Egyptian J. Anim. Prod. (2024) 61(1):31-37

ANTIOXIDANT CAPACITY OF CYSTEAMINE ON BUFFALO GRANULOSA CELL PERFORMANCE UNDER OXIDATIVE STRESS CONDITIONS

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Submitted: 27 11 2023; Accepted: 12/3/2024; Published:24/4/2024

DOI: 10.21608/ejap.2024.250268.1065

SUMMARY

The impact of antioxidant supplementation of cysteamine during in vitro culture of buffalo granulosa cells (GCs) was evaluated in relation to their viability and enzymatic activities under hydrogen peroxide (H_2O_2)-induced oxidative stress. Mural GCs were harvested, cultured for 24 h of confluence at 37.5ºC and then exposed to 500 µM H_2O_2 for 30 min followed by cysteamine supplementation (0, 100, 200, 300 and 400 µM) for further 24 h of culture and their counterpart-control groups were supplemented with cysteamine without H_2O_2 pre-treatment. Granulosa cell viability was assessed using neutral red assay, while the culture conditioned media were subjected to enzymatic analyses. Our results revealed that GCs viability rate was decreased by H_2O_2 pre-treatment; being 63.0±14.0, 61.8±14.1, and 61.8±10.3 % for 100, 300, and 400 µM of cysteamine, respectively, except increasing viability for 200µM cysteamine (80.0±10.0 %) as compared to control (65.2±4.0). *However, the viability was insignificantly enhanced by cysteamine levels under normal physiological conditions. For enzymatic analyses, comparable GSH-Px activity was monitored for all groups of the study. Catalase activity was consistent in 100 and 200 µM of cysteamine under H_2O_2 or normal physiological condition. While a significant reduction was observed in 300 µM of cysteamine under H_2O_2 pre-treatment, there were no significant differences between all groups of the study for malondialdehyde (MDA) levels. In conclusion, H_2O_2 negatively affected GCs viability; however, 200 µM of cysteamine ameliorated cells viability under oxidative stressful condition. The antioxidant enzymes GSH-Px/CAT acted in synergistic dynamics against oxidative stress.*

Keywords: Granulosa cells, Oxidative stress, Cysteamine, Hydrogen peroxide, GSH-Px

INTRODUCTION

Granulosa cells (GCs) are the main functional cellular mediator within ovarian follicle compartment regulating follicle growth and development in mammals (Deng *et al*., 2021 and Liu *et al*., 2023). However, GCs proliferation, differentiation, and steroid secretion abilities are the key drivers that reinforce the ovarian follicle towards either growth or atresia. Besides, it affects the oocyte capacity to acquire its competence for maturation as being determined by the follicular GCs function and performance. Granulosa cells provide essential nutrients, metabolic precursors, growth factors, and cytokines that are necessary for oocyte growth in functional bidirectional communication during follicular development (Whang *et al*., 2019 and Alam and Miyano, 2020). This led the researchers to use GCs phenotype aspects and growth intensity as indicator for studying the developmental competence of the embryo and pregnancy outcome (Assou *et al*., 2010).

Moreover, follicular GCs exposed their protective characteristics for the accompanying oocyte against different stress insults; e.g. heat and oxidative stress. Since GCs could exert different tolerance mechanisms related to apoptosis regulation, hormonal secretion, antioxidant and oxidative stress-related systems (Tripathi *et al*., 2013 and Faheem *et al*., 2021). Under oxidative stress condition, overabundance of reactive oxygen species (ROS) production is well-acknowledged which subsequently resulting in GCs apoptosis which underlies the imbalance of both survival and apoptotic regulator mechanisms inside the cell (Devine *et al*., 2012 and Yang *et al*., 2017). Meanwhile, cellular redox homeostasis is accomplished via its antioxidant defense system which is responsible for maintaining an adequate amount of ROS through their scavenging ability. This defense mechanism is regulated by two different antioxidant types; enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) as well as non-enzymatic antioxidants including cysteamine, vitamin C, vitamin E, glutathione, melatonin, and carotenoids (khan *et al*., 2021). Therefore, antioxidants play an integral role in suppressing oxidative stress damage.

Cysteamine is a non-enzymatic antioxidant with a low-molecular weight that contains thiol. Those thiol compounds provide a protective potential against oxidative stress mediated by either their radical scavenging properties or enhancing cysteine mediated GSH synthesis (Gasparrini *et al*., 2003 and Silva and Silva, 2023). The benefit of cysteamine has been revealed in enhancing nuclear oocyte

maturation, fertilization, embryos development to the blastocyst stage and their quality in different animal species (Wani *et al*., 2012 and Khazaei and Aghaz, 2017). While a unique species-specific effect was observed for those species that were more susceptible to oxidative stress due to their higher lipid content as the case in buffaloes (Gasparrini *et al*., 2003 and Gasparrini *et al*., 2006). However, there are no available reports revealing the impact of cysteamine supplementation on granulosa cells culture. Therefore, the present study was conducted to evaluate the impact of cysteamine, as an antioxidant, on buffalo granulosa cells viability and enzymatic activity, cultured in vitro under oxidative stress or normal conditions.

MATERIAL AND METHODS

Experimental design:

Two different culture conditions were utilized: (1) under oxidative stress condition induced by H_2O_2 treatment (500 µM) for 30 min and (2) under normal physiological condition without H_2O_2 pretreatment, to study the impact of cysteamine supplementation in five different concentrations (0, 100, 200, 300 and 400 µM) on buffalo granulosa cells viability, and antioxidant biomarkers including glutathione peroxidase (GSH-Px) and catalase (CAT), as well as lipid peroxidation marker malondialdehyde (MDA) in culture media.

Chemicals:

Unless stated otherwise, disposable cell culture ware and dishes used in this study were of tissue-culture grade and were obtained from Nunc, Roskilde, Denmark. Chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Ovaries collection and granulosa cells preparation:

Buffalo ovaries were harvested at a local abattoir then transported to the laboratory in a thermos containing normal physiological saline solution (0.9% NaCl) within 2 h post-slaughter at a temperature ranging from 34 to 37 °C. Ovaries were repeatedly washed with pre-warmed 0.9% NaCl, rinsed in 70% ethanol for 30 seconds, and washed twice with pre-warmed saline solution in the laboratory. Ovarian antral follicles with a diameter 2-8 mm were subjected for follicular fluid aspiration using an 18-gauge sterile needle with a rotational motion for permitting follicular GCs to be assembled. The follicular fluid, after excluding all cumulus oocyte complexes (COCs), was transferred immediately into a 15 ml sterilized falcon tube and centrifuged at 176 *g* for 10 min at room temperature. The supernatant was discarded, and the pellets of GCs were re-suspended in 1 ml of pre-warmed complete culture medium (TCM-199 HEPES containing 10% fetal bovine serum and 1% gentamycin) with gentle pipetting to break up cellular clumps and washed for a further 10 min centrifugation then GCs pellet was finally resuspended in 1 ml of complete culture medium. Total number and viable GC number were estimated in a hemocytometer using 0.4% trypan blue exclusion dye under a magnification 10 X (Inverted microscope, Leica, Germany).

Granulosa cells culture and H_2O_2 treatment:

Granulosa cells were seeded in a 96-well plate at a density of 2.0 – 2.5 X 10^5 cells per well in 250 µl of complete culture medium per well under 5% CO2 in humidified air at 37 °C. After 24 h of GCs culture when cells grew to 80 - 90% of confluence, the monolayer was exposed to 500 µM H_2O_2 for 30 min (oxidative stress condition) followed by the supplementation of cysteamine in five different concentrations (0, 100, 200, 300, and 400 µM) for further 24 h of culture and their counterpart-control groups (under normal physiological condition) were supplemented with cysteamine (0, 100, 200, 300 and 400 µM) without H_2O_2 pre-treatment.

By the end of culture, the spent culture media from both H_2O_2-treated and control groups were harvested and stored at - 40 °C for further enzymatic analyses. in addition, GCs viability was evaluated by measuring the uptake of the vital dye, Neutral Red (Repetto *et al*., 2008). Briefly, GCs monolayer was incubated with neutral red solution (40 µg/mL) in phosphate buffer saline (PBS) for 2 h at 37 °C. The cells were then washed twice with PBS (150 µl/well) followed by extracting the dye with acidified ethanol solution (1% acetic acid, 50% ethanol and 49% deionized water) for 10 min of incubation. Then the neutral red uptake was quantified at 540 nm.

Enzymatic analyses:

The GCs spent culture media from all groups of the study were exposed to the colorimetric method using a spectrophotometer instrument (Sunostk SBA 733 plus, Bio diagnostic - Egypt) to assess glutathione peroxidase (GSH-Px; Cat. No. GP 25-24) at a wavelength of 340 nm, catalase (CAT; Cat. No. CA 25-17) at a wavelength of 510 nm and malondialdehyde (MDA; Cat. No. MD 25-29) at a wavelength of 534 nm following the manufacturer's guideline for each assay (Bio Diagnostic, Giza, Egypt). The experiment was conducted in three biological replicates with at least three technical replicates for each group.

Statistical analysis:

GCs viability and enzymatic performance were analyzed using two-ways ANOVA model with interaction followed by Duncan's test to compare the means. Mean values were statistically different at $P \leq 0.05$ and data are expressed as mean ± standard error of the mean (SEM). All analyses were

performed using the IBM SPSS Statistics 22 program (SPSS 16.0, Inc., Chicago, Illinois, USA).

RESULTS

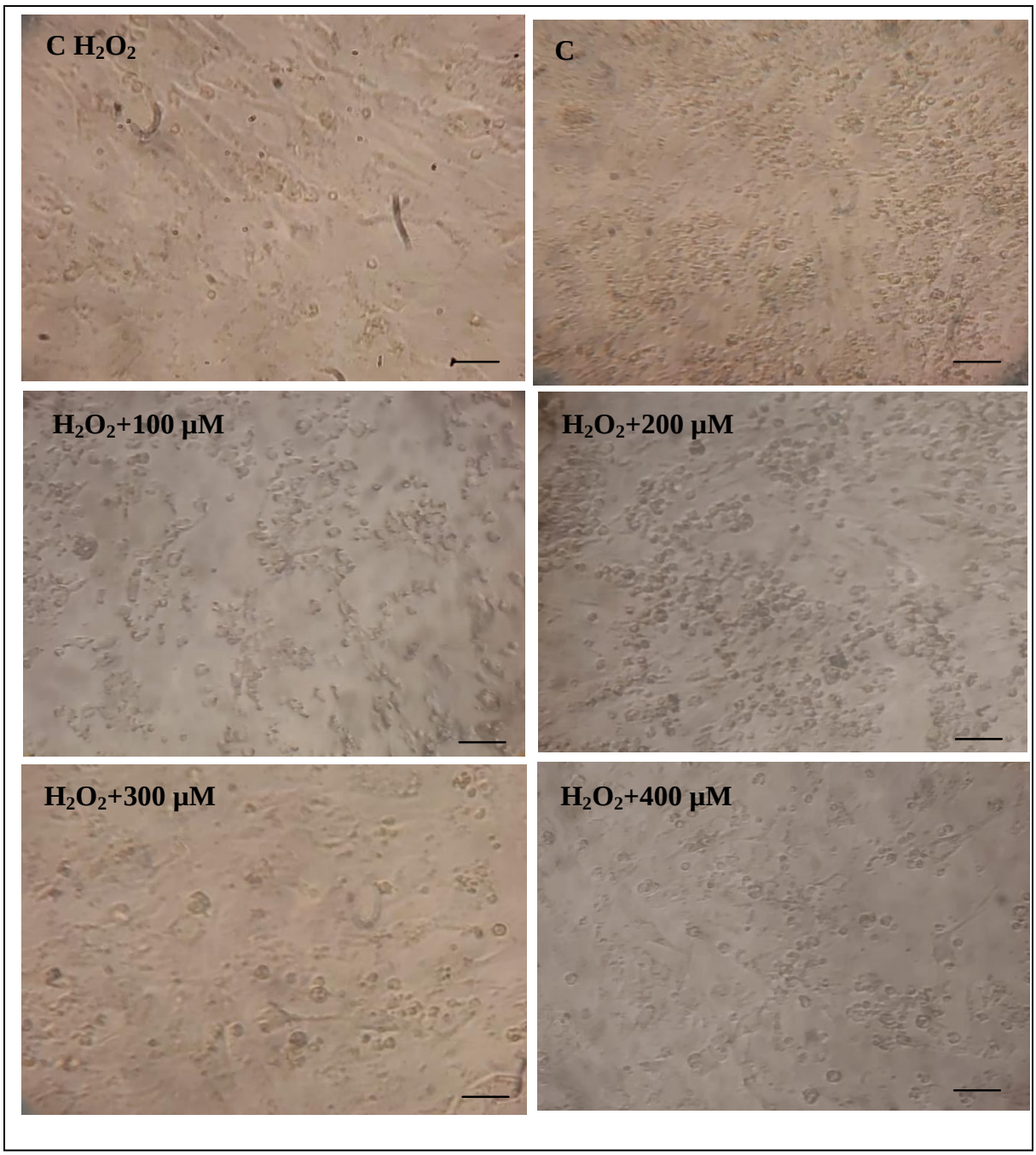
Effect of H_2O_2 and/or cysteamine supplementation on GCs viability:

Granulosa cells viability after H_2O_2 and/or cysteamine supplementation are presented in Table (1) and Figure (1). Post GCs culture viability was evaluated using neutral red assay. The effect of adding cysteamine in 200 µM of concentration after H_2O_2 treatment resulted in apparent improvement of viable buffalo granulosa cells (80.0±10.0 %) in compared to other groups as well as control (65.2±4.0 %) under H_2O_2 treatment without any significant differences. Moreover, the viability of GCs in normal physiological condition increased with the supplementation of cysteamine and recorded the highest percentage (98.3±1.7 %) with 300 µM concentration of cysteamine.

Table 1. Buffalo granulosa cells viability (%) post 48 h of *in vitro* culture

Culture conditions	Cysteamine concentrations				
	0 µM	**100 µM**	**200 µM**	**300 µM**	**400 µM**
H_2O_2 treatment	65.2^{bcd} ±4.0	63.0^{cd} ±14.0	80.0^{abcd} ±10.0	61.8^{d}±14.1	61.8^{d} ±10.3
Normal condition	85.5^{abcd} ±12.1	90.3^{abcd} ±5.8	94.7^{ab} ±3.9	98.3^{a} ±1.7	92.7^{abc} ±3.3

Numbers with different letters (a,b,c,e) differ significantly at $P < 0.05$.



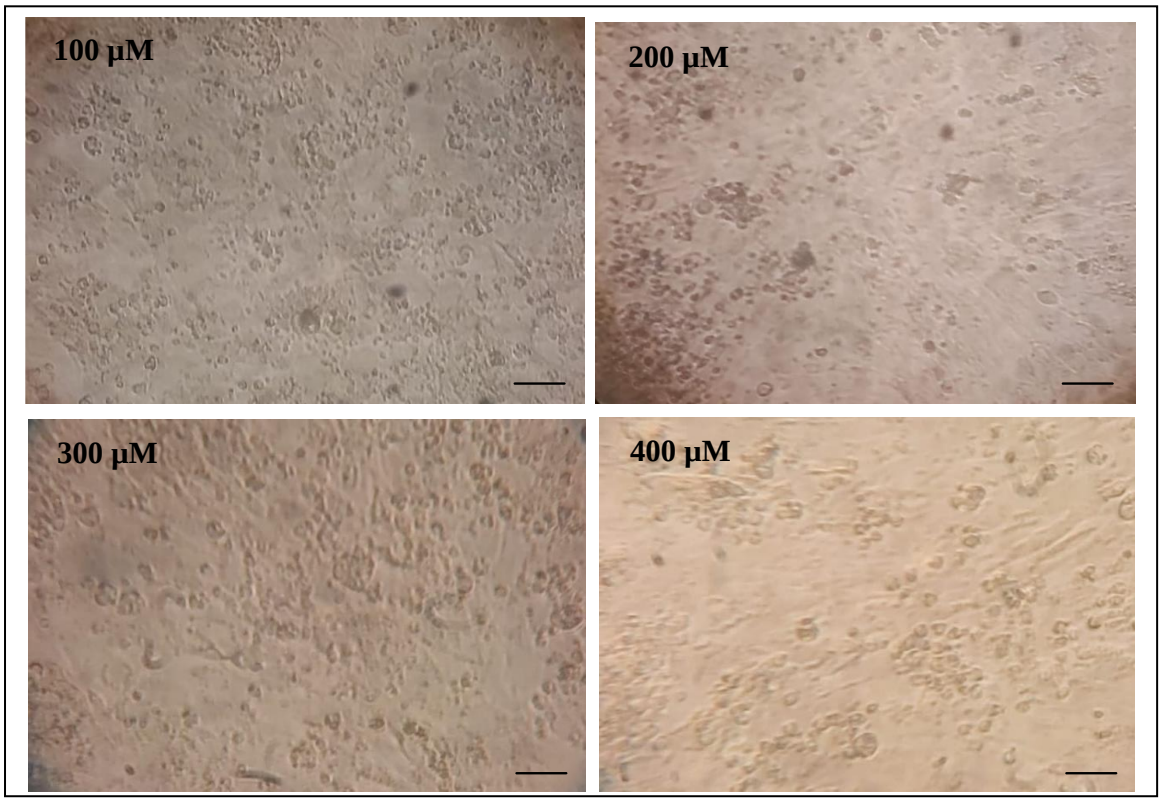


Figure 1. Buffalo granulosa cells viability cultured *in vitro* under oxidative stress (H_2O_2) and/or cysteamine supplementation (0, 100, 200, 300 and 400 µM). Control groups are the GCs that cultured without cysteamine supplementation (zero concentration) under either oxidative stress (C H_2O_2) or normal physiological condition (C Normal). Original magnification X200.

The activities of glutathione peroxidase, catalase and malonaldehyde in GCs conditioned media:

The effect of cysteamine supplementation on glutathione peroxidase (GSH-Px) activity in culture media was shown in Table (2). Under H_2O_2 treatment, the activity of glutathione peroxidase (GSH-Px) was obviously decreased in the control group (0.098±0.04 mU/mL) when compared to GCs supplemented with cysteamine in all other groups. The highest concentration of cysteamine (400 µM) leads to the highest activity of glutathione peroxidase (0.69±0.37 mU/mL), while inversely pattern was noticed with the decrease of cysteamine concentrations. In normal condition, glutathione peroxidase activity recorded higher values in all treated groups except for 100 µM cysteamine group (0.18±0.02 mU/mL) in compared to control (0.22±0.07 mU/mL).

Table 2. Glutathione peroxidase (GSH-Px, mU/mL) activity in buffalo GCs conditioned media

Culture conditions	Cysteamine concentrations				
	0 µM	**100 µM**	**200 µM**	**300 µM**	**400 µM**
H_2O_2 treatment	0.098±0.04	0.33±0.11	0.43±0.11	0.44±0.09	0.69±0.37
Normal condition	0.22±0.07	0.18±0.02	0.46±0.17	0.42±0.16	0.28±0.18

There are no significant differences between and within the groups.

The activity of catalase (as depicted in table 3) showed significant increase in the control group (60.91±5.87 U/L) under H_2O_2 treatment, when compared to the high concentrations of cysteamine (300 and 400 µM). The lowest level of catalase (9.71±2.12 U/L) was found in GCs treated with 300 µM of Cysteamine. In contrast, the activity of CAT under normal physiological condition was significantly higher in the group supplemented with 400 µM of cysteamine (49.74±4.76 U/L) in compared to the control group (16.74±2.15 U/L) under the same condition.

As shown in Table (4), the level of malondialdehyde in pre-treated groups with H_2O_2 as well as in normal physiological condition showed insignificant differences among all groups of the study.

Table 3. Catalase (CAT, U/L) activity in buffalo GCs conditioned media

Culture conditions	Cysteamine concentrations				
	0 µM	**100 µM**	**200 µM**	**300 µM**	**400 µM**
H_2O_2 treatment	60.91^{a}±5.87	37.01^{abc}±8.66	40.8^{abc}±17.26	9.71^{c}±2.12	20.21^{bc}±8.18
Normal condition	16.74^{bc}±2.15	41.08^{ab}±9.44	34.79^{abc}±12.96	38.91^{ab}±6.40	49.74^{a}±4.76

Numbers with different letters (a,b,c) differ significantly at $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Table 4. Malondialdehyde (MDA, nmol/ml) activity in buffalo GCs conditioned media

Culture conditions	Cysteamine concentrations				
	0 µM	**100 µM**	**200 µM**	**300 µM**	**400 µM**
H_2O_2 treatment	4.01±0.14	3.84±0.23	3.31±0.70	3.67±0.17	3.58±0.14
Normal condition	3.59±0.20	3.33±0.47	3.63±0.59	3.97±0.20	3.52±0.33

There are no significant differences between and within the groups.

DISCUSSION

The synergistic mechanism of follicular granulosa cells relying on their secretory, enzymatic activity and nutritive properties lead to promote the growth and development of ovarian follicles in different animal species. Moreover, oocyte maturity and subsequent developmental capability are functionally strengthened through granulosa cells performance. The supportive action of granulosa cells extends to protect the oocytes against oxidative stress damage (Yang *et al*., 2017). Oxidative stress is one of the most consequences that might occur under *in vitro* culture system of oocytes and embryos. Therefore, the impact of oxidative stress on buffalo GCs viability and enzymatic capacity as well as the functional role of cysteamine as an antioxidant to alleviate their negative influences was the aim of the present study.

The main factor underlying the threats of oxidative stress is triggering the excessive production of reactive oxygen species (ROS) inside the cell. There are two cellular approaches to eliminate ROS, through the activation of enzymatic antioxidant such as superoxide dismutase (SOD) and the nonenzymatic antioxidant such as glutathione (GSH), which is the main cellular antioxidant used to determine the degree of ROS (Buettner, 2011). In the present study, oxidative stress condition was induced for the *in vitro* cultured GCs by hydrogen peroxide treatment (500 µM H_2O_2) for 30 min. The viability of GCs was severely affected by H_2O_2 treatment either for control (without cysteamine addition) or cysteamine treated groups (61.8 – 65.2%) except for 200 µM concentration of cysteamine, the viability increased to reach 80.0% being comparable to the normal physiological condition of GCs culture (85.5 - 98.3%). Numerous studies have shown that external oxidative stress induced by H_2O_2 causes an increment of the ROS accumulation level, which are subsequently responsible for destructive cellular DNA, lipids, proteins, and mitochondrial activity and integrity (Chernyak *et al*., 2006; Schieber *et al*., 2014; Zhang *et al*., 2016; Yang *et al*., 2017, and Liu *et al*., 2023). These factors eventually compromise cell viability, GC dysfunction and even apoptosis (Liu *et al*., 2023).

It has been revealed that the addition of exogenous antioxidants enhances the antioxidant capacity of the cell against stress insults (Buettner, 2011). Yang *et al.* (2017) stated that H_2O_2 induced GC death and increased ROS level in dose- and time dependent manner, while with the addition of N-acetyl-cysteine (NAC), which is a cysteine precursor, dose-dependently prevented H_2O_2 negative effects. This is in agreement with our result for 200 µM of cysteamine addition depicted by enhancing GCs viability under oxidative stress. The mechanism of cysteamine to suppress the oxidative damage might be through the activation of cellular enzymatic defense system represented in the glutathione and catalase enzymes. It has been acknowledged that cysteamine potentially acts to restore the reduced intracellular glutathione levels through increasing the uptake of cysteine (Silva and Silva, 2023). This leads to an increase in glutathione synthesis; one of the most important means of cellular defense. The characteristics of cysteamine as a scavenger of free radicals in the culture media are also asserted (Roushandeh *et al*., 2012). In buffalo embryos, the up-regulation of anti-apoptotic genes expression and down-regulation of pro-apoptotic genes expression were the mechanism of action underlying cysteamine supplementation (Elamaran *et al*., 2012). Numerous studies showed that exogenous antioxidants have boosted H_2O_2-induced granulosa cell survival through the inhibition of ROS and MDA generation and enhancing GSH-Px, CAT and SOD activities (Hwang *et al*., 2019; Deng *et al*., 2021, and Wang *et al*., 2021). This was obviously demonstrated by the stability of MDA levels, the cellular marker for oxidative stress, with cysteamine supplementation being comparable under oxidative stress and normal physiological condition as our result indicated.

CONCLUSION

According to our findings, buffalo granulosa cells viability has been enhanced with cysteamine supplementation under normal physiological

condition. Hydrogen peroxide (H_2O_2)-induced oxidative stress negatively affected the granulosa cells viability; while 200 µM of cysteamine ameliorate cells viability under oxidative stress. The antioxidant enzymes GSH-Px/CAT act in synergistic mechanism against oxidative stress.

Ethics approval and consent to participate:

Institutional Animal Care and Use Committee (IACUC) at Cairo University, Egypt approved the experimental protocol of the present study (Protocol # CU-II-F- C-52-23).

ACKNOWLEDGEMENTS

The authors would like to thank the cell-culture lab staff in Cairo University Research Park (CURP), Faculty of Agriculture, Cairo University; Aya Kordy and Beshoy Sameh and the undergraduate students; Leen Latif, Mariam Ahmed, Mary Agaiby, Rahma Mohamed, Zahra Mohamed for their skilled technical assistance.

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قدرة السيستامين المضادة للأكسدة على أداء الخلايا الحبيبية للجاموس تحت ظروف الإجهاد التأكسدي

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يهدف هذا البحث إلي تقييم تأثيرإضافة السيستامين المضاد للأكسدة أثناء زراعة الخلايا الحبيبية للجاموس وتقييم حيويتها وأنشطتها الأنزيمية تحت ظروف الإجهاد التأكسدي الناتج عن بيروكسيد الهيدروجين. (H_2O_2) تم الحصول علي الخلايا الحبيبية الجدارية من الحويصلات المبيضية ذات الفراغ بمجرد سحب البويضات ثم تمت زراعتها عند درجة حرارة 37.5 درجة مئوية .بعد 24 ساعة من الزراعة ، تم تعريض الخلايا الحبيبية الجدارية إلى 500 ميكرومول من H_2O_2 لمدة 30 دقيقة تليها إضافة السيستامين في خمسة تركيزات مختلفة (0 ،100 ،200 ، 300 ,400) ميكرومولار لمدة 24 ساعة أخرى من الزراعة. بينما مجاميع التحكم المناظرة (في ظل ظروف فسيولوجية طبيعية) تم إضافة السيستامين (0 ،100 ،200 ، 300 ,400) ميكرومول (دون معالجة مسبقة بـ H_2O_2) تم تقييم حيوية الخلايا الحبيبية باستخدام صبغة neutral red بينما تعرضت بيئة الزراعة للتحليلات الأنزيمية لفحص أنشطة الجلوتاثيون بيروكسيديز(GSH-Px)، الكاتالاز (CAT) والمالونداياالدهيد .(MDA) أشارت النتائج إلي أن معدل حيوية الخلايا الحبيبية إنخفض بشكل كبير عن طريق المعالجة المسبقة بـ H_2O_2 لجميع مجموعات الدراسة 65.2٪ ±4.0، ±63.0٪ ،14.0± 61.8٪ 14.1± و61.8٪10.3± ، لـ 0 ،100 ، 300 و 400 ميكرومولار من السيستامين، على التوالي (باستثناء 200 ميكرومول من السيستامين 10.0 ± 80.0٪) .ومع ذلك، تم تعزيز الحيوية ($P \geq 0.05$) (في ظل الظروف الفسيولوجية الطبيعية) 85.5٪12.1± ، 90.3٪5.8± ،94.7٪3.9± ،98.3٪ 1.7± و92.7٪ 3.3± لـ (0 ،100 ،200 ، 300 و 400) ميكرومولار من تركيزات السيستامين، على التوالي. بالنسبة للتحليلات الأنزيمية، لوحظ نشاط GSH-Px مماثل لجميع مجموعات السيستامين تحت المعالجة المسبقة بـ H_2O_2 أو الظروف الفسيولوجية الطبيعية .ومع ذلك، أظهرت الخلايا الحبيبية المعالجة بـ H_2O_2 دون إضافة السيستامين أقل نشاط ل . GSH-Pxكان نشاط انزيم الكاتالاز ثابتًا في مجموعات 100 و 200 ميكرومول من السيستامين تحت المعالجة المسبقة بـ H_2O_2 أو تحت الظروف الفسيولوجية الطبيعية. ومع ذلك، فقد لوحظت إختلافات كبيرة ضمن مجموعات 300 و 400 ميكرومول من السيستامين، مما يعكس التأثير السلبي للمعالجة المسبقة بـ H_2O_2 بالمقارنة مع الظروف الفسيولوجية الطبيعية .علاوة على ذلك، لم تكن هناك فروق ذات دلالة إحصائية بين جميع مجموعات الدراسة لمستويات MDA. في الختام، فإن الإجهاد التأكسدي الناجم عن بيروكسيد الهيدروجين (H_2O_2) أثر سلبا على حيوية الخلايا الحبيبية، ومع ذلك، فإن تركيز 200 ميكرومول من السيستامين يعمل على تحسين حيوية الخلايا تحت الإجهاد التأكسدي .تعمل الإنزيمات المضادة للأكسدة GSH-Px/CAT في آلية تآزرية ضد الإجهاد التأكسدي.