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Ameliorating Effects of Kaempferol on Buffalo Oocytes Developmental Competence (Part- I)

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ABSTRACT

To our knowledge, the cytoprotective roles of Kaempferol on the *in vitro* matured buffalo oocytes and embryos are still unknown. The current research was conducted to investigate the ideal dose of kaempferol that could be used safely in *the vitro* embryo production technology of buffalo as well as investigates the kaempferol effects on buffalos· cumulus cell expansion, *in vitro* maturation, penetration, fertilization, cleavage, morula, and blastocyst rates. Tissue culture media-199 supplemented with Kaempferol at 0, 5, 10, and 15 µg/ml was used to mature cumulus-oocyte complexes (COCs). The current study revealed that kaempferol supplementation at 10 µg/ml in the *in vitro* maturation medium of buffalo oocytes significantly improved cumulus cell expansion (75.33±1.75%), in vitro maturation (87.08±1.16%), penetration (78.40±3.75%), fertilization (42.80±1.15%), cleavage (28.20±3.51%), morula (21.70±2.65%), and blastocyst rates (19.90±2.45%) of buffalos. In conclusion, the presence of kaempferol in buffalo oocytes maturation medium is potentially important to regulate its developmental competence. Moreover, 10µg/ml of Kaempferol is an ideal promising dose that could be used safely to augment the *in vitro* embryo production technology of buffalo.

1. INTRODUCTION

In mammals, the follicular dynamics including maturation, ovulation, and fertilization are crucial processes that must run successfully to ensure efficient reproductive potentials (Dai et al., 2017). Even though, after *in vitro* oocyte maturation or *in vivo* ovulation process the oocyte quality and its developmental competence decreases over time in a process called "oocyte aging" (Zheng et al., 2016). Oocyte aging entries a lot of changes like zona pellucida hardening (Diaz and Esponda, 2004 a&b), which decreases the rate of fertilization and induces abnormal embryo cleavage pattern and rate (Miao et al., 2009), consequently delaying the embryonic developmental potential (Tarín et al., 1999), due to abnormal spindles and chromosomal ultrastructure (Saito et al., 1993). Moreover, oocyte aging significantly affects mitochondrial function (Cui *et al.*, 2012). So, modulating the oocyte aging process in the *in vitro* embryo production techniques becomes a necessary request to ameliorate the whole procedure.

Kaempferol (KAE) is a promising flavonoid detected in a wide range of plants (Calderón-Montaño *et al*., 2011; Ding *et al*., 2013). Polyphenolic KAE is a bioactive substance that is crucial for primary and secondary follicles development with viable mitochondria (Yao *et al.*, 2019). KAE was reported to have promising effects on the *in vitro* cultured preantral ovine follicles (Santos *et al.*, 2019). Furthermore, KAE addition to the embryo development medium was found to increase COX2 and SOX2 mRNA expression but substantially decrease Caspase-3 expression levels (Kumar *et al.*, 2023). KAE was reported to have potential roles in alleviating the aging changes in porcine oocytes by improving the quality of oocytes (Yao *et al*., 2019). Additionally, it protects the mitochondrial membrane functional potential (MMP) and reduces the rate of blastocysts' apoptosis, so in turn improved the embryo developmental competencies besides improving the blastocysts' quality (Yao *et al*., 2019). Orlovschi et al. (2014) documented that kaempferol supplementation in the medium of the *in vitro* procedures significantly enhanced the oocyte maturation potentials and increased the proportion of morula-stage embryos.

The aim of the current study is to explore the ameliorative effects of Kaempferol addition to IVM of buffalo oocytes on their developmental competence.

2. MATERIAL AND METHODS

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

Approval Ethics

The experimental procedures adopted in the current study were authorized by the Faculty of Veterinary Medicine, Benha University, Egypt (institutional review board for animal experiments) which provided the current study its ethical approval number (BUFVTM 27-06-23).

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Oocytes recovery and In Vitro maturation

Buffalo ovaries from slaughtered houses were transported in a sterile Dulbecco's phosphate buffered saline (D-PBS) at 37°C where they were washed in new a sterile D-PBS several times, then finally washed in a sterile warmed normal saline. Follicles ranging in size between 4-8mm were chosen to aspirate the qualified COCs using an 18-gauge needle (Yousaf and Chohan, 2003). The aspirate was left warmed for 15 minutes until the settlement of the aspirated COCs. 5m1 of the sediment was recovered in a new sterile falcon petri dish where evenly granulated homogenous oocytes that were surrounded with several layers (at least,4 layers) of compact, granular, and homogenous cumulus (granulosa) cells were selected to conduct the current experiment. In a sterile warmed D-PBS, the selected COCs were washed 3 times and then in fresh pre-warmed TCM-199 with phenol red that was enhanced with 10% FCS, 10 µg/ml LH, 5 µg/ml FSH, 1µg/ml estradiol, 100 mg/ml of streptomycin, 2.2 mg/ml sodium pyruvate, and 100 IU/ml of penicillin. The maturation dish is prepared by pouring 100µL/well of maturation media (IVM) into a disposable falcon© petri dish which is covered with the sterile paraffine mineral oil and incubated at 38.5°C, 5% CO_2, with 90-95% humidity/ 1h before use.

In all experiments, Kaempferol (K0133, Pub Chem SID 24896195) was supplemented to the IVM media at 0 (Control), 5, 10, or 15 µg/ml. The selected COCs were matured in definite groups according to the different Kaempferol concentrations at a rate of 5-15-oocyte/well/group using 100µl of the in vitro maturation medium for 22 hr at 5% CO_2, 38.5°C in complete humidified air (Gasparrini et al., 2008).

Assessment of Buffalo Cumulus Cell Expansion

After 22 hours of *in vitro* maturation, the rate of cumulus cell expansion was evaluated subjectively using the stereomicroscope.

COCs expansion was classified into the following categories according to Neglia et al. (2013):

- No expansion
- Moderately expanded COCs (only the outer cumulus cells layer was moderately loosened).
- Fully expanded COCs (all cumulus cell layers were efficiently loosened).

In vitro fertilization

Semen preparation and insemination

Firstly, 1000µL IVF-TALP was used as the fertilization media in a sterile falcon petri dish, media was covered with sterile paraffin oil and incubated at 5% CO_2, 38.5°c with maximum humidity (90-95%). In a water bath, 3 straws of frozen buffalo semen were thawed for 30 sec /37°C, then evacuated. By centrifugation (2200 rpm/5 min) the sperm cryopreservation medium washed out. The supernatant was discarded and 1ml of fresh TALP medium was added again and mixed with the sperm pellets, second centrifugation was applied to completely wash out the cryopreservation medium.

Sperm capacitation

Sperm-TALP (S-TALP) media was used to capacitate the sperms *in vitro.* By swim-up technique for an hour, the highly mobile, highly active spermatozoa were trapped in the capacitation medium. The uppermost layer containing the spermatozoa was transferred into a small test tube containing 3ml of fresh prewarmed S-TALP medium using a sterile plastic Pasteur pipette (Parrish et al., 1986). After that 1 ml of the S-TALP medium supplemented with 10 mg/ml heparin was used for *in vitro* capacitation of buffalo sperms. The sperm pellets were mixed with the previous mixture and incubated for 10 min/38.5°c in 5% CO_2 with 90-95% humidity. Matured buffalo oocytes (Kaempferol treated or not) were washed twice in a warm IVF-TALP medium and then co-incubated with the capacitated sperms (20 µL/ dose) in the fertilization drop (10 oocytes/drop) for maximum 6 hours at 38.5°c in 5% CO_2 with 90-95% humid air.

Culturing of fertilized oocytes

The fertilized oocytes were denuded by gentle pipetting and then washed in the synthetic oviductal fluid media (SOF) three times before culturing in a new SOF media. IVC media was replaced every 48h with fresh media until the seventh-day post-fertilization to prevent the accumulation of the toxic by-product resulting from amino acids degradation (ammonium). The percentage of cleaved embryos was estimated after the first 48h post-fertilization. Starting from day 5 to 7 those developed to morula and blastocyst stages were recorded (Gasparrini et al., 2008). During the examination period, the culture dish gentle shaking was applied to allow a uniform media distribution among the developing embryos.

Statistical Analysis

One-way ANOVA was statistically applied to the current data using Graph Pad Prism software version 8.4 (Graph Pad Prism, San Diego, CA) to determine the degree of significance between the kaempferol-treated groups. Duncan's Multiple Range test (LSD) using Costat Computer Program (1986) was used to compare means. the Difference was considered significant at ($P<0.01$) and this was denoted by superscripted letters.

3. RESULTS

Table 1 shows a highly significant difference at ($P<0.01$) between the different kaempferol-treated buffalo oocytes groups regarding the percentage of the fully expanded cumulus cells around the oocyte after 22h of maturation. Where, there was a significant improvement in the percentage of the buffalo oocytes exhibiting full expansion with 10 µg/ml of kaempferol (75.33±1.75%), while control and the other kaempferol-treated oocytes groups (5 and 15 µg/ml) showed the lowest records (49.69±1.92, 61.13±3.72, and 59.76±2.42 %, respectively). The situation is vice versa concerning the percentage of moderately expanded cumulus cells where control, 5 and 15 µg/ml treated oocytes groups showed the highest recorded (48.56±2.25, 37.98±3.56 and 38.98±2.36 %, respectively) if compared with the oocyte group treated with 10 µg/ml of kaempferol (24.33±1.74 %).

Table 1 The effects of kaempferol (KAE) supplementation at different concentrations to buffalo oocyte maturation medium on the rate of buffalo cumulus cells expansion (Mean ± S.E).

	Moderate expansion %	*Full expansion %*
Control	48.56±2.25^{a}	49.69±1.92^{c}
5 µg/ml	37.98±3.56^{b}	61.13±3.72^{b}
10 µg/ml	24.33±1.74^{c}	75.33±1.75^{a}
15 µg/ml	38.98±2.36^{b}	59.76±2.42^{b}
P value =	(P<0.0001)	

ste seTuehT of each group were stated as mean ± SEM. The experiment was replicated four times /group. Means with different alphabetical superscript letters in the same column were statistically significant at $P<0.01$.

Table 2 shows a significant difference at (*P<0.01*) between the different kaempferol-treated groups of buffalo oocytes concerning the *in vitro* maturation (IVM) and fertilization potentials. Where, the 10 µg/ml kaempferol treated oocytes group showed the highest IVM % (87.08±1.16 %) if compared with control (0), 5, and 15µg/ml treated groups (66.32±5.39, 77.60±4.59, and 73.32±3.12 %, respectively). Furthermore, the highest percentage of fully expanded cumulus cells and the highest IVM % associated with the 10 µg/ml treated oocyte group were reflected in highly significantly improved penetration and fertilization rates (78.40±3.75 and 42.80±1.15 %, respectively) especially if it was compared with control (58.20±1.22 and 31.90±0.75 %, respectively), 5ug/ml (68.10±.0.75 and 37.80±3.13 %, respectively), and 15 ug/ml (59.20±0.84, and 30.30±3.29%, respectively).

Table 2 The effects of kaempferol (KAE) supplementation at different concentrations to buffalo oocyte maturation medium on its maturation and fertilization potentials (Mean ± S.E).

	In Vitro maturation %	Penetration rate %	Fertilization rate %
Control	66.32±5.39^{b}	58.20±1.22^{c}	31.90±0.75^{b}
5 µg/m	77.60±4.59^{ab}	68.10±.0.75^{b}	37.80±3.13^{ab}
10 µg/ml	87.08±1.16^{a}	78.40±3.75^{a}	42.80±1.15^{a}
15 µg/ml	73.32±3.12^{b}	59.20±0.84^{c}	30.30±3.29^{b}
P value =	P<0.01	P<0.0004	P<0.01

Table 3 shows a significant difference at (*P<0.01*) between the different kaempferol-treated oocytes groups concerning embryo cleavage, morula, and blastocyst formation rate. Where there were highly significant increases in the rate of embryo cleavage, morula, and blastocyst development % especially with the 10µg/ml treated group (28.20±3.51, 21.70±2.65 and 19.90±2.45 %, respectively), if compared with the control (16.40±1.21,9.68±0.34 and 6.69±1.98 %, respectively), 5 µg/ml (21.00±2.34,13.80±1.76, and 7.62±1.28 %, respectively), and 15 µg/ml (14.60±2.41, 8.66±1.09, and 6.74±1.33 %, respectively. From Table 3 and according to the pattern of results distribution, it was clear that 10 µg/ml of kaempferol is an ideal concentration that could be used safely to culture the fertilized buffalo oocyte *in vitro*.

Furthermore, from Tables 1, 2 and 3 and according to the pattern of results distribution it was clear that 10 µg/ml of kaempferol is the ideal dose that could be used safely to mature buffalo oocytes *in vitro*.

Table 3 The effects of kaempferol (KAE) supplementation at different concentrations to buffalo oocyte maturation medium on its embryo cleavage, morula, and blastocyst formation rate.

	Cleavage rate %	Morula %	Blastocyst %
Control	16.40±1.21^{b}	9.68±0.34^{b}	6.69±1.98^{b}
5 µg/m	21.00±2.34^{ab}	13.80±1.76^{b}	7.62±1.28^{b}
10 µg/ml	28.20±3.51^{a}	21.70±2.65^{a}	19.90±2.45^{a}
15 µg/ml	14.60±2.41^{b}	8.66±1.09^{b}	6.74±1.33^{b}
P value =	P<0.01	P<0.002	P<0.0005

4. DISCUSSION

Among the flavonoids, kaempferol stands out, especially due to its powerful antioxidant and excellent cytoprotective effect especially on the reproductive cells (Jamalan et al., 2016). Therefore, we hypothesize in the current study that kaempferol may exert cytoprotective effects on buffalo oocytes that matured in vitro. Where in the current research it was denoted that kaempferol fantastically improved cumulus cell expansion % rate of buffalo oocyte maturation, penetration, fertilization % as well as the rate cleavage, morula, and blastocysts %, especially with 10µg/ml. The current study results came in harmony with the results of the previous results that denoted that kaempferol improved zygotic development through the regulation of SOX2, COX2, and Caspase-3 expression (Zhao et al., 2020). It had been found that impairing SOX2, COX2, and Caspase-3 expression leads to impairment of oocyte maturation (IVM), fertilization (IVF), and embryo cleavage (Kim et al., 2015). The importance of the SOX2 gene which is expressed in the inner cell mass (ICM) of the blastocyst is represented in its great importance for cell pluripotency which is vital for early embryo development (White et al., 2016; Lee et al., 2021). Moreover, Caspase-3 plays a vital role in the process of early embryonic apoptosis (Asadi et al., 2022) it had been discovered that KAE significantly reduces its expression and so reduces the apoptosis of the early porcine embryos. Furthermore, Zhao et al. (2020) reported that supplementation of kaempferol (KAE) in the *in vitro* medium enhanced the early embryo division and blastocyst rates by regulating many of the growth factors that modify the embryo's developmental competence.

Additionally, it had been reported that KAE significantly improved the oocyte quality through its great ability to increase the GSH level and decreases the reactive oxygen species level (ROS) (Guo et al., 2015; El-Kott et al., 2020), which in turn prevents mitochondrial dysfunction (Wang et al., 2017; Yao et al., 2018; Zhao et al., 2020). In this trend, Yao et al. (2018) and Zhao et al.;2020) reported that KAE addition to the *in vitro* medium significantly enhances the mitochondrial membrane potential, besides it reducing the mitochondrial stress so preserving high early embryo developmental potential.

Also, it was documented that KAE effectively alleviates the aging dynamics of the porcine oocytes by reducing ROS production/generation, preserving mitochondrial membrane potential (MMP), and reducing apoptosis which in turn improves the oocyte quality and promotes subsequent embryo development (Choi, 2011; Yao et al., 2018; Chen et al., 2018; Yao et al., 2019).

5. CONCLUSION

The current study is considering the first report about the use of kaempferol in the *in vitro* maturation medium of buffalo oocytes. The current results prove that kaempferol is a promising additive in buffalo oocyte IVM medium which enhance significantly its overall developmental competence, especially at 10 µg/ml which could be used safely for in vitro embryo production technology of buffalo.

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