Original Article

Buffalo Bulletin (July-September 2026) Vol.45 No.3
https://doi.org/10.56825/bufbu.2026.4536075

QUERCETIN'S ROLE IN ENHANCING BUFFALO OOCYTE MATURATION AND *IN VITRO* EMBRYO PRODUCTION

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Received: 14 January 2025
Accepted: 30 September 2026

ABSTRACT

Reproductive inefficiency in *Nili-Ravi* buffaloes (*Bubalus bubalis*) presents a significant challenge to genetic progress and milk production in South Asia. *In vitro* embryo production is one of new technology but its media in indigenous *Nili Ravi* buffalo needs to be optimized to get promising results. This study explores the potential of Quercetin, a powerful antioxidant, to enhance *in vitro* oocyte maturation and embryo development, thereby improving reproductive outcomes. The objective was to evaluate the impact of varying Quercetin concentrations on *in vitro* oocyte maturation and *in vitro* embryo production. Total 200 oocytes were aspirated from ovaries of slaughtered female buffaloes and cultured in a maturation medium supplemented with Quercetin at concentrations of 0 µM, 5 µM, 10 µM, and 20 µM. Following fertilization, embryos were cultured to assess developmental progress. Results revealed that supplementation with 10 µM Quercetin significantly enhanced both *in vitro* oocyte maturation and blastocyst formation compared to the other concentrations ($P \leq 0.05$).

The 10 µM concentration yielded the highest embryo development, underscoring its potential in improving oocyte maturation and facilitating successful *in vitro* embryo production, can be an effective strategy to enhance *in vitro* reproductive efficiency in *Nili-Ravi* buffaloes. This study lays the foundation for integrating Quercetin into *in vitro* maturation media of *Nili Ravi* buffalo breeding programs, with promising implications for genetic improvement and enhanced reproductive success.

Keywords: *Bubalus bubalis*, buffaloes, Quercetin, *in vitro* oocyte maturation, *in vitro* embryo production

INTRODUCTION

Agriculture plays a crucial role in the economies of both industrialized and developing countries, with the livestock sector being particularly significant in regions such as South Asia. In Pakistan, buffaloes are a vital source of milk, contributing to over 60% of the country's

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total milk production. Buffaloes also play a critical role in neighboring countries like India, where they provide more than 50% of the milk supply. Despite their importance, current milk production is insufficient to meet consumer demand, especially when compared to global dietary recommendations. For example, adults are advised to consume approximately 732 mL of milk daily according to US dietary guidelines, while the average per capita milk availability in Pakistan is only 608 mL per day. This disparity highlights a pressing need to enhance buffalo milk production through improved breeding and reproductive strategies.

One promising avenue for addressing these challenges is through genetic selection and improved breeding programs for buffaloes. Among the river-type buffalo breeds, *Nili-Ravi* is particularly recognized for its high milk production capacity. However, despite advances in genetic selection, reproductive inefficiencies continue to limit the full genetic potential of buffalo populations. *In vitro* embryo production (IVEP) has gained attention as a powerful tool to accelerate genetic improvements in livestock by enabling the production of genetically superior offspring. A key challenge in IVEP is the *in vitro* maturation (IVM) of oocytes, which is highly sensitive to oxidative stress. The accumulation of reactive oxygen species (ROS) during IVM can lead to oocyte and embryo damage, reducing the efficiency of IVF procedures. Antioxidants have shown promise in counteracting oxidative stress, and Quercetin, a natural flavonoid, is known for its antioxidant properties. However, its potential to improve oocyte maturation and subsequent embryo development in buffaloes remains largely unexplored.

This study aims to investigate the effects of Quercetin supplementation on the *in vitro* maturation of buffalo oocytes and subsequent embryo development. Specifically, the objectives are to determine the optimal concentration of Quercetin that enhances oocyte maturation and improves embryo quality. By evaluating the impact of Quercetin on IVEP success rates, this study seeks to provide valuable insights into improving buffalo reproductive efficiency, thereby contributing to the genetic enhancement of buffalo populations and supporting more sustainable milk production systems.

MATERIALS AND METHODS

Ethical approval

The study was conducted in accordance with the ethical guidelines approved by the Ethical Review Committee of The University of Haripur, KP-Pakistan.

Chemicals and reagents

Chemicals and reagents, unless specified otherwise, were procured from Sigma-Aldrich (St. Louis, MO, USA). No live animals were used; all materials were obtained from slaughterhouses.

Collection of ovaries

Buffalo ovaries were collected from a local abattoir immediately post-slaughter and transported to the laboratory within 4 h in 0.9% (v/v) saline solution supplemented with streptomycin and penicillin at 38.5°C. Upon arrival, ovaries were washed 3 to 4 times with wash buffer and maintained in a water bath at 38.5°C.

Follicular oocyte aspiration and selection

Cumulus-oocyte complexes (COCs) were

aspirated from 2 to 8 mm diameter follicles using an 18-G needle attached to a syringe. The follicular fluid was transferred to a falcon tube, settled for 5 minutes in a warm bath, and washed three times in oocyte wash medium containing tissue culture medium (TCM-199). Oocytes were selected using a stereomicroscope (10 to 15× magnification) at 38.5°C. Grade 1 and 2 oocytes with uniform ooplasm and multiple cumulus layers were selected using a 10 µL pipette and transferred to a 35 mm dish with wash media. Irregular, naked, or atretic oocytes were excluded.

In-vitro maturation (IVM)

The IVM procedures were performed in TCM-199 medium supplemented with 20 µl each of FSH, LH, cysteine and estradiol. The COCs with at least three layers of surrounding cumulus cells and uniform ooplasm were placed in 20 µL droplets of pre-warmed maturation medium under mineral oil. The total 200 oocytes (10 oocytes/ droplet) were cultured in maturation medium with varying concentrations of Quercetin (0 µM, 10 µM, 20 µM, and 40 µM) at 38.5°C for 24 h in a humidified atmosphere of 5% (v/v) CO_2. Cumulus expansion was then assessed using a stereomicroscope.

In-vitro fertilization (IVF)

Frozen semen was thawed at 37°C for 30 seconds and layered beneath 3 mL of fertilization medium. After 30 minutes of incubation, motile sperm were isolated by the swim-up method and centrifuged at 1500 rpm for 5 minutes. Mature oocytes were washed in fertilization medium to partially remove cumulus cells and co-incubated with spermatozoa in 20 µL droplets of fertilization medium (Tyrode medium with 0.6% BSA, 2.5 mM caffeine sodium benzoate, and 20 mg/mL heparin) at 38.5°C and 5% CO_2 for 6 h.

In-vitro culture (IVC)

Presumptive zygotes were washed in Stroebech culture medium and incubated at 38.5°C in either a standard incubator (5% CO_2, 20% O_2) or a low-oxygen chamber (5% CO_2) for 7 days. Blastocyst development was monitored on days 5, 6, 7, and 8 post-insemination.

Vitrification of blastocysts

Blastocysts were vitrified as per (Yang *et al*., 2012) protocol. Briefly, blastocysts were equilibrated in equilibration solution for 5 minutes, then sequentially exposed to vitrification solutions A (10% ethylene glycol + 10% DMSO) and B (20% ethylene glycol + 20% DMSO + 0.5 M sucrose) for 30 seconds each. The blastocysts were loaded onto straws and rapidly plunged into liquid nitrogen.

Statistical analysis

Data on maturation, cleavage, and blastocyst development were analyzed using one-way ANOVA with Tukey's post-hoc test. Graphs were prepared using GraphPad PRISM 5 software.

RESULTS

In vitro maturation

The effects of Quercetin on the in vitro maturation rates of buffalo oocytes are shown in Figure 1. The maturation rate was significantly higher ($P \leq 0.05$) in oocytes treated with 10 µM Quercetin compared to the control, 5 µM, and 20 µM groups. However, no significant differences ($P \geq 0.05$) were observed among the control, 5 µM, and 20 µM concentrations. These results suggest that the optimal Quercetin concentration for oocyte maturation is 10 µM.

***In vitro* fertilization**

The *in vitro* fertilization rates of buffalo oocytes matured with varying concentrations of Quercetin are presented in Figure 2. Oocytes treated with 10 µM Quercetin showed fertilization rates comparable to those in the control and 5 µM groups ($P \leq 0.05$). In contrast, oocytes treated with 20 µM Quercetin exhibited significantly lower fertilization rates ($P \leq 0.05$) than the control, 5 µM, and 10 µM groups. These findings indicate that higher concentrations of Quercetin (20 µM) may negatively affect fertilization.

***In vitro* cleavage**

The impact of Quercetin on cleavage rates of buffalo oocytes is illustrated in Figure 3. Oocytes matured with 10 µM Quercetin demonstrated significantly higher cleavage rates ($P \leq 0.05$) compared to the control, 5 µM, and 20 µM groups. Conversely, oocytes in the control, 5 µM, and 20 µM groups showed significantly lower cleavage rates ($P \leq 0.05$) than those treated with 10 µM Quercetin. These results highlight the superior efficacy of 10 µM Quercetin in promoting cleavage.

DISCUSSION

The present study provides evidence that quercetin supplementation at an optimal concentration of 10 µM in the maturation medium significantly enhances the *in vitro* maturation (IVM), fertilization, cleavage, and blastocyst development rates of buffalo (*Bubalus bubalis*) oocytes. This finding highlights the potential of quercetin as a crucial additive to counteract oxidative stress during IVM. The improvement in developmental outcomes aligns with the well-established role of antioxidants in mitigating the deleterious effects of reactive oxygen species (ROS), which are known to impair oocyte quality and developmental competence (Takashi *et al*., 2012; Silva *et al*., 2015).

The study concludes that supplementation of the maturation medium with 10 µM quercetin optimally supports oocyte maturation, sperm fertilization, cleavage, and blastocyst formation. This is consistent with prior findings in human and murine studies, where intermediate doses of quercetin improved developmental competence through its antioxidative properties (Cao *et al*., 2020). Quercetin acts to enhance oocyte developmental potential by reducing intracellular ROS levels, improving mitochondrial activity, and increasing intracellular glutathione (GSH) content (Liu *et al*., 2017; Ishige *et al*., 2001). In contrast, suboptimal concentrations (5 µM and 20 µM) resulted in lower maturation, cleavage, and blastocyst rates. The decline at higher concentrations may be explained by quercetin's interaction with estrogen receptors, altering granulosa cell functions and disrupting the oocyte microenvironment (Orlovschi *et al*., 2014; Kubo *et al*., 2015). Such effects underscore the importance of determining an optimal balance between antioxidant benefits and potential cytotoxicity at higher doses.

These findings contribute to the understanding of oxidative stress management during IVM and support the inclusion of antioxidants like quercetin in assisted reproductive technologies (ART). Quercetin's ability to activate signaling pathways such as SIRT3 and NRF2 (Liu *et al*., 2017; Zhang *et al*., 2015) introduces a novel mechanism for enhancing mitochondrial biogenesis and reducing ROS accumulation (Bonay and Deramaudt, 2015; Fan *et al*., 2017). This challenges existing assumptions that solely rely on broader antioxidative strategies and

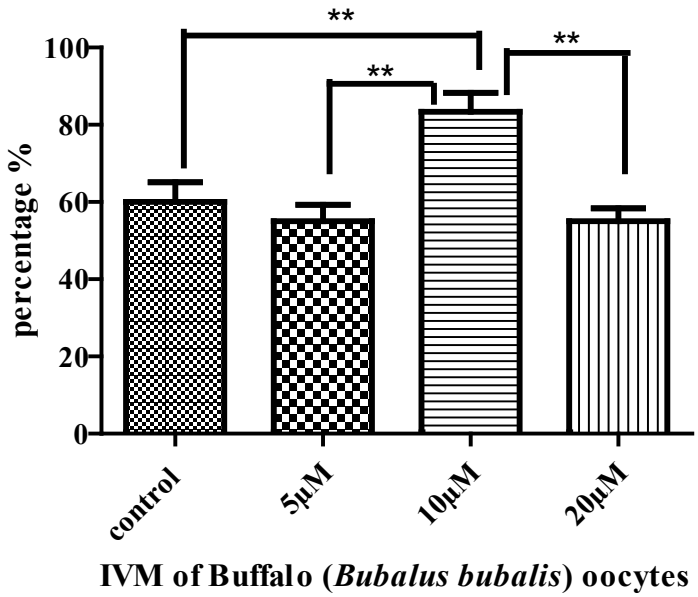

Figure 1. Percentage maturation rates of buffalo oocytes treated with different concentrations of Quercetin (P≤0.05; ns P>0.05).

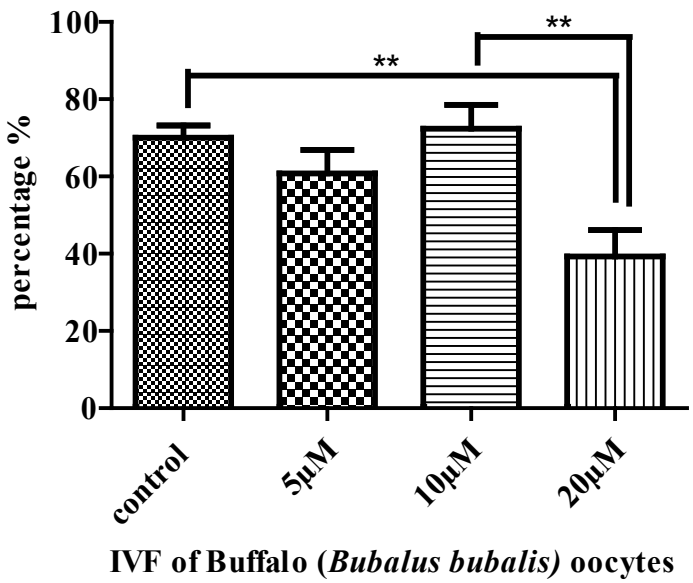

Figure 2. *In vitro* fertilization rates of buffalo oocytes treated with different concentrations of Quercetin (P≤0.05; ns P>0.05).

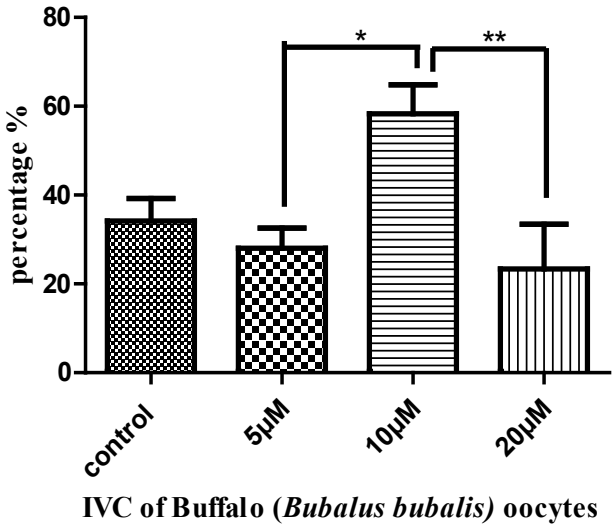

Figure 3. *In vitro* cleavage rates of buffalo oocytes at different concentrations of Quercetin (*P≤0.01; P≤0.05; ns P>0.05).

provides a more targeted approach for improving oocyte quality. Moreover, this study's findings are consistent with bovine, murine, and swine models, which have shown similar improvements in oocyte maturation and embryo development with quercetin supplementation (Maedomari *et al*., 2007; Albuquerque *et al*., 2021). The cross-species applicability of quercetin suggests that it may serve as a universal additive in ART protocols for livestock. Similarly, the observed decline in developmental outcomes at 20 µM suggests a threshold beyond which quercetin exerts cytotoxic effects. Future studies should explore the precise dose-response relationship and its molecular basis.

CONCLUSION

This study demonstrates that quercetin supplementation at 10 µM optimally enhances buffalo *in vitro* oocyte maturation, fertilization, and subsequent embryonic development, likely through its antioxidative and mitochondrial-supporting effects. The findings not only reaffirm the importance of oxidative stress management in ART but also introduce quercetin as a promising candidate for improving reproductive efficiency in livestock species. However, the biphasic response observed at higher concentrations highlights the need for careful optimization of antioxidant supplementation. Future research should focus on unraveling the molecular mechanisms and broader implications of quercetin's use in ART.

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