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Reactive Oxygen Species in Follicular Fluid as a Potential Biomarker of Oocyte Developmental Competence

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Abstract

Background: Reactive oxygen species (ROS) are critical factors for oocyte maturation and early embryogenesis; however, excessive ROS can induce oxidative stress, impairing mitochondrial function, DNA integrity, and embryo competence. The role of oxidative status in buffalo follicular fluid (FF) remains underexplored. This study assessed the relationship between total oxidant status (TOS) in buffalo FF and the developmental competence of oocytes retrieved by ovum pick-up (OPU) for subsequent in vitro fertilization (OPU-IVF).

Methods: Follicular fluid and cumulus–oocyte complexes (COCs) were collected from 62 healthy buffaloes. Oocytes were matured and fertilized *in vitro*, and the animals were classified based on blastocyst yield: G1 (no blastocysts), G2 (≥2 blastocysts), and G3 (>3 blastocysts). TOS was measured spectrophotometrically. The relationship between TOS and oocyte competence was analyzed by ROC (G1 *vs*. G3) and Spearman correlation (G1 *vs*. G2), with $p<0.05$ considered statistically significant.

Results: G2 group with >2 blastocyst exhibited lower TOS levels (1.10±0.51 *µmol* H_2O_2 Eq/L) than G1 (2.15±0.92; p=0.004), with higher follicle counts, oocyte yield, cleavage rate, and blastocyst production ($p<0.05$). ROC analysis identified a TOS threshold of 1.12 *µmol* H_2O_2 Eq/L (area under the curve [AUC]=0.851), and TOS inversely correlated with the proportion of high-quality blastocysts (r=-0.553, p=0.021).

Conclusion: Elevated oxidative stress in FF compromises oocyte developmental competence and embryo quality. TOS may serve as a predictive biomarker, supporting antioxidant-based optimization of assisted reproductive technology (ART) in buffalo.

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Introduction

Reactive oxygen species (ROS) are reactive oxygen_derived molecules of oxygen generated as by-products of cellular metabolism, primarily through mitochondrial oxidative phosphorylation and enzymatic pathways such as NADPH oxidase. While ROS play essential roles in physiological processes like oocyte maturation, fertilization, and early embryonic development, their overproduction can lead to oxidative stress, causing damage to lipids, proteins, and DNA.

This imbalance is a critical factor in reduced oocyte quality and impaired embryo development (1, 2).

The FF surrounding the oocyte provides a unique microenvironment, rich in nutrients, hormones, and signaling molecules, including ROS and antioxidants. This dynamic balance between ROS and antioxidants in FF is crucial for maintaining oocyte competence and subsequent embryo viability. Studies in humans and bovines have demonstrated that ROS levels in FF are closely associated with fertilization rates, embryo cleavage patterns, and blastocyst formation, making it a valuable biomarker for reproductive success (3, 4). The oxidative environment of buffalo FF and its influence on oocyte and embryo competence remains poorly understood. Oxidative stress, resulting from an imbalance between oxidants and antioxidants in follicular fluid, critically affects follicular function and can diminish overall reproductive performance (5).

Buffaloes are an economically significant livestock species, contributing substantially to global milk and meat production, particularly in developing countries (6, 7). ARTs, including ovum pick-up IVF, embryo transfer, and play a crucial role in addressing reproductive inefficiencies in buffaloes, such as low conception rates and extended calving intervals. Despite their potential, the application of ARTs in buffaloes faces challenges, including seasonal breeding patterns, lower oocyte quality, and reduced success rates compared to cattle. Optimizing ART protocols to account for the unique physiological and oxidative profiles of buffaloes is therefore essential for improving reproductive performance and meeting the increasing demand for high-quality animal products (8, 9). Furthermore, advancements in ART can enhance reproductive outcomes, increase milk and meat production, and support global food security. Identifying ROS and establishing cutoff values as biomarkers for embryo quality, alongside developing targeted antioxidant strategies, can further refine ART protocols, not only in buffaloes but also across other livestock species. These advancements hold the potential to drive progress in agricultural biotechnology and enhance overall livestock productivity (9, 10).

This study is the first to explore the link between follicular fluid oxidative status and oocyte developmental competence in buffalos. It specifically aimed to determine how variations in oxidative balance within the follicular environment affect oocyte quality.

Methods

This study was conducted at the Biofarm Research and Development Center of Avicenna Research Institute under the ethical approval number IR.SKU.REC.1403.015. All reagents, unless specified otherwise, were obtained from Sigma-Aldrich, USA. All experiments followed the relevant ethical rules. Following these ethical guidelines, oocytes were aspirated from 62 buffaloes via OPU technique for subsequent *in vitro* embryo production. During the OPU procedure, follicular fluid was collected to investigate the potential association between follicular TOS and embryonic developmental competence. Following collection, oocytes from each animal were matured *in vitro* for 22–24 *hr* and fertilized individually. Based on the blastocyst yield, the buffaloes were classified into three groups: those producing no blastocysts (G1), those producing more than two blastocysts (G2), and those producing more than 3 blastocysts (G3). Thereafter, TOS levels in follicular fluid were measured in these groups. Subsequently, the relationship between follicular TOS concentrations and the developmental potential of oocytes in supporting embryo production was evaluated.

Animals: Oocyte donors were 62 non-pregnant river buffaloes, aged 2–4 years, with an average body condition score of 3.0±0.25 (scale 1–5). Only animals deemed clinically healthy, with no previous history of illness (*e.g.*, infections, fever, digestive problems, or appetite loss), as confirmed by the farm veterinarian, were enrolled in the study. Donors received a total mixed ration formulated to meet the nutritional recommendations of the National Research Council (NRC).

OPU procedure: OPU protocol applied in this study is illustrated in figure 1. On day 0, dominant follicles were removed through dominant follicle removal (DFR). Subsequently, buffaloes received FSH (Cinnal-f; CinnaGen, Iran) on day 2 via intramuscular injection, administered in five descending doses (100, 75, 75, 60, and 60 *IU*) at 12-*hr* intervals. OPU was conducted 48 *hr* after the last FSH injection (day 6). The procedure was performed following the approach described in previous report (11). Prior to oocyte collection, heifers were restrained in an adjustable squeeze chute, and 5 *ml* of 2% lidocaine hydrochloride (Vetacaine; Aburaihan Pharmaceutical Co, Iran)

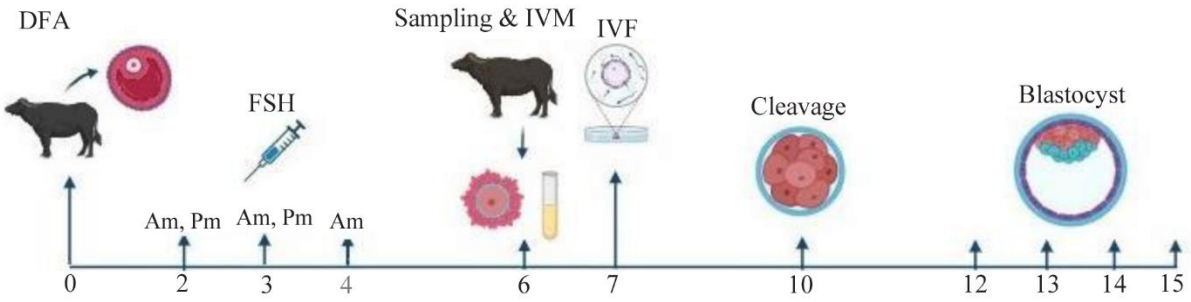

Figure 1. Timeline of the buffalo IVP process
DFA: Dominant Follicle Ablation, FSH: Follicle Stimulation Hormone, IVM: *In Vitro* Maturation, IVF: In Vitro Fertilization

was administered epidurally for anesthesia. The rectum was evacuated, and the vagina, vulva, and perineal region were disinfected with water and iodine prior to insertion of the ultrasound probe into the vagina. Antral follicles larger than 3 *mm* in diameter were aspirated using a disposable 18 G biopsy needle mounted on a needle holder attached to the ultrasound probe holder (iuStar-160 vet; United Imaging Healthcare, China), equipped with a micro convex probe (R11 MCA, 4–9 *MHz*). Aspiration was performed under a vacuum pressure of 50–70 *mmHg*, and the follicular fluid was collected into a 50 *ml* conical tube maintained at 38°*C*.

Follicular fluid sampling: Following OPU, follicular fluid was collected in conical tubes without flushing medium. The fluid was examined for the presence of COCs under a stereomicroscope and then centrifuged at 3,000×*g* for 10 *min* at 4°*C* to remove cumulus cells and cellular debris. The resulting supernatants were stored at −80°*C* until biochemical analysis.

In vitro embryo production (IVP)

Oocyte collection and in vitro maturation (IVM): Aspirated COCs were washed four times in washing medium (H-TCM199 supplemented with 10% fetal bovine serum) and cultured for 22–24 *hr* in maturation medium under mineral oil at 38.5°*C* in a humidified atmosphere containing 5% CO_2. The maturation medium consisted of TCM199 (M5017) supplemented with 2.5 *mM* sodium pyruvate, 1 *mM* L-glutamine, 10% (*v/v*) fetal bovine serum, 0.05 *IU/ml* FSH, 1 *mg/ml* estradiol-17β, and 0.1 *mM* cysteamine (12).

In vitro fertilization (IVF): The IVF procedure was conducted according to previously described protocols (13). Briefly, matured COCs were washed four times in fertilization medium prior to transfer into droplets of the same medium (Fert-TALP supplemented with 0.2 *mM* sodium pyruvate, 5 *mg/ml* fatty acid-free bovine serum albumin (BSA), and 10 *µg/ml* heparin). Motile sperm from frozen–thawed semen was isolated using a density gradient centrifugation (PureSperm 40% and 80%) at 500×*g* for 5 *min* at room temperature. Fertilization was carried out through co-incubating COCs with sperm at a final concentration of 1×10^6 *sperm/ml* in each droplet and cultured for 18-20 *hr* at 38.5°*C* in a humidified atmosphere containing 5% CO_2.

In vitro culture (IVC): Following IVF, cumulus cells were removed from presumptive zygotes by gentle pipetting using an appropriate pipette. The presumptive zygotes were then cultured in drops of synthetic oviductal fluid (SOF) medium supplemented with essential amino acids (MEM; 20 *µl/ml*, M7145), non-essential amino acids (BME; 10 *µl/ml*, B6766), and 8 *mg/ml* fatty acid-free BSA under mineral oil in a humidified atmosphere of 5% CO_2 and 90% N_2 at 38.5°*C* (14). The cleavage rate was determined on day 3 post-insemination (IVF=day 0) by calculating the proportion of zygotes reaching the 2–16 cell stage relative to the total number of oocytes subjected to IVM. Blastocyst formation rate was calculated as the number of blastocysts divided by the initial number of oocytes used for IVM.

TOS determination: TOS was quantified spectrophotometrically using a commercial colorimetric assay kit (Navand Salamat, Iran) following the manufacturer's protocol. In brief, 30 *µl* of follicular fluid or standard solution was dispensed into each well of a 96-well microplate, followed by the addition of 200 *µl* of reagent 1. After thorough mixing, 10 *µl* of reagent 2 was added, and the mixture was incubated at ambient temperature for

20 *min* under controlled conditions. Optical density was measured at 530 *nm* (or alternatively at 490 *nm*) using a calibrated microplate reader. TOS concentrations were calculated based on a standard curve generated with hydrogen peroxide equivalents and expressed as *µmol* H_2O_2 equivalents per liter (*µmol* H_2O_2 Eq/L) (15). All measurements were performed in duplicate to ensure reproducibility.

Statistical analysis: Descriptive statistics were reported as frequencies and percentages for qualitative variables, and as means, standard deviations, medians, and quartiles for quantitative variables. Due to the non-normal distribution of the quantitative data (assessed using the Shapiro-Wilk test), non-parametric tests were employed. The Independent-Samples Mann-Whitney U test was used to compare the two groups, while Spearman's rank correlation coefficient was applied to evaluate relationships between quantitative variables. Receiver operating characteristic (ROC) curve analysis was performed to identify the optimal cut-off value. All statistical analyses were conducted using SPSS software, version 21 (IBM, USA) with a statistical significance level of 5% ($p<0.05$).

Results

The study compared TOS and parameters of oocyte developmental competence between two groups of buffalos subjected to ovum pick-up and in vitro fertilization: G1 (n=12, no blastocyst formation) and G2 (n=17, ≥2 blastocysts). Descriptive statistics for these parameters are presented in table 1. To visualize the differences in TOS between the groups, a bar plot was generated (Figure 2).

The results demonstrated that G2 had a significantly lower total oocyte score (TOS; mean±SD: 1.10±0.51) compared to G1 (2.15±0.92; p=0.004). Additionally, G2 showed superior outcomes in the number of follicle count (p=0.010), oocyte yield (p=0.002), cumulus-oocyte complexes at the germinal vesicle stage (p<0.001), cleavage embryos on day 3 (p<0.001), and blastocyst production. In contrast, G1 had a higher number of poor-quality oocytes (GD), with no significant difference in denuded oocytes (p=0.787).

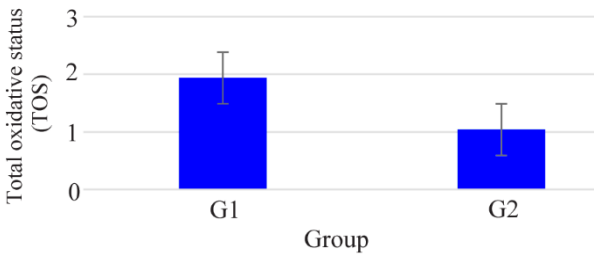

Figure 2. Mean TOS in G1 (no embryo) and G2 (>2 embryos) buffalos. Bar plot showing mean TOS with ±SD error bars for each group

ROC analysis: The ROC analysis was conducted to assess the diagnostic performance of TOS in distinguishing between G1(n=12, no embryos) and G3 (n=14, >3 embryos). The results are summarized in table 2, with the ROC curve presented in figure 3.

The optimal cut-off point for TOS was determined to be 1.12, with AUC of 0.851, indicating good discriminatory ability (p=0.002). The performance of the TOS cut-off point (1.12) was evaluated, as shown in table 3.

The TOS cut-off point of 1.120 demonstrated a sensitivity of 83.3% and a specificity of 64.3%. The PPV was 66.7%, and NPV was 81.8%. The overall diagnostic accuracy was 73.1%.

Correlation analysis: The relationship between

Table 1. Comparison of TOS and OPU-IVP outcomes between G1 and G2

	G1					G2					p [1]
	Mean	SD	Median	Q25	Q75	Mean	SD	Median	Q25	Q75	
TOS	2.15	0.92	2.31	1.18	2.64	1.10	0.51	1.07	0.75	1.46	**0.004**
Follicles	8.75	6.54	6.50	3.75	11.75	14.00	5.54	12.50	10.00	17.25	**0.010**
Oocytes	4.83	4.71	3.00	2.00	6.25	11.33	6.08	11.00	7.75	12.75	**0.002**
COCs	2.83	3.01	1.50	1.00	4.50	10.33	5.17	9.50	7.50	12.75	**<0.001**
Denuded oocytes	2.00	1.91	2.00	1.00	2.00	1.72	1.49	1.50	0.75	2.25	0.787
GD oocytes	3.17	2.62	3.50	0.25	5.00	0.28	0.46	0.00	0.00	1.00	
Cleaved oocytes	2.25	2.67	1.00	0.00	4.50	9.50	4.05	9.00	7.00	12.25	**<0.001**
Total blastocysts	0.00	0.00	0.00	0.00	0.00	5.78	2.98	5.00	4.00	7.00	

Note: Values represent means, standard deviations (SDs), medians, and interquartile ranges (Q25–Q75). p-values were derived from Independent-Samples Mann-Whitney U Test. GD = Grade D Oocytes (poor-quality oocytes not used for culture). TOS is expressed in µmol H_2O_2 Eq/L; all other parameters are expressed as number of oocytes

Table 2. Results of ROC and AUC for TOS

Cut-off point	AUC	S. E	95%CI		p-value
			Lower	Upper	
1.12	0.851	0.077	0.700	0.992	0.002

Note: AUC represents the area under the ROC curve for TOS, with the optimal cut-off point at 1.12
CI = confidence interval

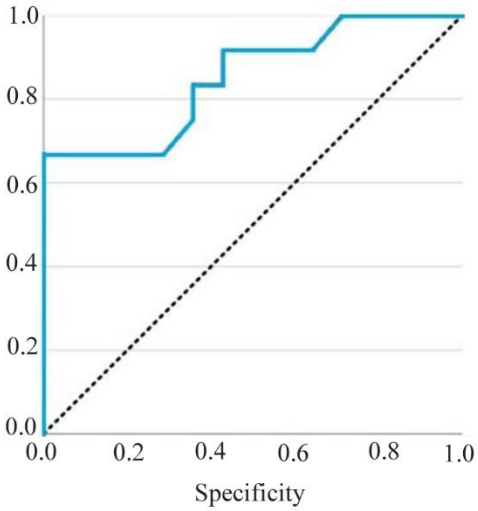

Figure 3. ROC curves for TOS

TOS and blastocyst development on different days, as well as blastocyst grades in G2, was assessed using the Spearman correlation test, as shown in table 4.

Discussion

This study provides novel insights into the role of TOS in follicular fluid as a biomarker of oocyte developmental competence in buffalo for ovum pick-up and *in vitro* embryo production. By evaluating follicular fluid from 62 buffalos, categorized into G1 (n=12, no embryos) and G2 (n=17, ≥2 embryos), significantly lower TOS levels were found in G2 (mean±SD 1.10±0.51) compared to G1 (2.15±0.92; p=0.004). Additionally, G2 exhibited superior reproductive outcomes, including significantly higher follicle counts, oocyte yields, cumulus-oocyte complexes at the germinal vesicle stage, cleavage rates on day 3, and blastocyst production, whereas G1 showed a significantly higher proportion of poor-quality oocytes (grade D).

These findings suggest that elevated oxidative stress, as indicated by TOS, may impair oocyte developmental competence, likely through mitochondrial dysfunction, which compromises ATP production and disrupts cellular energy homeostasis. This conclusion is supported by studies demonstrating that oxidative stress-induced mitochondrial impairment correlates with reduced oocyte quality and developmental potential (16). Additionally, oxidative stress can cause DNA fragmentation, disrupt spindle assembly and chromosome alignment, thereby increasing the risk of

Table 4. Correlation of TOS with blastocyst development and grades in G2

Parameters	Correlation coefficient	p-value
Bl (D5)	0.036	0.890
Bl (D6)	-0.271	0.293
Bl (D7)	-0.327	0.200
Bl (D8)	-0.371	0.143
Bl (I)	-0.553	0.021
Bl (II)	-0.384	0.128
Bl (III)	0.250	0.332

Note: A significant inverse correlation was observed between TOS and grade I blastocysts (Bl (I), r=-0.553, p=0.021). No significant correlations were found between TOS and blastocyst development on days 5, 6, 7, or 8, or with grade II and III blastocysts (p>0.05).

Table 3. Sensitivity and specificity of TOS cut-off point

TOS cut-off point	Group	Frequency	% TOS cut-off point	% Group
≤ 1.12	G3	10	66.7%	83.3%
	G1	5	33.3%	35.7%
> 1.12	G3	2	18.2%	16.7%
	G1	9	81.8%	64.3%

Note: Sensitivity (83.3%), specificity (64.3%), positive predictive value (PPV, 66.7%), and negative predictive value (NPV, 81.8%) are reported for the TOS cut-off point of 1.120. Overall diagnostic accuracy is 73.1%

meiotic errors during oocyte maturation (17, 18).

These cumulative alterations markedly reduce oocyte competence, impairing their capacity for successful maturation, fertilization, and subsequent embryonic development, consistent with previous studies linking ROS to decreased reproductive outcomes in mammals (18, 20).

This is the first study to directly assess TOS in follicular fluid as a predictor of oocyte quality and embryo production in buffalos, addressing a critical gap in species-specific reproductive research. While oxidative stress has been extensively studied in species such as cattle, sheep, and humans (10, 21), buffalos, a key livestock species in tropical and subtropical regions, have been largely neglected in this context. Previous buffalo studies have explored oxidative stress in relation to endometritis or seasonal variation in follicular metabolomics (22, 23), but none have linked TOS to OPU-IVP outcomes. Our findings establish TOS as a quantifiable marker for optimizing ARTs in buffalos, potentially enhancing embryo production in regions reliant on buffalo for dairy and meat industries.

The ROC analysis supports TOS's diagnostic potential, with AUC of 0.851 and an optimal cut-off point of 1.12, yielding a sensitivity of 83.3%, specificity of 64.3%, and diagnostic accuracy of 73.1%. This performance is highly promising for clinical applications, particularly given the specific grouping of our G3 subgroup (producing more than three blastocysts) which was designed to maximize the contrast between high and low reproductive outcomes. These observations align with evidence from human IVF studies indicating that ROS-related biomarkers serve as reliable predictors of fertilization outcomes and embryo developmental competence (2, 24). Similarly, Liu et al. observed that reduced oxidative stress in porcine follicular fluid improves meiotic maturation and enhances embryo quality (25). These cross-species parallels suggest that TOS could be integrated with other markers, such as lipid peroxidation or antioxidant enzyme levels, to improve diagnostic precision, as recommended in recent reviews (5, 18).

The significant inverse correlation between TOS and grade I blastocyst development in G2 ($r = -0.553$, $p=0.021$) emphasizes the targeted effects of oxidative stress on high-quality embryo production, whereas no significant associations were observed for blastocyst timing (days 5–8) or lower-grade blastocysts (II and III). This aligns with evidence that ROS induce mitochondrial dysfunction and ATP depletion, cell cycle arrest and apoptosis, thereby directly impairing embryo quality. Higher ROS concentrations correlate with increased apoptosis, leading to greater embryo fragmentation and reduced blastocyst development (21, 26). Environmental factors, particularly heat stress, exacerbate oxidative damage in ruminant follicles and impair both steroidogenesis and embryo viability. Under heat stress conditions, follicular fluid exhibited altered oxidative stress markers, including superoxide dismutase (SOD) and ROS, aligning closely with our findings on TOS (27).

Dietary antioxidants, such as green tea extract, have been shown to alleviate oxidative stress and enhance oocyte competence in buffalos (28). Similarly, supplementation of *in vitro* culture systems with antioxidants, including melatonin, resveratrol, N-acetylcysteine, astaxanthin and glutathione, has been shown to attenuate oxidative stress, thereby enhancing oocyte maturation and its developmental competence. These agents also contribute to improved blastocyst yield, cryotolerance, and the regulation of genes critical for early embryogenesis (29-33). These findings indicate that targeted modulation of TOS, through antioxidant supplementation or optimized management of the follicular microenvironment, may represent a promising strategy to enhance OPU-IVP protocols in buffalo. Approaches such as enriched culture media or pre-treatment interventions could further support oocyte quality and subsequent embryonic development, underscoring the critical role of oxidative balance in reproductive success.

Despite providing valuable insights, this study was subject to some limitations. For instance, the sample size was relatively small (n=29), and although TOS provides an integrative measure of the overall oxidant burden, assessing total antioxidant capacity (TAC) alongside TOS would have yielded a more comprehensive understanding of the redox status. To enhance the robustness and generalizability of these findings, future research should involve larger cohorts, integrate multi-omics approaches (34), and validate TOS thresholds across heterogeneous buffalo populations. Furthermore, longitudinal studies evaluating pregnancy and live birth outcomes following embryo transfer are warranted to more definitively establish the clinical utility of TOS as a predictive marker in assisted reproductive protocols. Collectively, addressing these limitations will strengthen

the translational relevance of oxidative stress assessments in improving buffalo reproductive management.

Conclusion

Our findings indicate that elevated oxidative stress, as reflected by TOS levels, is associated with reduced buffalo oocyte quality and impaired developmental competence. These results suggest that targeted modulation of the redox balance, through antioxidant supplementation or optimization of the follicular microenvironment, may help improve OPU-IVP outcomes. Future studies addressing current limitations and validating predictive biomarkers such as TOS are warranted to further support the translation of these findings into effective reproductive management strategies.

Conflict of Interest

The authors declare that there is no conflict of interest that could have influenced the outcomes or interpretation of this study.

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