Glutathione Peroxidase (GPx) Activity in Anatolian Buffalo's Oocytes Is Unaffected by Culture Temperature

Uğur ŞEN[1]*, Hasan ÖNDER[2], Cem TIRINK[3]

***Abstract*—** Temperature is a well-established fundamental variable in the cellular environment that directly influences cellular metabolism. Cellular metabolic activity is closely associated with oxidative processes and the regulation of intracellular antioxidant enzyme systems. Glutathione peroxidase (GPx) is a selenium-dependent antioxidant enzyme that plays a critical role in maintaining intracellular redox homeostasis. Accordingly, the present study aimed to evaluate the effects of low (36.5 °C) and conventional (38.5 °C) incubation temperatures during in vitro maturation (IVM) on glutathione peroxidase activity in oocytes obtained from Anatolian buffaloes. Oocytes were matured for 22 hours in medium 199 under either low or conventional incubation conditions. Antioxidant enzyme activity was assessed by spectrophotometric measurement of glutathione peroxidase activity at 340 nm. No significant differences in antioxidant enzyme activity were observed between oocytes cultured at low and conventional incubation temperatures. These findings indicate that variations in IVM temperature within the tested range do not affect glutathione peroxidase activity, suggesting that the antioxidant defense capacity of Anatolian buffalo's oocytes is maintained under both incubation conditions.

***Keywords*—** Anatolian Buffalo, Temperature, Antioxidant Activity, Oxidative Stress, Oocytes

I. Introduction

Various strategies have been explored to enhance oocyte maturation, including the supplementation of in vitro maturation (IVM) media with follicular fluid, co-culture systems, growth factors, and gonadotropic hormones [1, 2]. While the composition of the culture medium is a critical determinant of oocyte developmental competence, incubation conditions such as gas composition, humidity, and temperature also play a pivotal role in successful IVM [3]. Conventionally, oocyte maturation in vitro is conducted at core body temperature in domestic animals. However, accumulating evidence indicates that the temperature within preovulatory follicles is approximately 1.5–3 °C lower than that of the surrounding ovarian stroma or core body temperature [3, 4, 5, 6]. This phenomenon of follicular cooling raises important questions regarding the optimal thermal environment for oocyte maturation and whether reduced temperatures may confer developmental advantages.

Recent advances in reproductive biotechnology, particularly in vitro embryo production (IVP), have significantly expanded their application in domestic species [7]. Despite decades of research, however, culture conditions for IVP systems remain insufficiently standardized, mainly because in vitro environments cannot fully replicate the complex physiological conditions encountered in vivo [2, 3]. Multiple factors influence the efficiency of IVP, including media composition, hormonal supplementation, humidity, gas balance, and incubation temperature [8]. In buffaloes, although IVP technologies have been implemented, overall success rates remain relatively low, emphasizing the need for further optimization. Since successful embryonic development depends on both nuclear and cytoplasmic maturation of oocytes, refining IVM conditions is particularly important.

Notably, conventional IVM protocols for large ruminants such as cattle and buffaloes typically employ incubation temperatures of 38.5–39 °C, reflecting core body temperature rather than the cooler follicular microenvironment observed in vivo [5, 6, 8]. This discrepancy suggests that standard IVM temperatures may exceed the physiological conditions under which oocyte maturation naturally occurs, supporting the hypothesis that reduced incubation temperatures could be beneficial for oogenesis or subsequent embryonic development.

Reactive oxygen species (ROS) play a complex and controversial role during IVM and early embryogenesis. While physiological levels of ROS are associated with normal cellular signaling and healthy oocyte development [9, 10], excessive ROS accumulation can lead to meiotic arrest, impaired embryonic development, apoptosis, and cell death [3, 11]. To counteract oxidative stress, in vitro systems rely on both enzymatic antioxidants—such as superoxide dismutase, glutathione peroxidase, and catalase—and non-enzymatic scavengers including glutathione, α-tocopherol, ascorbic acid, and β-carotene [12, 13]. The extent of oxidative damage ultimately depends on the balance between ROS generation and antioxidant defense capacity [14]. Since ROS production is closely linked to cellular metabolic activity, a reduction in

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[1]Uğur Şen, The Ondokuz Mayıs University, Agricultural Faculty, Department of Agricultural Biotechnology, 55139, Samsun, Türkiye

[2]Hasan Önder, The Ondokuz Mayıs University, Agricultural Faculty, Department of Animal Science, 55139, Samsun, Türkiye

[3]Cem Tırınk, The Iğdır University, Agricultural Faculty, Department of Animal Science, 76000, Iğdır, Türkiye

metabolic rate—potentially achieved through lower incubation temperatures—may decrease oxidative stress during IVM.

Therefore, the present study aimed to investigate the effects of low (36.5 °C) and conventional (38.5 °C) incubation temperatures during in vitro maturation on antioxidant activity, specifically glutathione peroxidase activity, in oocytes obtained from Anatolian buffaloes.

II. Material and Methods

Ovaries were obtained from Anatolian buffaloes at a local slaughterhouse and transported to the laboratory under standard hygienic conditions. Cumulus–oocyte complexes (COCs) were aspirated from antral follicles measuring 2–8 mm in diameter using an 18-gauge needle attached to a 10 mL syringe. Recovered COCs were collected in 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).

For in vitro maturation, COCs were cultured in sodium bicarbonate-buffered Medium 199 (Sigma, M4530) with 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 luteinizing hormone (LH), 0.5 µg/mL follicle-stimulating hormone (FSH), and 10 ng/mL epidermal growth factor (EGF). Approximately 25–35 COCs were placed in 500 µL of maturation medium per well, overlaid with 300 µL mineral oil, and cultured in four-well dishes (Nunc, Roskilde, Denmark). Maturation was carried out for 22 h in a humidified atmosphere of 5% CO_2 in air at either 36.5 °C (low temperature) or 38.5 °C (conventional temperature). Maturation culture was repeated 5 times for each temperature group.

Cumulus cell expansions of cumulus-oocyte complexes were evaluated at the end of maturation period under a stereomicroscope. Cumulus-oocyte complexes with fully expanded cumulus cell layer considered as maturated oocytes [10].

At the end of the maturation period, cumulus cells were completely removed by vortexing, and oocyte nuclear maturation was assessed based on the presence of the first polar body. Denuded oocytes were washed three times in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) supplemented with 1 mg/mL polyvinyl alcohol, aliquoted into microtubes (approximately 25 oocytes per 10 µL), and stored at −80 °C until enzymatic analysis [10].

Nuclear phases of the oocytes was determined using fluorescent bisbenzimide (Hoechst) DNA staining. Nuclear phases were examined under a fluorescence microscope with UV filter at four hundred time magnification and oocytes reached to M II stage considered as maturated oocytes [10].

For enzyme extraction, frozen oocytes were subjected to ultrasonication for 30 min, followed by centrifugation at 10,000 × g for 20 min at 4 °C. The resulting supernatants were used for the determination of glutathione peroxidase (GPx) activity. Enzymatic activity was quantified using a commercially available GPx assay kit (Northwest Life Science Specialties, LLC, Vancouver, WA, USA) according to the manufacturer's instructions and measured spectrophotometrically at 340 nm [10].

Statistical analysis of GPx activity data was performed using Duncan's multiple range test, and differences were considered statistically significant at $P < 0.05$.

III. Results and Discussion

Maturation traits of Anatolian buffalo's oocytes matured at 36.5 °C or 38.5 °C are presented in Table 1. Low and conventional incubation temperatures in IVM had a similar effect on percentages of oocytes with expanded cumulus. Similarly, there were no significant differences between good or moderate quality cumulus-oocyte complexes matured at 36.5 °C or 38.5 °C with regard to reached to metaphase II (M II) stages.

TABLE 1. MATURATION TRAITS OF ANATOLIAN BUFFALO'S OOCYTES MATURED AT 36.5 °C OR 38.5 °C

Temperature	CE (%)	MII (%)
36.5 (n= 158)	93,5	65,2
38.5 (n= 161)	94,8	64,9

CE= cumulus expansion, MII= metaphase II stage

In the present study an attempt was made to carry out IVM of Anatolian buffalo's oocytes at low temperature of 36.5°C, which is considered to be the temperature of preovulatory follicles and at which supposedly in vivo oocyte maturation occurs. The results for the oocytes showed that cumulus expansion rate and the proportion of oocytes reaching the M II stage were similar between the tested two maturation temperatures (36.5°C or 38.5°C). This suggests that low incubation temperature of 36.5°C during the maturation process does not alter the progress of bovine nuclear maturation. Similarly, previous studies have shown that low incubation temperature during IVM did not affect the rate of cumulus expansion [12, 13], first polar body formation [12, 13, 14] and proportion of oocytes that reached to M II stage [12, 13, 15]. Ye et al [6] have reported that addition of follicular fluid to the maturation media did not alter nuclear maturation level of porcine oocytes matured at 35.5°C, compared to counterparts matured at 39.0°C and 37.0°C. Moreover, supplementation of estrous cow serum into the maturation media did not affect nuclear maturation level of bovine oocytes cultured at 37°C and 39°C [6]. Overall, these studies suggest that serum may protect oocytes from possible negative effects, if any, of low incubation temperatures. It has been suggested previously [15] that, lower follicular ambient temperature is advantageous to complete oocyte maturation or development, within the follicular microenvironment.

Glutathione peroxidase enzyme (GSH-Px) activity of Anatolian buffalo's oocytes matured at 36.5 °C or 38.5 °C culture temperature are shown in Figure 1. There were no

significant difference between oocytes matured at 36.5 °C (0.19±0.025 mU/ml) or 38.5 °C (0.21±0.036 mU/ml) culture temperature in terms of Glutathione peroxidase enzyme (GSH-Px) activity.

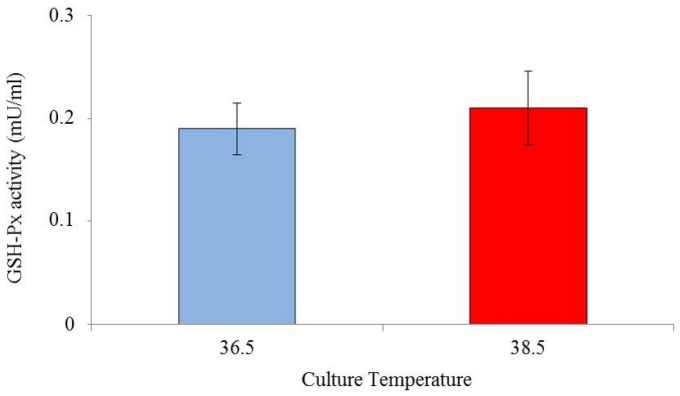

Fig. 1. Glutathione peroxidase enzyme (GSH-Px) activity of Anatolian buffalo's oocytes matured at 36.5 °C or 38.5 °C.

Reactive oxygen species (ROS) are unavoidable by-products of aerobic metabolism and are primarily generated during electron transfer reactions occurring in the mitochondrial respiratory chain and the endoplasmic reticulum [11, 12]. Under in vitro conditions, oocytes and early embryos are often exposed to elevated oxidative stress due to artificial culture environments that differ markedly from physiological conditions, leading to increased ROS production [3]. Because embryonic cells are particularly sensitive to oxidative imbalance, effective protection against excessive ROS accumulation is considered essential for successful oocyte maturation and subsequent embryonic development in vitro [12].

To counteract oxidative stress, cells rely on a tightly regulated antioxidant defense system composed of enzymatic and non-enzymatic components. Among these, glutathione peroxidase (GSH-Px) is a key selenium-dependent enzyme that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides into water and molecular oxygen, thereby preventing the accumulation of harmful ROS such as superoxide anions and hydroxyl radicals [11, 12]. In mammalian cells, GSH-Px plays a central role in maintaining intracellular redox homeostasis and protecting cellular structures from oxidative damage [16]. A reduction in GSH-Px activity has been associated with impaired redox balance, increased ROS accumulation, and heightened oxidative stress, which may adversely affect oocyte quality and developmental competence [17].

In the present study, glutathione peroxidase activity in Anatolian buffalo's oocytes was not significantly affected by incubation temperature during in vitro maturation, as similar enzyme activity levels were observed at both low (36.5 °C) and conventional (38.5 °C) temperatures ($P > 0.05$). These findings suggest that within the examined temperature range, the antioxidant defense capacity of buffalo oocytes is preserved and that moderate reductions in IVM temperature do not compromise intracellular redox regulation. This may indicate a degree of physiological adaptability of buffalo oocytes to thermal variations during maturation, potentially reflecting species-specific metabolic or antioxidative characteristics.

Our results contrast with those reported by Şen [10], who observed significantly lower GSH-Px activity in bovine oocytes matured at reduced incubation temperatures compared with those cultured under conventional conditions. This discrepancy may be attributed to interspecies differences in metabolic rate, mitochondrial activity, and intrinsic antioxidant capacity between cattle and buffalo oocytes. Buffalo oocytes are known to exhibit distinct physiological and metabolic features, including differences in lipid content and oxidative metabolism, which may influence their response to environmental stressors such as temperature fluctuations during in vitro culture.

Furthermore, it has been proposed that lowering incubation temperature may reduce cellular metabolic activity, potentially leading to decreased ROS generation. In such a scenario, unchanged GSH-Px activity, as observed in the present study, could reflect a balanced redox state rather than an impaired antioxidant response. This balance may explain why reduced incubation temperature did not adversely affect antioxidant enzyme activity in buffalo oocytes. However, since ROS production and antioxidant responses are dynamic and multifactorial, evaluating additional oxidative stress markers and developmental endpoints would be valuable to further elucidate the biological significance of temperature modulation during IVM.

IV. Conclusion

In conclusion, the present findings indicate that altering incubation temperature during in vitro maturation within a physiologically relevant range does not affect glutathione peroxidase activity in Anatolian buffalo's oocytes. These results contribute to a better understanding of species-specific responses to culture conditions and suggest that modest reductions in IVM temperature may be applied without compromising the antioxidant defense system of buffalo oocytes.

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