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Kaempferol motivative effects on mitochondrial antioxidant function and intrinsic antioxidant enzymes of buffalo oocytes (Part-II)

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ABSTRACT

Many of Kaempferol's (KAE) biological functions have been widely investigated. However, its role in animal reproduction still needs great attention and exploration, especially in assisted reproductive techniques, due to its promising mechanisms in modulating oxidative stress. The current study aimed to explore its roles in maintaining the redox hemostasis of buffalo oocytes that matured *in vitro* in the presence of different KAE concentrations (0,5,10 and 15 µg/ml). The current study revealed for the first time that KAE at ten µg/ml has great capability to scavenge the generated free radicals such as H2O2 (26.4±2.27 ng/ml) and nitric oxide (25.9±2.63 nmol/L). Moreover, it significantly attenuates the rate of lipid peroxidation (1.77±0.17 nmol/L) in buffalo oocytes matured *in vitro*. Firstly, by enhancing the scavenging mechanisms. Secondly, by increasing the intrinsic antioxidants, enzymes synthesis such as super oxide dismutase (SOD) (33.70±2.96 U/ml) and glutathione (GSH) (5.51±0.37 nmol/l). Additionally, the current results revealed that KAE can protect the oocyte mitochondria from apoptosis by increasing the intra-mitochondrial antioxidants such as SOD (4.46±0.21 nmol/mg) which prevents mitochondrial dysfunction. In conclusion, KAE addition to the *in vitro* maturation media of buffalo oocytes significantly ameliorates its developmental competence by increasing its quality and developmental potentials as a whole, controlling the redox hemostasis by scavenging actions as well as by enhancing the production of intrinsic antioxidant enzymes, and finally by protecting the mitochondrial functions.

1. INTRODUCTION

Exposure of oocytes to in vitro conditions could generate a high level of ROS, which could have effects on antioxidant enzymes such as glutathione (GSH) and/or damage mitochondria, leading to poor oocyte quality (Chappel, 2013; Succu et al., 2014). So, antioxidants must be added to counteract the negative effects of oxidative stress (Khan *et al.,* 2018). Exogenous antioxidants primarily work to strengthen the intrinsic antioxidant system or scavenge free radicals to protect cells (Liao *et al.,* 2016). Recently, there has been a surge in interest in Kaempferol's (KAE) antioxidant potential (Han *et al.,* 2018). Malondialdehyde (MDA), a measure of lipid peroxidation that affects the integrity of cell membranes (Ayala *et al.,* 2014), was demonstrated to be produced at lower levels when KAE was added *in vitro* (Jamalan *et al.,* 2016; EL-Raey and Azab, 2022). Kaempferol can produce its antioxidant effects by reducing lipid peroxidation and enhancing the production or activity of antioxidant enzymes like catalase (CAT), heme-oxygenase (HO), and glutathione (GSH) (Zhou *et al.*, 2015; EL-Raey and Azab, 2022). GSH is a powerful intracellular ROS scavenger that is important in protecting cells against oxidative stress (EL-Raey and Azab, 2022).

Choi (2011) documented that (KAE) directly affects mitochondria by enhancing SOD synthesis and activating the mitochondrial thioredoxin reductase (TrxR) pathway. This TrxR system is crucial for cell viability and for maintaining the redox equilibrium of cellular thiol (Myers and Myers, 2009). Thus, oxidative stress can proceed into cell death as a result of TrxR system malfunction (Choi, 2011). According to Yao *et al.* (2019b), KAE maintains and improves mitochondrial function; hence, it can alleviate mitochondrial membrane potential that has been impaired by oxidative stress (OS) leading to a sufficient production of energy and aid in the promotion of early embryonic development. KAE considerably lowers DNA damage and attenuates the decrease in mitochondrial membrane potential in oocytes that have been exposed to H_2O_2 (Yao *et al.,* 2019b). KAE has been shown to reduce H_2O_2-induced oxidative stress by raising levels of nuclear 2-related factor antioxidant-related element (Nrf_2), SOD, and catalase (Kumar *et al.,* 2016). Superoxide anion, one of the ROS generates by the reduction

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of O_2, which dismutates into H_2O_2 by SOD. Later, H_2O_2 was detoxified into water molecules by CAT and glutathione peroxidase (Liao et al., 2016). Glutathione donates electrons to H2O2 and converts to its oxidized form, glutathione disulfide (GSSG). GSSG can be reduced back to GSH by glutathione reductase (GR). This process is one of the most essential antioxidative defense mechanisms, known as glutathione metabolism (Liao *et al.*, 2016). The current study aims to explore the KAE effect on the antioxidant capacity of *in vitro* matured buffalo oocytes.

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).

2.1. Oocyte recovery and maturation protocol

Buffalo ovaries from slaughtered houses were transported in a sterile Dulbecco's phosphate buffered saline (D-PBS) supplemented with 100 lU/mL penicillin and 100 µg/mL streptomycin sulfate at 25-30 °C. At the laboratory, they were washed in a sterile D-PBS several times, then finally washed in a sterile warm normal saline. Follicles ranging in size between 4-8mm were chosen to aspirate the qualified cumulus-oocyte complex (COCs) using an 18-gauge needle (Yousaf and Chohan, 2003). The aspirate was left warm at (35-37°C) for 15 minutes until the settlement of the aspirated COCs. The sediment was recovered in a new sterile Falcon petri dish where evenly granulated homogenous oocytes that were surrounded with several layers (≥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 three times in fresh pre-warmed TCM-199 and then cultured in TCM-199 that was enhanced with 10% FCS, ten µg/ml LH, five µg/ml FSH, one µg/ml estradiol, 2.2 mg/ml sodium pyruvate, 100 mg/ml of streptomycin, and 100 IU/ml of penicillin. In a disposable Falcon© petri dish, the maturation media was prepared by pouring 100µL of media/well, covered with sterile paraffine oil, and then 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 in vitro maturation (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 10-15 oocytes /well/group using 100µl of the in vitro maturation medium for 22 h. at 5% CO_2, 38.5°C in complete humidified air (Gasparrini et al., 2008).

2.2. Oxidant and antioxidant parameters estimation

2.2.1. Superoxide dismutase concentration (U/ml)

According to the manufacturing procedures of Labtest Diagnóstica S.A., Brazil, SOD was determined using a Fructosamine Vet Assay Kit (1019). The absorbance rate was measured calorimetrically at 530 nm.

2.2.2. Hydrogen peroxide (H_2O2) assay (ng/ml)

Cayman's hydrogen peroxide assay Kit (Ann Arbor, MI, USA-600050) provides a simple method for the sensitive quantitation of extracellular H2O2 produced by cultured cells. H2O2 in the tested samples was measured calorimetrically at 550 nm optical density according to manufacturing procedures.

2.2.3. Glutathione concentration (GSH)

GSH concentration in the tested samples was measured according to Ellman (1959) and Hu (1994). The absorbance rate was estimated at 412 nm (Costa et al., 2006). The concentration of sulfhydryl groups was calculated using glutathione standards, and the results were reported as nmol/l.

2.2.4. Malondialdehyde concentration (nmol/L)

Malondialdehyde concentration was determined by mixing 1 ml of the tested sample with 2 ml of TCA-TBA-HCL (15% trichloroacetic acid, 0.375% Thiobarbituric acid, 0.25N hydrochloric acid), the mixture was heated in a water bath (100 °C/15 min). The solution was left to cool at room temperature to give the precipitate, which was centrifugated at 1,000 ×g /10 min. The supernatant absorbance was spectrophotometrically measured at 535 nm using the Spectronic 601 reader (Milton Roy). The concentration of Thiobarbituric acid-reactive substances (TBARS) was calculated based on the coefficient of molar absorptivity of the product (E535 = 1.56 × 10 −5 M −1 cm −1), and the results were reported as nmol/L. The standard curve was obtained using the stock solution of 10 mM malondialdehyde prepared from tetramethoxypropane (Sigma-Aldrich). The concentrations of MDA in the tested samples should show good linearity with the standard.

2.2.5. Nitric oxide (NO) measurement (nmol/L)

NO was measured by measuring the total nitrite and nitrate concentrations in the tested samples using the Griess method that was described by Archer (1993). The absorbance was measured at 545 nm.

2.2.6. Assessment of mitochondrial SOD production by the

A microtiter plate assay for superoxide using the MTT reduction method (MTT test) according to Madesh and Balasubramanian (1997).

MTT assay has been used as a common tool to measure cell proliferation/viability, drug cytotoxicity, and mitochondrial/metabolic activity of cells (Lee et al., 2014). MTT-derived formazan is usually measured at 570 nm.

2.3. 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 KAE-treated groups of buffalo oocytes concerning H_2O_2 and NO generation levels after *in vitro* maturation process. Where it was clear that 10µg/ml of KAE-treated oocyte group presented the lowest H_2O_2 and NO levels (26.40±2.27 ng/ml and 25.9±2.63 nmol/L, respectively), especially if compared with control which shows the highest records (39.90±1.88 ng/ml and 37.0±2.45 nmol/L, respectively), followed by 15µg/ml (32.90±2.08 ng/ml and 35.0±1.99 nmol/L, respectively), and 5µg/ml (34.60±2.58 ng/ml and 33.0±0.16 nmol/L, respectively). Moreover, Table 1 shows a highly significant difference at (*P<0.01*) between the different KAE-treated oocytes concerning the MDA level. Where KAE at 5µg/ml and 10µg/ml concentrations efficiently reduced the rate of MDA in buffalo oocytes (2.75±0.08 nmol/L and 1.77±0.17 nmol/L, respectively), especially if compared with control (4.01±0.29 nmol/L), and 15µg/ml KAE-treated group (3.15±0.14 nmol/L). Furthermore, it was clear from the rate of differences in Table 1 between H_2O_2, NO, and MDA that KAE was more effective in protecting buffalo oocytes from the hazardous effects of the reactive oxygen species (ROS) by combating the lipid peroxidation cascades in the oocytes during the maturation process.

Table 1 The effect of different Kaempferol concentrations on hydrogen peroxide ($H2O_2$), Nitric Oxide, and Malondialdehyde (MDA) generation potentials in the in vitro matured buffalo oocytes.

	MDA *nmol/L*	Nitric Oxide *nmol/L*	H_2O_2 *ng/ml*
Control	4.01±0.29^{a}	37.0±2.45^{a}	39.9±1.88^{a}
5 µg/ml	2.75±0.08^{b}	33.0±0.16^{ab}	34.6±2.58^{ab}
10 µg/ml	1.77±0.17^{c}	25.9±2.63^{b}	26.4±2.27^{b}
15 µg/ml	3.15±0.14^{ab}	35.0±1.99^{ab}	32.9±2.08^{ab}
P value	.0002	.0136	.0168

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

Table 2 shows that there was a highly significant difference at (*P<0.01*) between the different KAE-treated oocytes concerning SOD, GSH synthesis level, and intra-mitochondrial synthesis level of SOD. Where, when 10µg/ml of KAE was added to the maturation media of buffalo oocytes efficiently enhanced the production of the intrinsic antioxidant enzymes SOD and GSH (33.70±2.96 U/ml and 5.51±0.37 nmol/l, respectively), followed by 5 µg/ml (22.00±1.79 U/ml and 3.20±0.26 nmol/l, respectively). While control (16.50±1.92 U/ml and 2.93±0.06 nmol/l, respectively), and 15 µg/ml treated group (15.10±1.10 U/ml and 2.59±0.29 nmol/l, respectively) presented the lowest values of their intrinsic enzymes. Furthermore, 10µg/ml of KAE efficiently improved the rate of mitochondrial SOD production (4.46±0.21 nmol/mg), followed by 5 µg/ml treated group (3.29±0.24 nmol/mg). While, the control (2.32±0.17 nmol/mg), and 15 µg/ml KAE-treated oocyte group (2.41±0.11 nmol/mg) showed the lowest level of mitochondrial SOD production. Additionally, from Table 2 it was clear that KAE supplementation to the *in vitro* maturation media of buffalo oocytes acts significantly to enhance the production and function of the intrinsic antioxidant enzymes (SOD and GSH) not only on the oocyte ooplasm level but also on the mitochondrial level.

Table 2 The Effect of different Kaempferol concentrations on cytoplasmic and mitochondrial intrinsic antioxidants enzymes (SOD and GSH) production potentials in the in vitro matured buffalo oocytes.

	SOD *U/ml*	GSH nmol/l	MTT nmol/mg
Control	16.50±1.92^{b}	2.93±0.06^{b}	2.32±0.17^{b}
5 µg/ml	22.00±1.79^{b}	3.20±0.26^{b}	3.29±0.24^{b}
10 µg/ml	33.70±2.96^{a}	5.51±0.37^{a}	4.46±0.21^{a}
15 µg/ml	15.10±1.10^{b}	2.59±0.29^{b}	2.41±0.11^{b}
P value	.0008	.0003	.0007

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

4. DISCUSSION

Kaempferol is one of the most common natural flavonoids, it was extracted from various fruits and plants (Holland et al., 2020). Recently such flavonoid was found to exert a lot of biological, biochemical, and pharmacological roles and activities in modulating cellular health and functions (Periferakis et al., 2022, Yang et al., 2022, Almatroudi et al., 2023, Dong et al., 2023). Lately, KAE gained great interest to be used during in vitro embryo production technology. To our knowledge, the current study is the first report that explored KAE effects on the redox state of buffalo oocytes that matured *in vitro*. The current study revealed for the first time that KAE supplementation to IVM media at 10 µg/ml significantly scavenged the free radicals especially H_2O_2 (26.4±2.27 ng/ml), and Nitric Oxide (25.9±2.63nmol/l). The current results came in harmony with Middleton et al. (2000), Kampkötter et al. (2007), and Yao et al. (2019a) who reported that KAE is a pioneer ROS scavenger especially superoxide due to its unique chemical structure which possesses the ability to inhibit xanthine oxidase activity and so scavenge superoxide. H_2O_2 negatively affects cellular viability by destroying the cytoplasmic membrane and distributing cellular homeostasis (Kagan and Li, 2003).

The current study for the first time clarified the potent effects of KAE on the rate of lipid peroxidation where it has been discovered that 10 µg/ml potentially reduced the rate of MDA production (1.77±0.17 nmol/L) thus protecting the cytoplasmic membrane of buffalo oocytes that were rich in polyunsaturated fatty acids (PUFAs). The current results came in harmony with Nirmala and Ramanathan (2011), Kulanthaivel et al. (2012), and El-Raey and Azab (2022), who stated that the rate of lipid peroxides synthesis significantly decreased when KAE was used. On the other hand, Lee et al. (2010), and El-Raey and Azab (2022), reported that KAE alleviates ROS generation and accumulation partially.

The current study revealed that KAE can control the redox homeostasis of buffalo oocytes in two ways: the first is by scavenging the generated ROS (H_2O_2 and Nitric oxide), protecting the cellular membrane from disintegration, and consequently protecting the intracellular constituents from loss and maintaining the normal biological functions of the matured oocytes *in vitro*. The second mechanism is illuminating as it proves clearly how KAE is a promising natural flavonoid that could be used efficiently to enhance the in vitro embryo production technology, where the current study discovered that KAE at 10 µg/ml can significantly enhance the intrinsic antioxidant enzymes of the buffalo oocytes that matured in vitro, especially SOD (33.70±2.96 U/ml) and GSH (5.51±0.37nmol/l). The current result came in harmony with Kim et al. (2008a), Kim et al. (2008b), Kumar et al. (2016), and El-Raey and Azab (2022) who

stated that KAE could relieve H_2O_2-induced oxidative stress by increasing the levels of SOD, and catalase that significantly reduced ROS. Moreover, Hong et al. (2009) reported that KAE upregulates Nrf2-mediated HO-1 expression. Furthermore, Zhao et al. (2020) and El-Raey and Azab (2022) stated that KAE significantly increased the rate of GSH synthesis, which increased the quality of the oocytes and sperms by increasing their ability to resist oxidative stress.

Concerning the role of KAE on the mitochondrial function of buffalo oocytes that matured *in vitro*, the current study and for the first time revealed that KAE supplementation to IVM of buffalo oocytes enhanced the mitochondrial SOD production, especially at 10µg/ml (4.46±0.21 nmol/mg) which in turn preventing the mitochondrial dysfunction. the current study results came in agreement with Choi (2011), Chen et al. (2018), Yao et al. (2019a), and Yao et al. (2019b) who documented that KAE supplementation to the IVM and IVC media of the aged oocytes and embryos significantly enhanced their quality by improving the mitochondrial membrane potential ($\Delta\psi m$) so retarding the process of mitochondrial dysfunction and reducing apoptosis process. Maintaining healthy MMP is an essential request to provide a sufficient energy supply that promotes early embryo development and DNA damage (Ott et al., 2007). Moreover, Yang et al. (2019) stated that KAE can regulate the mitochondrial Sirtuin (Sirt-3); which is essentially important in regulating the different cellular hemostasis, especially redox homeostasis, and aging process (Cimen et al., 2010). Additionally, Saw et al. (2014) and Zhang et al. (2019) illustrated another mechanism by which KAE can enhance mitochondrial activity by finding the synergistic relationship between it and the Nrf2-ARE pathway, which is necessary to regulate the mitochondrial function, therefore KAE could reduce the mitochondrial dysfunction and apoptosis. In addition, Guo et al. (2015) reported that KAE attenuated the loss of mitochondrial membrane potential ($\Delta\psi m$) and modulated the release of cytochrome-c (cyt-c).

5. CONCLUSIONS

Finally, supplementation of KAE to IVM media of buffalo oocytes especially at 10 µg/ml significantly improved its developmental competence firstly by improving the oocyte and embryo developmental quality (Bahgat et al., 2023); as well as by modulating the redox homeostasis of buffalo oocyte either by the scavenging the generated free radical species (ROS) and by enhancing the intrinsic antioxidant enzymes synthesis (SOD and GSH), besides it protecting the healthy potentials of the oocyte mitochondria, which in turn improving the embryo yield from in vitro embryo production techniques.

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