1500 IU SOD) under mineral oil and cultured in a humidified atmosphere of 5% CO2, 5% O2 and 90% N2 in air at 38.5°C. İn vitro fertilization was considered as 0 day. Zygotes cleaved were recorded on days 3 of development. Morula and blastocyst development the proportion of zygotes were evaluated on days 5 and 8, respectively. Embryos develop to blastocyst were washed 3 times in Ca2+ and Mg2+ -free PBS supplemented with 1 mg/ml polyvinyl alcohol and were stored in microtubes (approximately 5 blastocyst / 10 µl) at -80 °C until enzyme activity analysis of glutathione peroxidase.

The freezing–thawing oocytes and blastocyst were broken by ultrasonication during 30 min for enzymatic extracts. They then centrifuged at 4 °C and 10,000 g for 20 min. Supernatants were used to determine enzymatic activity. Glutathione peroxidase activity was determined using a commercial sensitive glutathione peroxidase activity kit as suggested by the manufacturer (Glutathione Peroxidase (GPx) Assay, Northwest Life Science Specialties, LLC, and Vancouver, WA USA). Glutathione peroxidase activity was recorded spectrophotometrically at 340 nm.

Effect of temperature during IVM and antioxidants in embryo culture on developmental competence of embryos was analyzed after arcsine-transformation transformation using the General Linear Model (GLM) of the SPSS package program. Significant differences between glutathione peroxidase activity in oocytes and blastocysts were tested using Duncan's test. The statistical significance was determined at the level of 0.05.

III. Results and Discussion

The cleavage and blastocyst formation rates of oocytes maturated 36.5 or 38.5 °C temperatures and cultured in SOFaa with or without antioxidants following fertilization are presented in Figure 1.

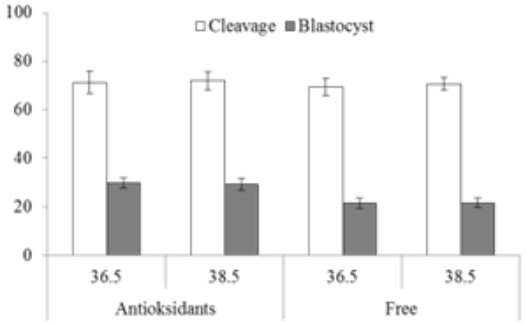

Fig 1. The cleavage (open bars) and blastocyst formation (black bars) rates of oocytes maturated 36.5 or 38.5 °C temperatures and cultured in SOFaa with or without antioxidants following fertilization. The bars with different letters are significantly different at $P < 0.05$.

There were no significant differences between embryos obtained from oocytes maturated 36.5 °C or 38.5 °C incubation temperatures in terms of cleavage rate and percentage of these embryos developed to blastocysts stages. Previous studies showed that low (36.5 °C or 37.0°C) or conventional (38.5 °C or 39.5 °C) incubation temperature during IVM had similar effect on embryonic development until development to the blastocyst stage in different farm animal species [4, 13]. Antioxidant supplementation (a mixture of GSH and SOD) increased ($P<0.05$) ratio of embryos developed to blastocyst stage. Similarly, Luvoni et al. [10] and Uysal et al. [14] reported that GSH and SOD supplementation improve embryo development rate until the blastocyst stage.

Glutathione peroxidase enzyme (GSH-Px) activity of in vitro matured bovine oocytes and blastocysts are shown in Figure 2 and 3, respectively.

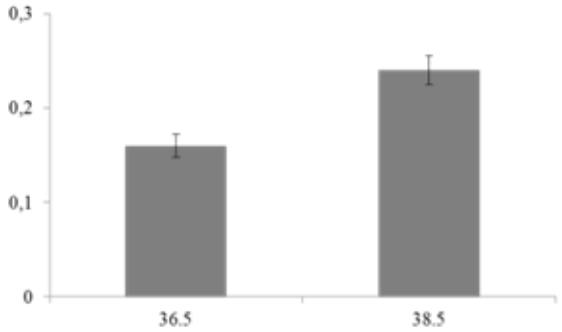

Fig. 2. Glutathione peroxidase (GSH-Px) activity of in vitro matured bovine oocytes at 36.5 or 38.5 °C incubation temperatures. The error bars represent the standard error of means and bars with different letters are significantly different at $P < 0.05$.

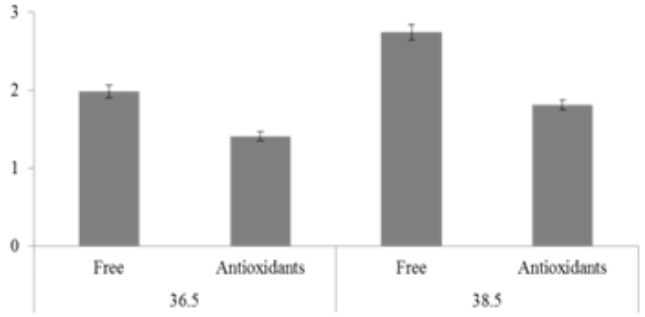

Fig. 3. Glutathione peroxidase (GSH-Px) activity of blastocysts obtained in vitro matured bovine oocytes at 36.5 or 38.5 °C temperatures and cultured with antioxidants or antioxidants free embryo culture media after fertilization. The error bars represent the standard error of means and bars with different letters are significantly different at $P < 0.05$.

ROS production is an inevitable consequence of oxidative reactions within cells when electron transfer reactions to the oxygen occur in the mitochondrial respiratory chain and endoplasmic reticulum [7, 10]. In vitro conditions usually increase ROS production in oocytes and embryonic cells [3]. Protection of embryos from oxidative stress may be a necessity for a successful development in vitro because of more sensitivity to excessive concentration of ROS in embryonic cells [10]. GSH-Px converts ROS such as superoxide anion and hydroxyl radical into water and molecular form of oxygen [7, 10]. Especially, in mammalian cells it plays a critical role to protect them from oxidative stress [15]. Low activity of GSH-Px can increase the amount of reduced gluthatione and allow a rise in ROS concentrations and oxidative stress [16]. In the present study, maturation of oocytes at low (36.5 °C) incubation temperature decreased glutathione peroxidase enzyme activity, contrarily conventional (38.5 °C) incubation temperature increased glutathione peroxidase enzyme activity ($P<0.05$). Also GSH-Px activity of blastocysts from oocytes maturated 36.5 °C was significantly lower ($P<0.05$) than those of oocytes matured 38.5 °C incubation temperature. Additionally, supplementation of antioxidants into embryo culture media decreased ($P<0.05$) GSH-Px activity in blastocysts obtained oocytes maturated at either 36.5 °C or 38.5 °C incubation temperature. Moreover, antioxidants were more effective in reducing GSH-Px activity in blastocysts from oocytes maturated at 36.5 °C incubation temperature compared to blastocysts from oocytes maturated at 38.5 °C incubation temperature ($P<0.05$).

These results indicated that low incubation temperature (38.5 °C) which represents preovulatory follicles temperature may decrease oxidative metabolism due to low metabolic activity of bovine oocytes and blastocysts after in vitro fertilization. Unfortunately, observations regarding to the metabolic activity were not measured in the present study, low metabolic activity of oocytes may lead to drop in ROS

production and it’s may cause decrease in GSH-Px activity. Because, oocyte and early embryo function at a lower temperature could encourage the expression of a low metabolism than core body temperature in in vitro [3].

IV. Conclusion

In conclusion, the results of this study indicate that bovine oocytes can complete process when cultured at 36.5 °C with some degree of developmental competence to the blastocyst stage. Additionally, maturation of bovine oocytes at low temperature may cause low ROS production in both oocytes and blastocysts resulting in low metabolic activity. Moreover, low temperature maturated oocytes may have strong enzymatic antioxidant system and they don’t need of an additional antioxidant system during embryo culture after fertilization.

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Bovine oocytes matured low temperature could be overcome the increase in ROS because of the presence of their own enzymatic antioxidant system without the need of an additional antioxidant system even after fertilization.