2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(2): 146-159, June 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj19

Effects of Curcumin Supplementation on Viability and Antioxidant Capacity of Buffalo Granulosa Cells under *In Vitro* Culture Conditions

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ORGINAL ARTICLE
pii: S232245682000019-10
Received: 02 Apr 2020
Accepted: 15 May 2020

ABSTRACT

The current study was conducted to investigate the possible protective effect of curcumin supplementation on buffalo granulosa cells (GCs) under *in vitro* culture condition. Buffalo ovaries were collected from local abattoir in physiological saline solution and transported directly to laboratory. Follicular fluid containing GCs and cumulus-oocyte-complexes were aspirated from antral follicles with diameter 2-8 mm. The collected GCs were seeded (Approximately 375,000 viable cells) in an 8-well culture plate containing tissue culture medium-199 (TCM-199) and kept at 37 °C in a humidified atmosphere of 5% CO_2. The curcumin was supplemented to TCM media at levels of 1, 2.5, 5 and 10 µM for 24 and 48 h at 37 °C or kept without treatment as control group. The viability of cells was determined using the trypan blue test. Intracellular reactive oxygen species (ROS) level was assessed by measuring the fluorescent intensity of 6-carboxy-2′,7′-dichlorodihydro fluorescein diacetate (H_2DCFDA). In addition, mitochondrial activity of GCs was determined. The results of the present study indicated that the viability of GCs under culture conditions was significantly decreased in groups treated with 1, 2.5, 5 and 10 µM curcumin (86.0%, 86.26%, 83.0% and 74.0%, respectively) compared to control group (93.60 %). The two groups of granulosa cells cultured with 2.5 and 5 µM curcumin recorded greater level of mitochondrial activity than the groups cultured with 1 µM and 10 µM curcumin. Moreover, there was a significant increase in ROS level in group cultured with 10 µM curcumin, compared to control and other experimental groups. The enzyme activity of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was increased after treating *in vitro* cultured granulosa cells with 5 µM of curcumin. However, the enzymatic activity of CAT, SOD, GSH and DPPH was declined significantly 48 h post-curcumin treatment. In conclusion, supplementation of curcumin at low concentration (2.5 µM) for 24 h to *in vitro* cultured GCs improved intracellular metabolic activity and antioxidant protective system, whereas it could not sustain this action for 48 h. Moreover, supplementation of curcumin at high concentration and for long duration may negatively affect viability of GCs under *in vitro* culture condition via induction of oxidative stress.

Key words: Antioxidant, Buffalo, Granulosa cells, Oxidative stress, Viability.

INTRODUCTION

Oxidative stress, mediated by oxygen-derived free radicals (also known as reactive oxygen species, ROS) is a frequent state affecting nearly all living organisms because of suboptimal environmental conditions. In homeostatic situation, there is stability between the production of ROS and scavenging power of cells through the cellular antioxidant system (Panieri et al., 2016). Nevertheless, when the production of ROS overcomes the cellular antioxidant capability, it perhaps contributes to a problem referred to oxidative stress (Agarwal et al., 2005). The ROS level could be elevated endogenously during many physiological and reproductive procedures including ovulation (Agarwal et al., 2005; Gupta et al., 2010). Moreover, the use of oxygen as a respiratory substrate was reported to produce oxidative stress throughout the aerobic metabolic process and energy production (Frisard and Ravussin, 2006). Furthermore, other endogenous sources (mitochondria, inflammatory cell activations, plasma membrane nicotinamide adenine dinucleotide phosphate, oxidase, lysosomes, and peroxisomes) may affect the production of ROS in mammalian cells (Klaunig et al., 2009).

Incidence of oxidative stress mediated by ROS was found to be having a negative effect on female reproductive system and finally causes infertility (Agarwal et al., 2012). Ovarian granulosa cells (GCs), the major cellular constituent in a follicle, have two vital functions in female reproduction: steroid production and defend the oocyte throughout ovulation (Yada et al., 1999; Sohel et al., 2013; Cinar and Sohel, 2015). At the end of follicular growth, GCs in the dominant follicle are differentiated into luteal cells by an ovulatory luteinizing hormone (LH) surge (Duffy and Stouffer,

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2003). This method is crucial for successful ovulation and formation of corpus luteum to keep the pregnancy. On the other hand, all through ovulation after the pre-ovulatory rise of LH, inflammatory cells particularly neutrophils and macrophages are vastly hired to produce ROS to facilitate follicular rupture and the release of the oocyte (Shkolnik et al., 2011), indicating exposure of GCs to some sort of oxidative stress during ovulation. In addition to an endogenous source, environmental sources of ROS could make the situation more complex.

One of the plants efficiently used in folk medicine is Curcuma *longa Linn* (Hatcher et al., 2008). In this herb, curcumin component has the highest proportion (Aggarwal et al., 2007). Curcumin is a yellow polyphenol compound found in turmeric (Esatbeyoglu et al., 2012), and its chemical structure is *1, 7-bis (4-hydroxy-3- methoxyphenyl)-1, 6-eptadiene-3, 5-dione* (Nadkarni, 2007; Kádasi et al., 2012). It has proven to be a highly effective anti-carcinogenic, antiviral, antioxidant (Steward et al., 2008; Correa et al., 2013; Tapia et al., 2013), and anti-inflammatory substance in human and animal models (Epstein et al., 2010; Sung et al., 2012).

Curcumin acts as an antioxidant since it scavenges reactive oxygen and nitrogen species (Barzegar and Moosavi-Movahedi, 2011; Trujillo et al., 2013 ; Mohebbati et al. 2017) and induces cytoprotective enzymes such as glutathione-Stransferase (GST), γ-glutamyl cysteine ligase (γ-GCL) and heme oxygenase-1 (HO-1) (Dinkova-Kostova et al., 2008; Reyes-Fermín et al., 2012). It is able to scavenge hydrogen peroxide, peroxyl radicals, superoxide anion, hydroxyl radicals, singlet oxygen, nitric oxide, and peroxynitrite anion (Trujillo et al., 2013). It has been revealed that curcumin causes endogenous antioxidant defense systems by modulating transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Tapia et al., 2012; Liu et al. 2016; Zhang et al., 2019a; Zhang et al., 2019b; Zhu et al., 2020), activator protein-1 (AP-1), and nuclear factor kappa B (NFκB) (Pinkus et al., 1996). Therefore, the aim of this study was to investigate the effects of curcumin supplementation to *in vitro* culture media of buffalo GCs on their viability and enzymatic defense system.

MATERIALS AND METHODS

Experimental groups

A primary culture of GCs was used as a basic technique to study the effects of curcumin supplementation on buffalo GCs cultured *in vitro* in TCM-199 medium. Primary cultures of GCs were grown in six groups. The groups were divided as the following: group 1: untreated (control), group 2: untreated control and add dimethyl sulfoxide (DMSO) (it is the dissolving solution for curcumin), group 3: treated only with 1 µM curcumin, group 4: treated only with 2.5 µM curcumin, group 5: treated only with 5 µM curcumin and group 6: treated only with 10 µM curcumin. The recovered cells were grown until they reached up to 40–50% confluency before being allocated into the different treatment groups. A minimum of 10 ovaries were used in each biological replicate. Three biological replicates of GCs were used for each experimental assay done in this study.

Collection of ovaries and granulosa cells

Granulosa cells were collected and cultured according to the procedure described by Sohel et al. (2017). A total of 120 buffalo ovaries were obtained from a local slaughterhouse, and transported in 0.9% saline solution at 37 °C within 2 h of collection. A minimum of 10 ovaries were used in each replicate in order to obtain a sufficient number of GCs for different assays. Ovaries were washed twice with 0.9% saline solution and then washed once with 70% ethanol. The follicular contents (follicular fluid containing GCs and cumulus-oocyte complexes) were aspirated from antral follicles of 2–8 mm in diameter by an 18-gauge needle attached to a 5-mL syringe and placed in a 50-mL sterile falcon tube containing 10-mL TCM-199 medium (Sigma- Aldrich, M5017, Steinheim, Germany). After collection, tubes were left for 15 min at 37 °C to allow the oocyte-cumulus complexes and cellular debris to settle at the bottom of the tube. The upper liquid containing GCs was then collected in a 15-mL falcon tube, and centrifuged at 1800 rpm for 5 min to obtain the GCs. The collected GCs were washed with 5 ml of phosphate buffer saline (PBS) that is free from calcium magnesium by repeat pipetting followed by centrifugation at 1500 rpm for 10 min. Finally, 3 ml of trypsin was added and the tube incubated at 37 °C for 3 min, then 5 ml of TCM-199 was added to inactivate trypsin by repeat pipetting of GCs followed by centrifugation at 1500 rpm for 10 min.

In vitro culture and treatment of granulosa cells

Approximately 375,000 viable cells were seeded in an 8-well culture plate (Corning Incorporated, Kennebunk, ME, USA), *in vitro* culture in medium containing TCM-199 medium (Sigma-Aldrich, D6046, Steinheim, Germany) supplemented with 10% fetal bovine serum (vol/vol), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma Aldrich, P4333, Steinheim, Germany) and kept at 37 °C in a humidified atmosphere of 5% CO_2 until reached 40–50% confluency. The curcumin was added to the TCM-199 medium at the following levels (Control, DMSO, 1 µM, 2.5 µM, 5 µM and 10 µM) for 48 h with change the medium once after 24 h.

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Cell morphology and viability

After treatment, GCs from different treatment groups were observed using inverted microscopy for confluency and changes in morphology. The viability of cells was determined using the trypan blue exclusion test as described by Strober (2015) with some modifications. Briefly, after the treatment, both adherent and floating cells from each treatment group were collected and resuspended in one mL of *in vitro* culture medium. Following that, 100 µL of cell suspension and 100 µL of 0.4% trypan blue were mixed into a micro-centrifuge tube and incubated for 1–2 min at room temperature. Ten microliters of cell mixture/ trypan blue were applied to the hemocytometer and placed under a microscope (Inverted Microscope, Leica DMI 3000B, Wentzler, Germany) at magnification of 20X for counting live and dead cells. GC viability was calculated as a percentage of viable cells from total cell count.

Cytotoxicity assay

In the present study, the neutral red uptake assay supplies a quantitative estimation of how many feasible cells in culture. It is one of the most applied cytotoxicity tests with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The granulosa cells were seeded in 96-well tissue culture plates and were treated for the appropriate period. The plates were incubated for 2 h with a medium containing neutral red. The cells were subsequently rinsed with media, the dye was removed properly and the absorbance was read using a spectrophotometer. Once the cells have been treated, the assay can be completed in <3 h (Repetto et al., 2008).

Intracellular reactive oxygen species detection

Intracellular ROS accumulation in different treatments and control group was assessed by 6-carboxy-2′, 7′-dichlorodihydro fluorescein diacetate (H_2DCFDA, Sigma-Aldrich, USA) according to the protocol described by Sohel et al. (2017). The GCs from each group were incubated with 400 µL of 15 µM H_2DCFDA for 20 min in the dark at 37 °C. Cells were then washed twice with PBS and images were immediately captured with a Nikon Eclipse Ti-S microscope (Nikon Instruments Inc., Tokyo, Japan) using a green-fluorescence filter at excitation/emission: ~492–495/517–527 nm and images were acquired by NIS Elements software. For quantitative analysis, the mean fluorescence intensity of five non-overlapping fields in each well was measured using Image J software (Rueden et al., 2017). Data are presented as mean ± standard deviation.

Mitochondrial activity

Mitochondrial activity of buffalo GCs was determined using MitoTracker Red CMXRos (M7512, Invitrogen, Karlsruhe, Germany) according to the previous published protocol (Prastowo et al., 2017) with small modifications. The GCs from each group were incubated with 15 µL of 200-nM MitoTracker red dye for 45 min, followed by two washings with PBS and were then fixed overnight at 4 °C with 4% formaldehyde. The mitochondrial activity of GC samples was visualized under a laser scanning confocal microscope (LSM 710; Carl Zeiss, Germany) using specific excitation lasers at 579– 599 nm. A constant level of laser gain (master gain = 700), pinhole (1 µm) and pixel size (1024 × 1024) were applied during image acquisition aim to allow image fluorescence signal comparison. Resulted images were then processed using ZEN 2011 software (Carl Zeiss, Germany). For quantitative analysis, the mean fluorescence intensity of five non-overlapping fields in each well was measured using Image J software. Data are presented as mean ± SD.

Enzyme activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay

The ability of different extracts to act as hydrogen donors was measured by DPPH radical scavenger activity. The assay was carried out according to method of Blois (1958). The DPPH, a stable free radical, contains an odd electron, which is responsible for the absorbance at 515-517 nm and for a visible deep purple color. When DPPH accepts an electron from an antioxidant compound, it is reduced to 1,1-diphenyl-2-picrylhydrazine (decolorized non-radical, DPPH2).

Determination of superoxide dismutase (SOD) activity

SOD activity was assayed in the liver tissue by the method of Marklund and Marklund,(1974) at 420 nm for 1 min on a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). Activity was expressed as the amount of enzyme that inhibits the autoxidation of pyrogallol by 50%, which is equal to 1 U/mg protein.

Determination of catalase (CAT) activity

The activity of CAT was measured by spectrophotometric method based on the decomposition of H_2O_2 as described by Aebi (1984).

Determination the profile of GSSG and GSH by HPLC

The thiols compounds of oxidized and reduced glutathione were detected by HPLC using the method of Jayatilleke and Shaw (1993). Glutathione (oxidized and reduced) reference standard purchased from Sigma-Aldrich Chemical Co (G4376, USA). The powder of glutathione was dissolved in 75% methanol in a stock of 1 mg/ml and diluted before application to HPLC. The HPLC system of Agilent (Santa Clara, USA) consisted of quaternary pump, a column oven,

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Rheodine injector and 20µl loop, UV variable wavelength detector. The report and chromatogram taken from Chemstation program purchased from Agilent. Synerji RP Max column 3.9 at wavelength 210 nm with flow rate 2ml/min was used. Pot. Phosphate buffer - acetonitrile at pH 2.7 was used as an isocratic mobile phase.

Determination of adenosine tri-phosphate content in media by HPLC

The detection of adenosine tri-phosphate (ATP) by HPLC was done according to the method of Teerlink et al. (1993)

Statistical analysis

A minimum of three biological replicates was used in each experiment per each essay (number of each replicate = 3n and total number of replicates = 72n). Statistical differences of means were compared between different experimental groups and were analyzed by applying one-way ANOVA, followed by Duncan's multiple range test that was used to detect differences among means. Differences in values of means were considered significant at $P \leq 0.05$. The General Linear Model (GLM) procedure on SAS Software (SAS, 2004) was used for statistical analysis. Data are supposed to be normally distributed and were expressed as mean ± SD of three biological replicates.

The parameters were analyzed according to the following model:

$Y_{ijk} = \mu + A_i + e_{ij.}$

Y_{ijk} = the measured trait.

μ = Overall means.

A_i = The Effect of different levels of curcumin.

e_{ij} = Experimental error.

RESULTS

Viability

The viability of *in vitro* cultured granulosa cells (figure 3) was significantly decreased ($p \leq 0.05$) on groups treated with DMSO (88.0±1.6%), 1 µM curcumin (86.0±1.6%)**,** 2.5 µM curcumin (86.26±1.6%)**,** 5 µM curcumin (83.0±1.6%) and 10 µM curcumin (74.0±1.6%) compared to control group (93.60±1.6 %).

Mitochondrial activity

There were no significant differences in mitochondrial activity of granulosa cells cultured with DMSO, 2.5 µM curcumin and control group (Figures 2 and 4). In addition, there was no significant difference in the activity of mitochondria between the group cultured with 2.5 µM curcumin and 5 µM curcumin. Moreover, the two groups cultured with 1 µM curcumin and 10 µM curcumin did not show differences on mitochondrial activity. However, the two groups of granulosa cells cultured with 2.5 µM and 5 µM curcumin recorded higher level of mitochondrial activity than the groups cultured with 1 µM and 10 µM curcumin.

Reactive oxygen species (ROS) level

There were no significant differences on ROS level of granulosa cells cultured with DMSO, 1 µM and 2.5µM curcumin group (Figures 1 and 5). However, there was a significant ($p \leq 0.05$) increase in ROS level in group cultured with 10µM curcumin compared to control and other treatments. In addition, the group of granulose cells cultured with 5µM curcumin recorded higher level of ROS than the groups cultured with 1µM, 2.5µM and 10µM curcumin.

Activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in in vitro cultured granulosa cells

There were no significant differences on DPPH radical scavenger activity of granulose cells cultured with DMSO, 2.5µM after 48 h, 5µM after 48 h, 10µM after 48 hand 1µM after 24 h cultured of curcumin (Figure 6). In addition, there was no significant difference between granulose cells cultured with 1µM after 48 h, 10 µM after 24 h and 2.5 µM after 24 h cultured of curcumin. In addition, there was a significant difference between the granulose cells cultured as a control group and 5µM curcumin group for 24 h. However, the two groups of granulose cells cultured with 5µM curcumin for 24 hours and 1 µM curcumin for 48 h recorded high level of DPPH radical scavenger activity in compared with other groups.

Activity of superoxide dismutase (SOD) in in vitro cultured granulosa

There were significant differences on SOD activity between the granulose cells cultured in control group and experimental groups supplemented with curcumin (Figure 7). The maximum activity of SOD was recorded in granulose cells cultured with 5 µM curcumin for 24 h compared to control group that had the lowest activity of this enzyme. In addition, there were no significant differences on SOD activity of granulose cells cultured with DMSO, 1µM curcumin for 24 h, 2.5 µM, 5 µM, and 10 µM curcumin for 48 h. In addition, too no significant difference of granulose cells cultured 2.5 µM curcumin for 24 h, 5 µM curcumin for 24 h and 1 µM curcumin for 48 h, As noted the group of granulose cells cultured with 5 µM curcumin for 24 h recorded higher level of SOD activity than other experimental groups.

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Activity of catalase in in vitro cultured granulosa cells

There were significant differences on CAT activity between the granulose cells cultured as a control group, DMSO and 5 µM curcumin groups for 24 h (Figure 8). In addition, no significant differences on CAT activity of granulose cells cultured DMSO, 1 µM curcumin for 24 h, 2.5 µM curcumin for 24 h, 10 µM curcumin for 24 h, 1 µM, 2.5 µM, 5 µM, and 10 µM curcumin for 48 h. As noted, the group of granulose cells cultured with 5 µM curcumin for 24 h recorded higher level of SOD activity than the others groups.

Activity of glutathione in in vitro cultured granulosa cells

There were significant differences ($P < 0.05$) on GSH activity between the granulose cells cultured control group, DMSO and 5 µM curcumin for 24 h (Figure 9). As noted, the group of granulose cells cultured with 5 µM curcumin for 24 h recorded higher level of SOD activity than the others groups.

Activity of oxidized glutathione in in vitro cultured granulosa cells

There was a significant ($P < 0.05$) decline in GSSG level on groups treated with curcumin at concentration of 5 µM and 10 µM after 24 h of culture in addition the same trend was observed in granulosa cells treated with curcumin at concentration of 1 µM after 48 h compared to all experimental groups (Figure 10). However, the highest level of this enzyme was recorded in control group.

Intracellular adenosine triphosphate content in in vitro cultured granulosa cells

There were significant differences ($P < 0.05$) on CAT activity between the granulose cells cultured in control group, DMSO and 5 µM curcumin for 24 h (Figure 11). The content of ATP was increased gradually and significantly ($P < 0.05$) in ascending pattern in GCs and reached the maximum profile after 24 H in the group cultured with 5 µM. After that, the profile of ATP was decreased ($P < 0.05$) in GCs cultured with curcumin at concentration of 5 and 10 µM for 48 h. The lowest profile of ATP was recorded in control group.

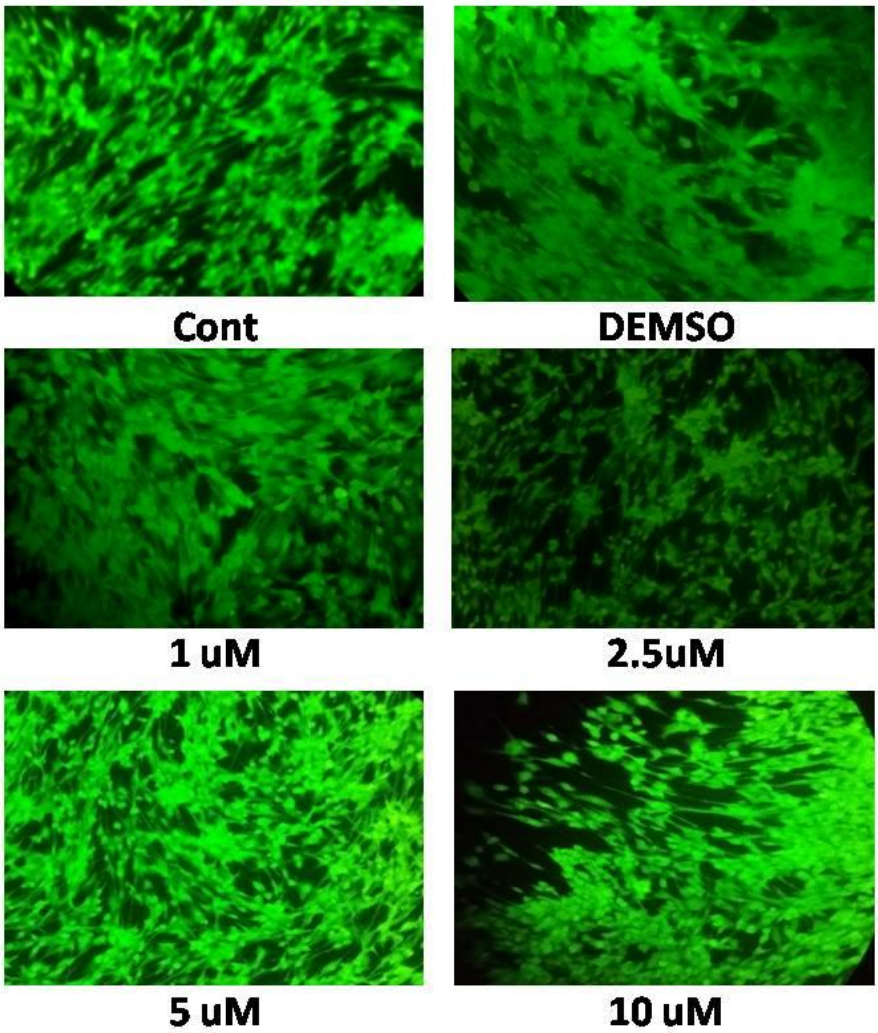

Figure 1. Image of *in vitro* cultured granulosa cells stained with H_2DCFDA measuring the level of reactive oxygen species (ROS) after supplementation with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h. The image was taken by inverted microscope (Leica DMI 3000B, Wentzler, Germany) at magnification of 20X.

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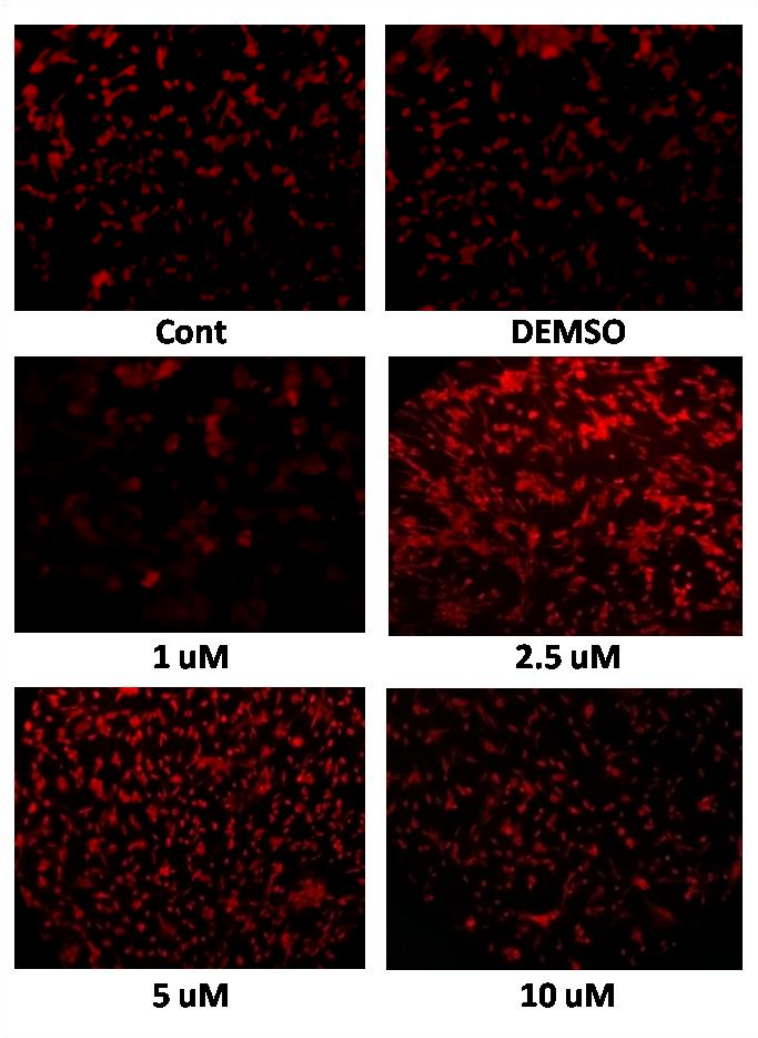

Figure 2. Image of *in vitro* cultured granulosa cells stained with mitotraker red measuring mitochondrial activity after supplementation with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h. The image was taken by inverted microscope (Leica DMI 3000B, Wentzler, Germany) a magnification of 20X.

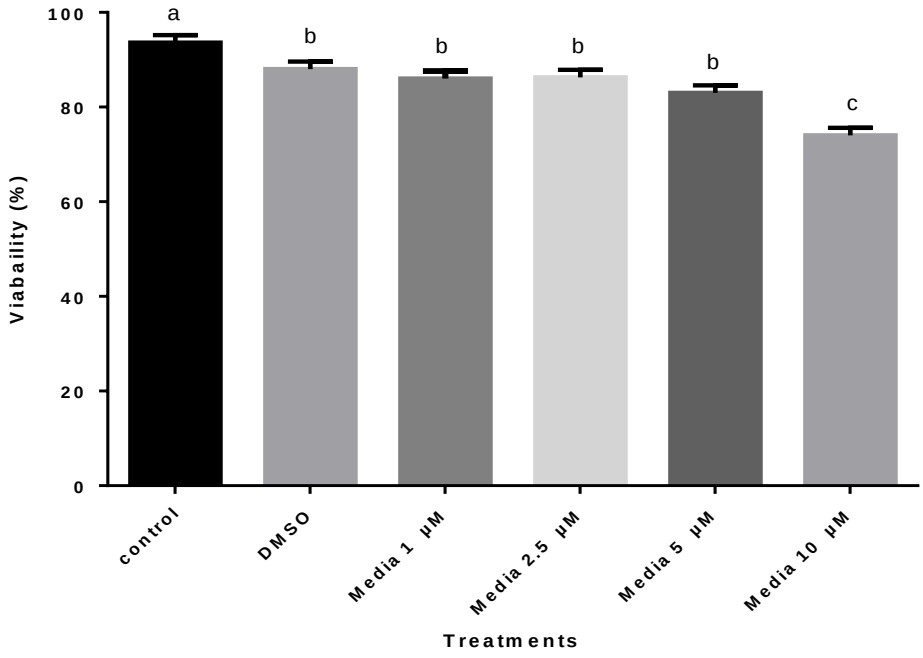

Figure 3. Viability of *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

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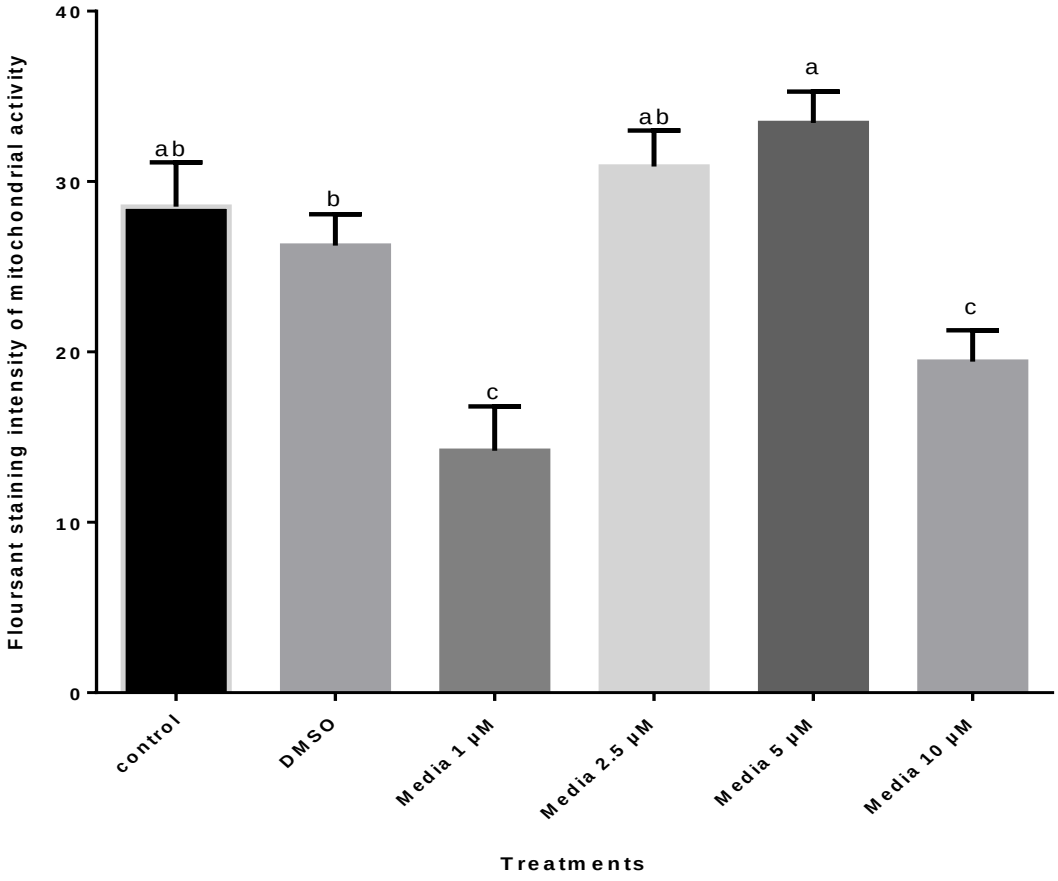

Figure 4. Mitochondrial activity of i*n vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

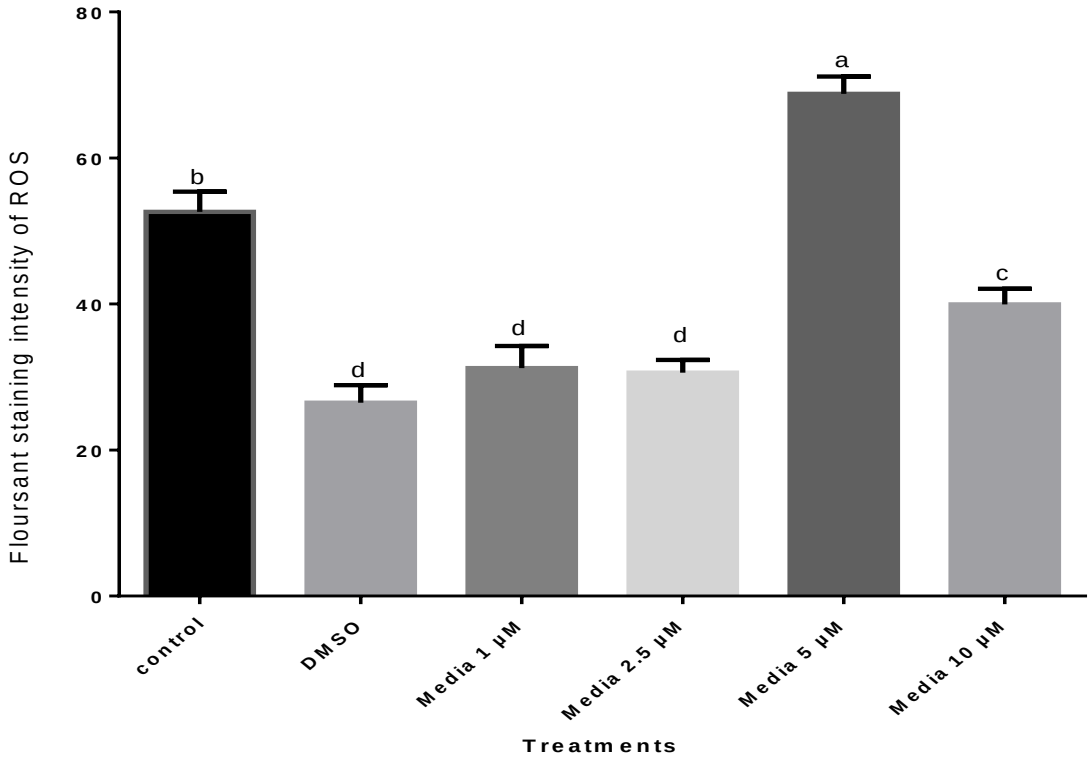

Figure 5. Intracellualr reactive oxygen species level of *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

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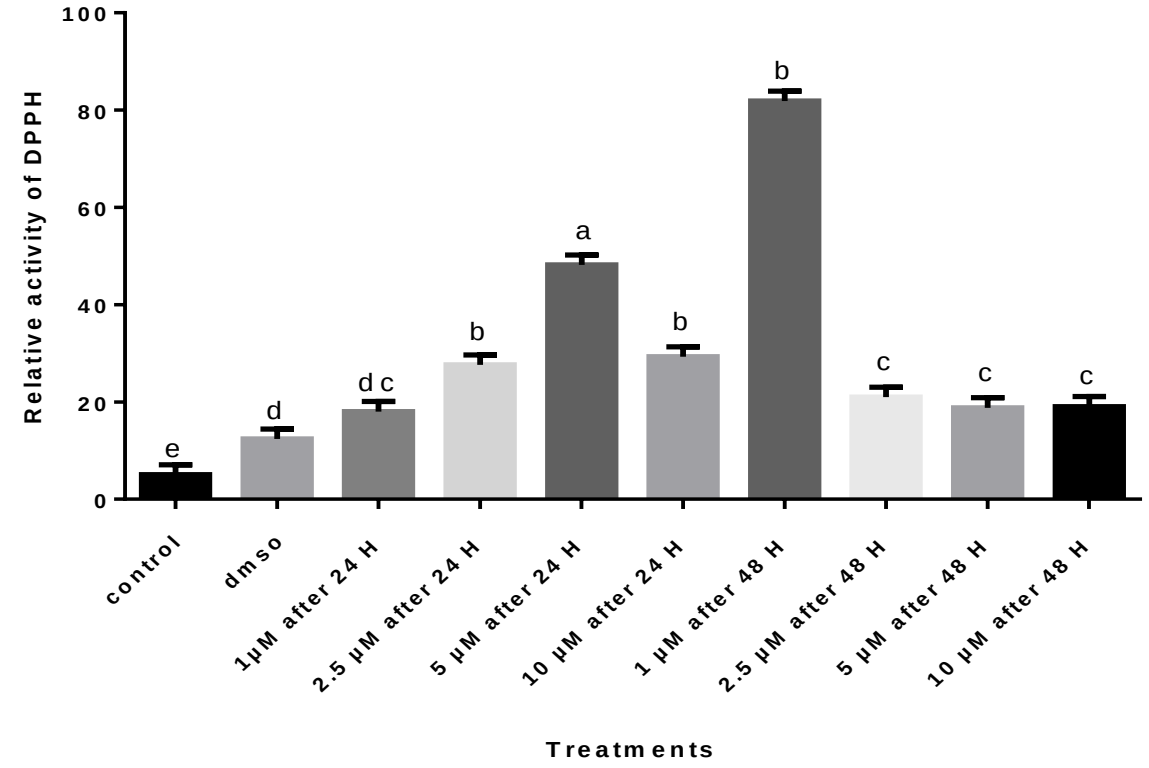

Figure 6. Enzymatic activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

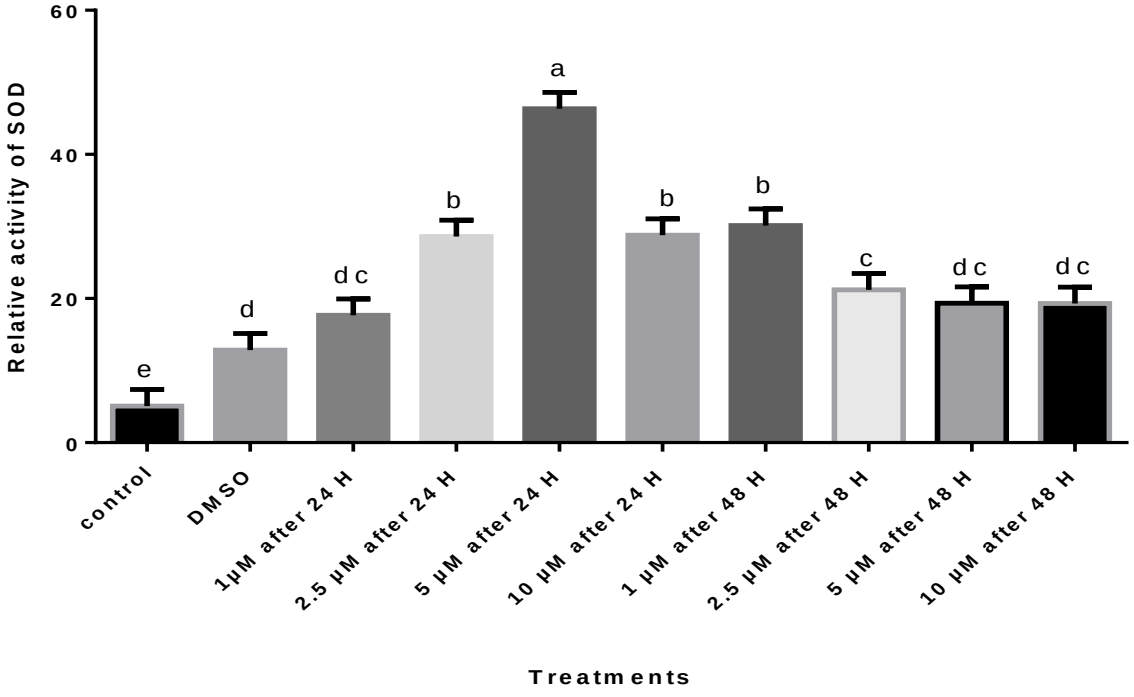

Figure 7. Enzymatic activity of superoxide dismutase (SOD) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

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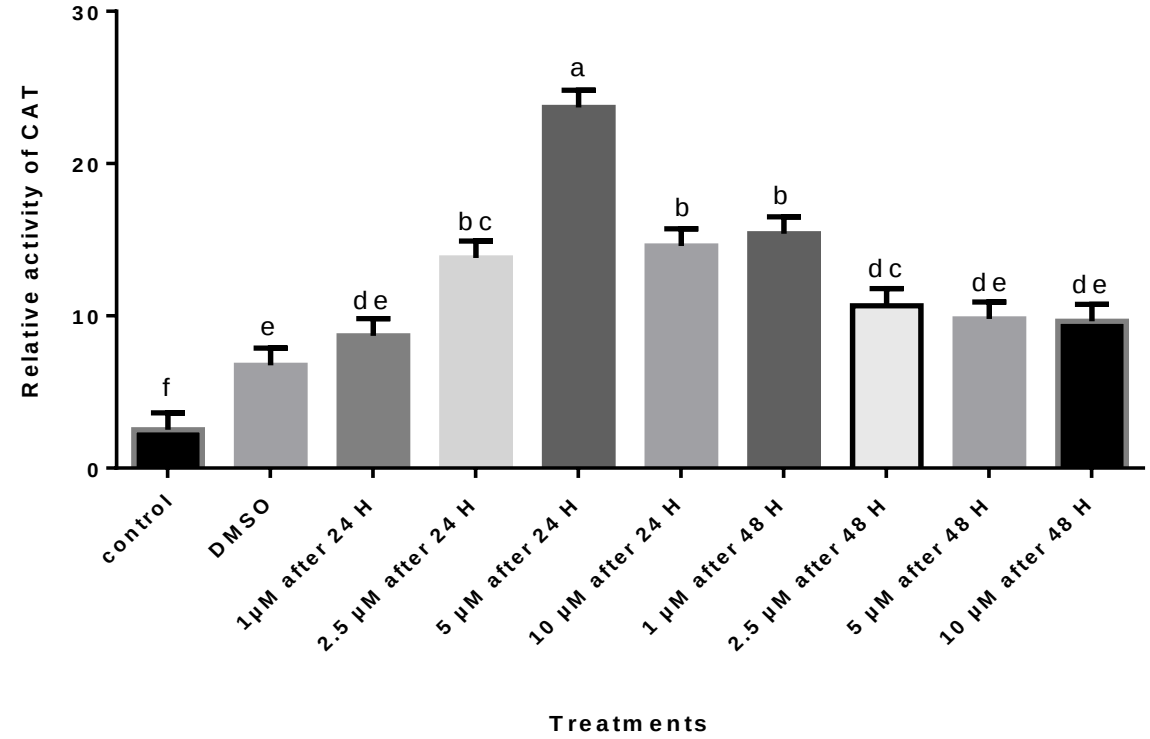

Figure 8. Enzymatic activity of catalase (CAT) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

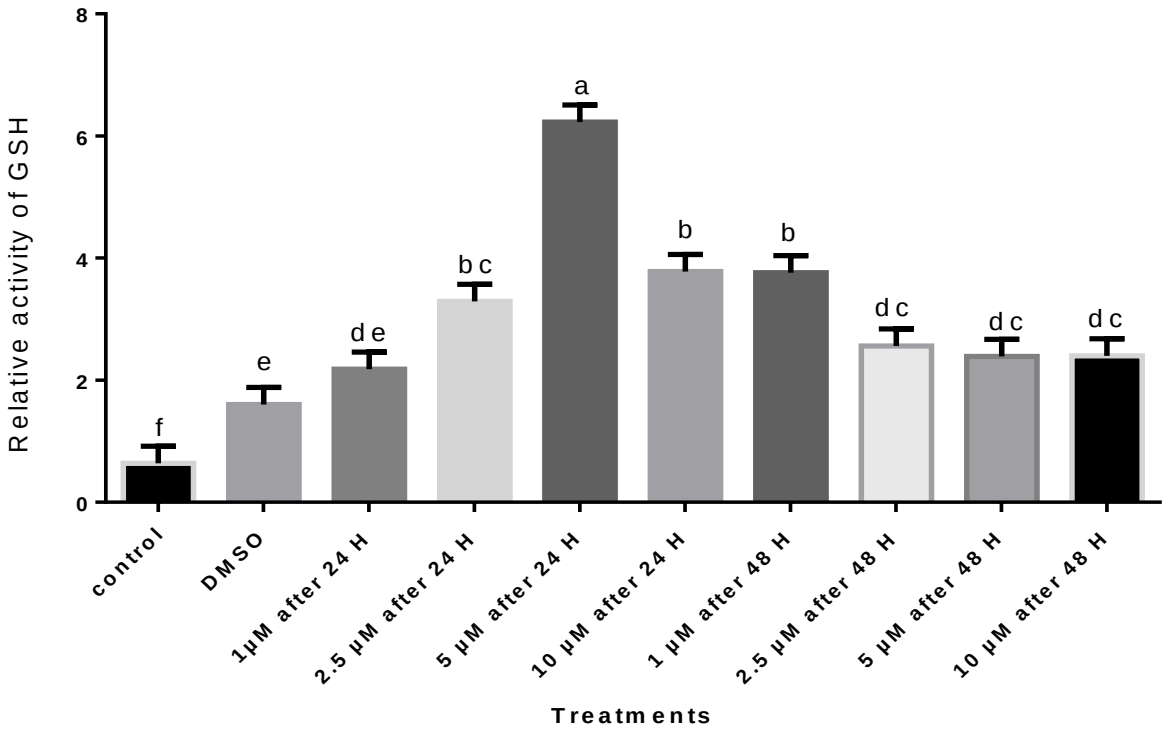

Figure 9. Enzymatic activity of glutatione (GSH) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

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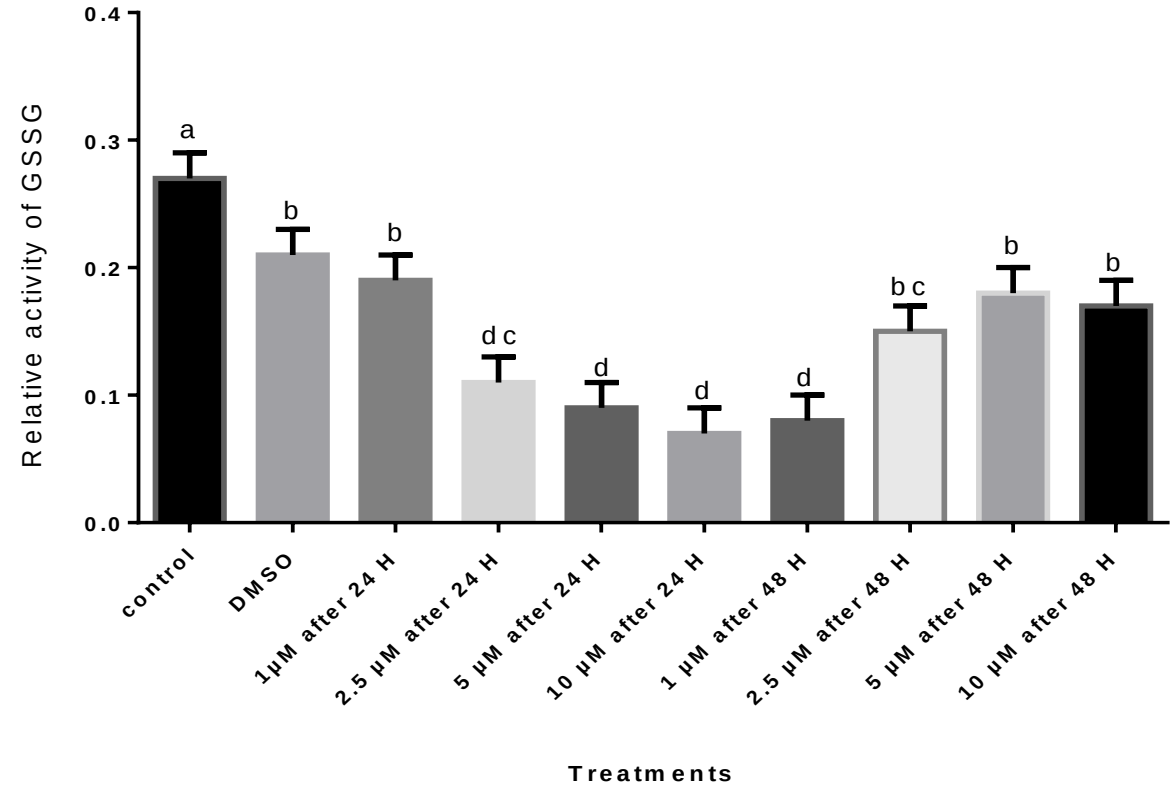

Figure 10. Enzymatic activity of oxidized glutatione (GSSG) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h and 48h.

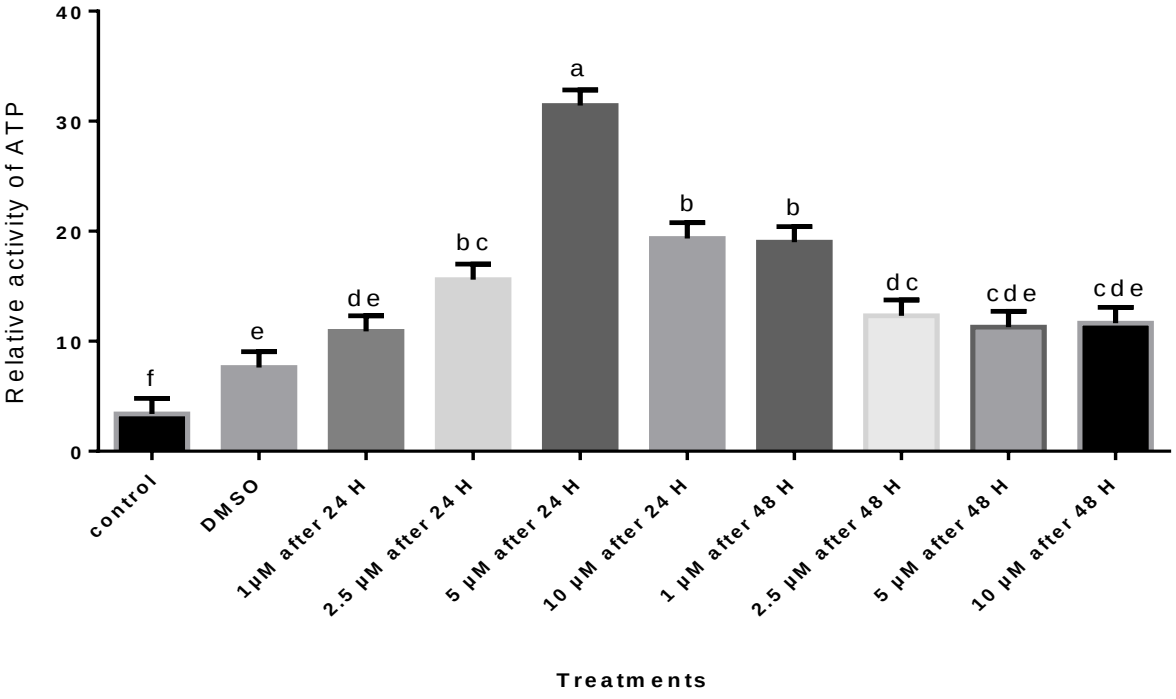

Figure 11. Intracellular adenosine triphosphate (ATP) content in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) for 24h and 48h.

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DISCUSSION

Granulosa cells represent ovarian somatic cells that are in direct contact with oocytes. The GCs supports the oocyte via secretory activity, protective and nutritive effects. Therefore, GCs play a major role in acquisition of oocyte development potential and ovulation process (Buccione et al., 1990; Joyce et al., 2001; Su et al., 2009). However, these cells are also affected by oxidative stress that could be induced by ROS produced either by normal metabolic activity or as a result of *in vitro* culture conditions (Aggarwal et al., 2005). The induction of oxidative stress could subsequently lead to apoptosis of livings cells (Al Dhaheri et al., 2014).

In the current study, the culture medium was supplemented with different concentrations of curcumin to alleviate the excessive ROS accumulated in GCs under *in vitro* culture condition. Results of the present study indicated reduction of granulosa cells viability cultured on groups treated with DMSO (88.0%), 1 µM curcumin (86.0%), 2.5 µM curcumin (86.26%), 5 µM curcumin (83.0%) and 10 µM curcumin (74.0%) compared to control group (93.60 %). In accordance with our results, Kádasi et al. (2012 and 2017) reported reduction in growth of *in vitro* culture swine granulosa cells after curcumin supplementation with 10 and 100 $\mu g.mL^{-1}$ compared to control and 1 $\mu g.mL^{-1}$. In addition, it was reported that curcumin down-regulated proliferation of colon cancer cells (Hanif et al., 1997). This negative effect of curcumin in cultured cells is exerted through apoptosis induction (Bhaumik et al., 1999; Liduan et al., 2004; Voznesens' ka et al., 2010). On contrast, Aktas et al. (2012) have shown a positive proliferative effect of curcumin on mice ovarian follicular cells by preventing apoptosis. Indeed, the variation in curcumin effect observed in our study and other investigations could be explained by type of cells under investigations, conditions of culture, dose and duration of treatment (Kádasi et al., 2012 and 2017).

The reduced viability of granulosa cells was coupled with increased level of ROS in groups treated with 5 µM of curcumin compared to other experimental groups in the current study. Although, curcumin is a well-known antioxidant (Mantzorou et al., 2018) that is used for reducing incidence of oxidative stress (Santos-Parker et al., 2017). However, high concentration of curcumin could induce cell death (Raza et al., 2008). Nevertheless, when the production of ROS overcomes the cellular antioxidant capability, this may lead to a problem referred to as oxidative stress (Agarwal et al., 2005). The ROS level could be elevated endogenously during many physiological procedures including ovulation (Agarwal et al., 2005). However, during *in vitro* cell culture, the ROS could be elevated to the level that cause oxidative stress (Rizzo et al., 2012; Castro et al., 2014; Hatami et al., 2014). Interestingly, our results indicated that the level of ROS and mitochondrial activity were elevated on granulosa cells cultured with 5 µM of curcumin which was linked with reduced viability of this group, confirming harmful side effects of increasing level of this compound during cell culture. Oxidative stress that occurred due to supplementation of exogenous oxidants has induced apoptosis in different types of mammalian cells, including hepatocytes (Haidara et al., 2002), epithelial cells (Jungas et al., 2002), and fibroblasts (Ran et al., 2004). On the other side, curcumin has maintained the mitochondrial respiratory function as well as redox status of PC12 cell line without influencing ROS and viability of cells (Raza et al., 2008). This in accordance with our results that demonstrated increased mitochondrial activity and ATP content in GCs supplemented with curcumin for 24 h during *in vitro* culture however, that was coupled with reduced cellular viability.

Several intracellular enzymes comprise the defense systems of mammalian cells. For example, SOD, GPX1 and CAT, GSSG and DPPH are contributing to scavenging capacity of cells to reduce the harmful effects of oxidative stress induced by ROS (Qin et al., 2015). In the current study, the enzyme activity of CAT, SOD, GSH and DPPH was increased after treating cultured granulosa cells with 5 µM of curcumin however all these enzymes were declined significantly reduced after 48 h. A recent study done by Qin et al., (2015) demonstrated a protective effect of curcumin on alleviating oxidative stress of porcine granulosa cells by rescuing the activity of antioxidant enzymes. However, the present study indicated that although curcumin increase the level of different antioxidant enzymes after 24 h of *in vitro* cell culture but it could not sustain this biological action after 48 h and cell viability was reduced due to increased ROS level.

CONCLUSION

The present findings indicated negative effect of *in vitro* culture on granulosa cell viability and redox status. Antioxidant compound namely curcumin increased the negative effect of *in vitro* culture when added at higher concentration (10 µM). However, low concentration (2.5 µM) of curcumin could maintain metabolic activity as well as defense system by up-regulation of antioxidant enzymes for short duration.

DECLARATIONS

Author's contributions

All authors have contributed to Lab work, the experimental design, writing and revision of the manuscript.

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Acknowledgement
All authors express their thanks to Ms/Fatma Sultan for her technical assistance during *in vitro* culture of GCs.

Competing interests
All authors declare no competing interests that might interfere with the data provided in the current manuscript.

Consent to publish
All the authors approved and agreed to publish the manuscript.

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