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ALTERATIONS IN PHOSPHATASES AND ANTIOXIDANT ENZYMES' ACTIVITY IN FOLLICULAR FLUID AND GRANULOSA CELLS DURING FOLLICULAR ATRESIA IN GOAT (*CAPRA HIRCUS*) OVARY

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ABSTRACT: Ovarian follicular physiology plays an essential role in female reproduction. Healthy ovarian antral follicles act as an indicator of reproductive health that possess high tendency to undergo follicular atresia. Follicular fluid and granulosa cells form a physiological milieu around oocyte with direct interaction. Thus, the present study aims to understand the biochemical variations within follicular fluid and granulosa cells lysates during different stages of follicular atresia. Qualitative and quantitative analysis of Phosphatases and Antioxidant enzymes revealed a significant correlation with progression of follicular atresia in healthy antral follicles. Acid phosphatase activity (µmoles/ml) was found to increase significantly to 0.801±0.004 and 0.869±0.002 in slightly atretic and late atretic follicular fluid in contrast to healthy antral follicles (0.503±0.002). The activities of alkaline phosphatase, superoxide dismutase, catalase and glutathione peroxidase declined to 0.157±0.002, 0.551±0.001, 0.300 ±0.002 and 0.558±0.002 in follicular fluid of atretic follicle in comparison with 0.268±0.002, 0.671±0.001, 0.543±0.003 and 0.808±0.002 in healthy follicles respectively. Similar decreasing trend of enzymatic activity was observed within granulosa cell lysate (µmoles/100mg) from healthy to slightly atretic and atretic follicles. This suggests the role of biochemical alteration and oxidative stress in follicular components during follicular atresia that could be beneficial for maintenance of fertility in mammals.

KEYWORDS: Follicular atresia, phosphatases, Antioxidant enzymes, granulosa cells.

INTRODUCTION

Ovary is an extremely dynamic organ where complicated phenomenon of folliculogenesis and atresia occurs, bringing the study of ovarian follicular dynamics to the great interests of researchers and clinicians these days[1]. Folliculogenesis is characterized by different developmental stages of an ovarian follicle from primary to preantral, antral and pre ovulatory; whereas, follicular atresia involves breaking down of ovarian follicles at different developmental stages[2,3] that correlate with the infertility among mammals. Within a follicle, oocyte development is dependent on the physiological interaction of oocyte and granulosa cells that defines the development and maintenance of a follicle; moreover, the surrounding follicular fluid forms a nutritive milieu around oocyte forming an osmotic gradient to recruit fluid from the thecal vasculature[4]. Thus, changes in structural, biochemical, molecular or genetic level of follicular fluid and granulosa cells will impede developmental progression of an

ovarian follicle. It has already been documented that Granulosa cell apoptosis is recognized as a hallmark majorly contributing to the follicular atresia[5,6,7]. Follicular fluid relates with the metabolic activity of follicular cells influencing follicular development, oocyte maturation and follicular atresia[8,9]. This focuses on the necessities to study biochemical milieu of the follicular components including ions, metabolites and enzymatic profiles that can emphasize on mechanistic reasons of follicular atresia benefitting in infertility treatment and other assisted reproductive techniques[10]. Biochemical profiling has been done in ovarian follicles of cattle[11], pig[12] and buffalo[13]. In some studies, biochemical profiling has been done on follicles of super ovulated goats[14] but reports on biochemical characteristics of follicular fluid and surrounding granulosa cells in atretic follicles of goat is scanty and need further investigation. Most importantly, the role of enzyme activities that control most of physiological functions within follicles must be entailed. Studies suggest various Phosphatase and antioxidant enzymes are involved in follicular atresia. In bovine antral follicles, acid phosphatases were found to initiate whereas alkaline phosphatases were found to impede follicular atresia[15]. Similarly, various antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were found to be associated with granulosa cell apoptosis mediated follicular atresia[6,7]. These enzymes are thought to imbalance oxidant–antioxidant level bringing atresia by generation of Reactive oxygen species[16]. SOD dismutates superoxide anion radicle to form hydrogen peroxide that is degraded into water and oxygen molecule by CAT and GPx[17]. Thus, the present study aimed to gain insights into the changes in enzymatic activities of phosphatases and antioxidant enzymes of follicular fluid and granulosa cells with the progression of follicular atresia in caprine antral follicles. The study will evaluate the role of different enzymes and oxidative stress in initiating atresia that will be beneficial to combat fertility troubles in reproductive biology.

MATERIALS AND METHODS

Collection of ovaries and classification of follicles

Ovaries from Jammnapari breed of goat were collected from slaughter houses of Chandigarh and brought to the laboratory in normal saline at 4ºC. The follicles were manually separated from ovaries using fine forceps and were classified as healthy, slightly atretic and atretic on morphometric bases of colour, turbidity of follicular fluid and vascularity[18,19] for biochemical studies.

Biochemical analysis

For the biochemical investigations, the follicular fluid was aspirated out from the healthy, slightly atretic and atretic follicles that were centrifuged at 1000 rpm for 15 minutes to obtain the clear follicular fluid and granulosa cells pellet. The follicular fluid and

granulosa cells were quantified and stored at 4ºC for subsequent use in following different enzyme analyses.

The acid phosphatase (EC 3.1.3.2) and Alkaline Phosphatase (EC 3.1.3.1) activity was estimated spectrophotometrically using the method of Linhardt and Walter[20]. The superoxide dismutase (EC 1.15.1.1) activity was assayed by the method of Marklund and Marklund[21] based on rate of auto-oxidation of Pyrogallol. Catalase (EC 1.11.1.6) activity was estimated using the method of Aebi[22] after processing the homogenates as described by Cohen and coworkers[23]. The protein concentration of sample homogenate was measured by the method of Lowry and coworkers[24]. Glutathione peroxidase (EC 1.11.1.9) was assayed by the method of Rotruck and coworkers[25] with modifications.

RESULTS AND DISCUSSION

The bio-chemical studies of follicular fluid and granulosa cells of different categories of antral follicles undergoing atresia namely healthy, slightly atretic and late atretic, revealed prominent changes in several enzyme activities emphasizing its relation with progression of atresia within healthy follicles.

The acid phosphatase activity was recorded as 0.503 ± 0.002, 0.801 ± 0.004, and 0.869 ± 0.002 μmoles/ml in the follicular fluid of healthy, slightly atretic and atretic follicles respectively (Fig. 1) showing an increase in enzyme activity from healthy to atretic follicles. Similarly in granulosa cells, the enzyme activity increased significantly from 0.558 ± 0.004 in healthy antral follicles to 0.724 ± 0.010, and 0.761 ± 0.010 μmoles/100mg in slightly atretic and atretic follicles (Fig 1). The alkaline phosphatase activity was estimated as 0.268 ± 0.002, 0.260 ± 0.003 and 0.157 ± 0.002 μmoles/ml in the follicular fluid of healthy, slightly atretic and atretic follicles respectively (Fig. 2) indicating the decrease in alkaline phosphatase activity as follicle advanced in the stage of atresia. Similarly, in the granulosa cells, the enzyme activity declined in the same manner as 0.964 ± 0.002, 0.933 ± 0.003, and 0.412 ± 0.003 μmoles/100mg in healthy, slightly atretic and atretic follicles (Fig. 2,).

The activity of SOD significantly declined with increasing stages of atresia. It was found to be 0.671 ± 0.001, 0.654 ± 0.001, and 0.551 ± 0.001 μmoles/ml in the follicular fluid and 1.940 ± 0.009, 1.119 ± 0.004, 1.080 ± 0.012 μmoles/100mg in the granulosa cells of healthy, slightly atretic and atretic follicles respectively (Fig. 3) showing a decrease in enzyme activity from healthy to slightly atretic to atretic follicles respectively. Like wise, Catalase activity was estimated as 0.543 ± 0.003, 0.307 ± 0.002, and 0.300 ± 0.002 μmoles/ml in the follicular fluid and 9.843 ± 0.219, 3.160 ± 0.049 and 2.427 ± 0.022 μmoles/100mg in the granulosa cells of healthy, slightly atretic and atretic follicles respectively (Fig. 4) showing a decrease in

enzyme activity from healthy to atretic follicles.

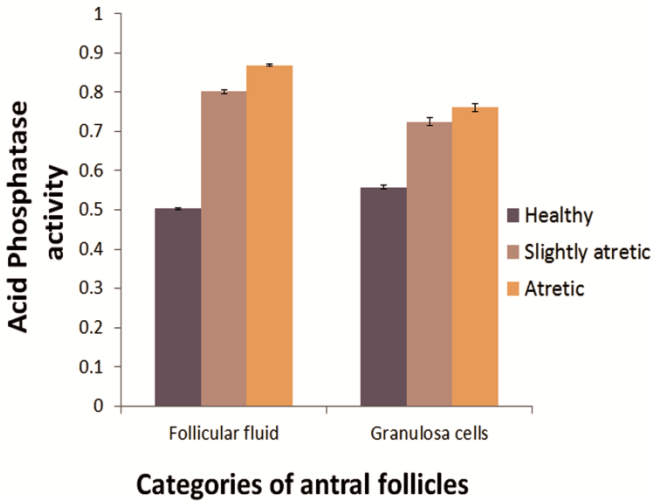

Figure 1. Variations in Acid Phosphatase activity in follicular fluid (µmoles/ml) and granulosa cells (µmoles/100mg) in different categories of antral follicles.

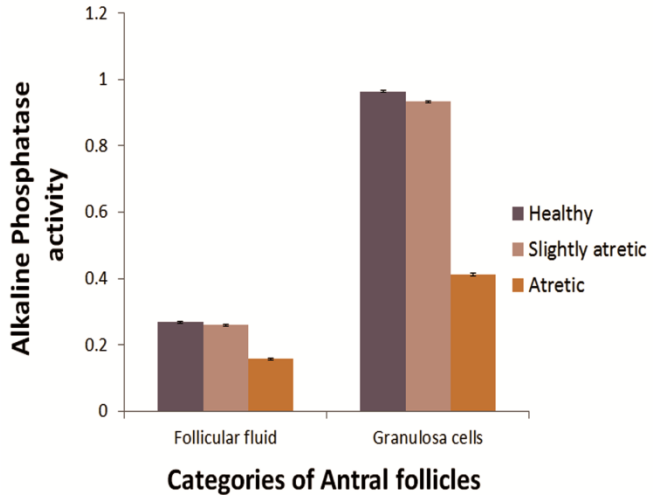

Figure 2. Variations in Alkaline Phosphatase activity in follicular fluid (µmoles/ml) and granulosa cells (µmoles/100mg) in different categories of antral follicles.

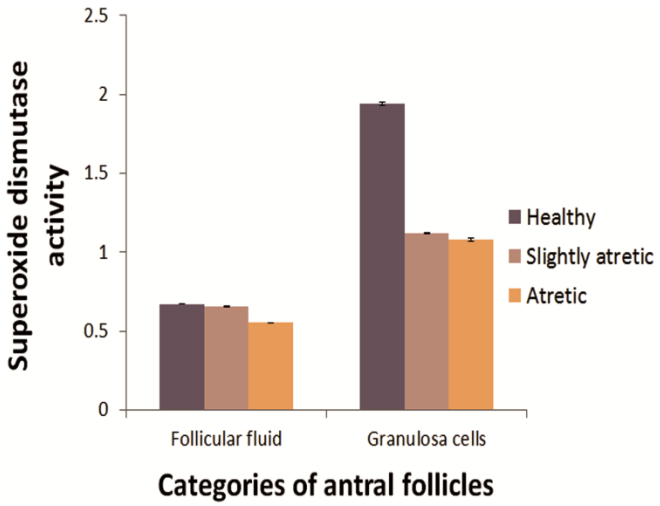

Figure 3. Variations in Superoxide dismutase (SOD) activity in follicular fluid (µmoles/ml) and granulosa cells (µmoles/100mg) in different categories of antral follicles.

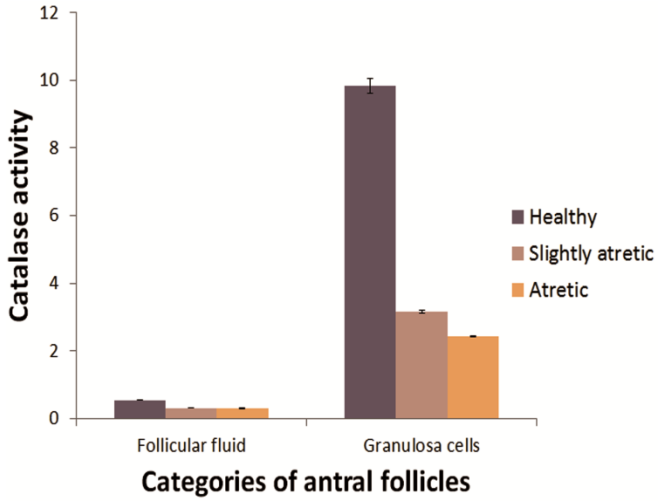

Figure 4. Variations in Catalase (CAT) activity in follicular fluid (µmoles/ml) and granulosa cells (µmoles/100mg) in different categories of antral follicles.

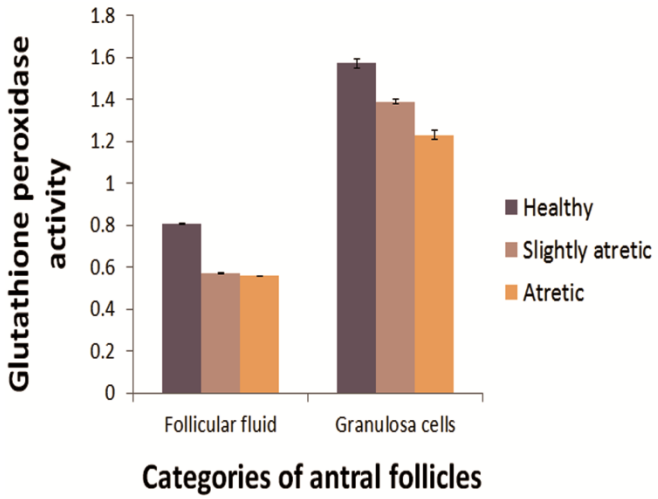

Figure 5. Variations in Glutathione peroxidase (GPx) activity in follicular fluid (µmoles/ml) and granulosa cells (µmoles/100mg) in different categories of antral follicles.

The results significantly revealed the relation of declining enzymatic (Antioxidative) activity with progression of atresia within a healthy antral follicle indicating the role of oxidative stress during follicular atresia. A significant positive correlation ($p<0.05$) was observed between all the Antioxidative enzyme activities (Alkaline Phosphatase, Superoxide Dismutase, Catalase and Glutathione Peroxidase) and atretic stages.

The fertility potential in female is directly proportional to the healthy ovarian antral follicles. The biochemical characteristics of the follicular fluid and granulosa cells surrounding the oocyte in the ovarian follicle play a critical role in maintaining the health and quality of a follicle[4]. However, a significant number of antral follicles undergo atresia during each menstrual cycle due to natural and unnatural reasons. In the present investigation, analysis of several biochemical parameters revealed the association of different stages of follicular atresia with changes in phosphatase and antioxidant enzyme activity within follicular fluid and granulosa cells of healthy and atretic follicles.

The results exhibited an increasing and declining trend of Acid and Alkaline phosphatase activity respectively both in follicular fluid and granulosa cells from healthy to slightly atretic and atretic follicles. Acid and alkaline phosphatase are lysosomal enzymes which catalyze various reactions in the body and are involved in the active transport of phosphates across the cell membrane, synthesis of protein and DNA turnover in nucleus[8]. Acid phosphatases are known to inactivate estrogen receptors by dephosphorylation that, in follicles, inhibits

the stimulation of estrogen receptors in response to FSH[15] making the follicle atretic. In follicular fluid, acid phosphatase acts as a phosphor-protein, inactivating cyclic AMP-dependent protein kinases, that further limits the ability of antral follicles to respond to gonadotropin stimulation[15]. The increase in Golgi complex and lysosomes in atretic follicles/cells is also possibly attributable to the rise in acid phosphatase activity. The ultrastructural studies have revealed extensive lysosomal accumulation, participating in lysis and formation of apoptotic vesicles, signifying increase in acid phosphatase activity in the granulosa cells as well[26]. The increase in acid phosphatase enzyme activity observed in the follicular fluid and granulosa cells of atretic follicles may also be related to some mechanism for the secretion of steroids[27]. The present biochemical estimation of alkaline phosphatase activity focuses on the possible role of alkaline phsophatase in active transport of nutrients and secretary material across the membrane[28]. The association of alkaline phosphatase positive sites with theca interna indicates the involvement of this enzyme in steroid metabolism and transport[29]. Shift in the follicular hormonal milieu from androgen dominant to estrogen dominant with the follicle development probably resulted in declined alkaline phosphatase activity[9]. The decline in levels of alkaline phosphatase in follicular fluid and granulosa cells of atretic follicles also may be due to increased vascularity and changed morphology and biochemistry of granulosa cells for steroid hormone synthesis[9,29,30].

Furthermore, the present findings also depicted a decreasing trend in the activity of various antioxidant enzymes namely SOD, CAT, GPx in follicular fluid and granulosa cells from healthy to slightly atretic and atretic follicles, elucidating the role of oxidative stress and decreasing anti-oxidative power in bringing follicular atresia. Declining antioxidative enzyme activity, either in follicular fluid or granulosa cells, facilitates the generation of several reactive oxygen species like superoxide radicles, hydroxyl radicles and hydrogen peroxide that influence the cellular integrity inflicting serious damages to cellular DNA, lipids, proteins and other macromolecules resulting in cell apoptosis[31]. This ultimately triggers follicular atresia as comprehensive granulosa cell apoptosis has been found to be underlying cause of follicular atresia[5,32].

SOD is a family of metallo-enzymes that scavenges and dis-mutates the superoxide anion radical (O^{2-}.) to hydrogen peroxide (H_2O_2) whereas catalase is an ubiquitous enzyme, associated with SOD, that degrades H_2O_2 to water and O_2 (33). SOD exists in three forms- manganese-associated form of SOD is localized in mitochondria of cells, whereas the copper and zinc associated form are generally found in the cytoplasm and all the three forms of SOD are expressed in ovary and their pattern of expression appears to be correlated with gonadotropin induced follicular development and luteal

steroidogenesis and regression[27]. Decrease in SOD and CAT generates ROS that creates nicks in DNA by disrupting its sugar moiety, causes chromatin condensation and makes the follicular cells permeable to several pro-apoptotic signalling molecules easing initiation and execution of apoptosis[34,35]. Weakening of antioxidant defences also occurs in granulosa cells that have been associated with down-regulation of Cu/ZnSOD, MnSOD and CAT genes and accumulation of oxidative damage mainly involving mitochondria[36].

Another antioxidative enzyme, Glutathione peroxidase (GPx), catalyses the oxidation of glutathione to detoxify ROS such as hydrogen peroxide into water; thus, decline in its activity causes oxidative stress that may the possible cause of follicular atresia in antral follicles[37]. This supports the present findings where GPx activity decreased in follicular fluid and granulosa cells of slightly atretic and atretic follicles as compared with healthy antral follicles. Such pattern of enzymatic defences of SODs, CAT and GPx in follicular fluid and granulosa cells fails to scavenge ROS toxicity allowing them to rapidly diffuse into the oocyte elevating oocyte and follicular atresia[7,30]. In addition, Granulosa cells are steroidogenically active cells within antral follicles that require high level of energy and thus, produces higher amount of ROS resulting in the observed decline in antioxidative enzyme activities[38,39]. Consequently, the present study suggested that antral follicles undergo distinct biochemical changes, both in follicular fluid and granulosa cells lysate, during follicular atresia. These alterations enhance the oxidative stress and ROS generation within the healthy antral follicle making the follicles atretic. Follicular fluid and surrounding cells involves several physiological processes associated with fertility, thus, knowledge of its biochemical composition will facilitate future in vivo and in vitro research concerning fertility troubles.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

REFERENCES

1. Yang, D.Z., W. Yang, Y. Li & Z. He, 2013. Progress in understanding human ovarian folliculogenesis and its implications in assisted reproduction. *J. Assist. Reprod. Genet.,* 30(2): 213-9.
2. Cheng, G., Z. Weihua, S. Mäkinen, S. Mäkelä S. Saji, M. Warner, J.A. Gustafsson & O. Hovatta, 2002. A role for the androgen receptor in follicular atresia of estrogen receptor beta knockout mouse ovary. *Biol. Reprod.,* 66(1): 77-84.
3. Sharma, R.K. & J.K. Bhardwaj, 2007. Granulosa cell apoptosis in situ in caprine ovary. *J. Cell. Tiss. Res.,* 7(2): 1111-14.
4. Revelli, A., L.D. Piane, S. Casano, E. Molinari, M. Massobrio & P. Rinaudo, 2009. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod. Biol. Endocrinol.,* 7: 40-52.

5. Bhardwaj, J.K & P. Saraf, 2014. Malathion-Induced Granulosa Cell Apoptosis in Caprine Antral Follicles: An Ultrastructural and Flow Cytometric Analysis. *Microsc. Microanal.*, 20: 1861-68.

6. Bhardwaj, J.K & P. Saraf, 2016 Granulosa Cell Apoptosis by Impairing Antioxidant Defense System and Cellular Integrity in Caprine Antral Follicles Post Malathion Exposure. *Environ. Toxicol.*, 31(12): 1944-54.

7. Bhardwaj, J.K & P. Saraf, 2017. N-Acetyl Cysteine-Mediated Effective Attenuation of Methoxychlor-Induced Granulosa Cell Apoptosis by Counteracting Reactive Oxygen Species Generation in Caprine Ovary. *Environ. Toxicol.*, 47(1): 156-66.

8. Mishra, O.P., J.N. Pandey, & P.G. Gawande, 2003. Study on biochemical constituents of caprine ovarian follicular fluid after superovulation. *Asian. Aust. J. Anim. Sci.*, 16: 1711-15.

9. Nandi, S., V.G. Kumar, B.M. Manjunatha, & P.S.P. Gupta, 2007. Biochemical composition of ovine follicular fluid in relation to follicle size. *Develop. Growth. Differ.*, 49: 61-6.

10. Iwata, H., J. Inoue, K. Kimura, T. Kuge, T. Kuwayama & Y. Monji, 2006. Comparison between the characteristics of follicular fluid and the developmental competence of bovine oocytes. *Anim. Reprod. Sci.*, 91: 215-23.

11. Tabatabaei, S., M.A. Moghadam, M. Mamouei, K. Mirzadeh & A. Aghaei, 2014. Hormonal Profile of Ovarian Follicular Fluid and Blood Plasma during Different Stages of Estrous Cycle in Holstein Cattle. *Iran. J. Appl. Anim. Sci.*, 4(2): 263-8.

12. Huang, W.T., S.G. Lu, P.C. Tang, S.C. Wu, S.P. Cheng, & J.C. Ju, 2002. Biochemical compositions of follicular fluid and the effects of culture conditions on the in vitro development of pig oocytes. *Asian. Aust. J. Anim. Sci.*, 15: 1403-11.

13. Patra, B.K., D.N. Mohanty, R.K. Das, G.R. Sahoo, D.K. Kama & L. Sahoo, 2015. Total protein, glucose and steroid profile in the ovarian follicular fluid of buffaloes of coastal Odisha. *Buffalo. Bulletin.*, 34(4): 423-8.

14. Deshpande, S.B. & M.M. Pathak, 2010. Hormonal and Biochemical Profiles In Follicular Fluid of Unovulated Follicles in Superovulated Goats Ovaries. *Vet. World.*, 3(5): 221-3.

15. Henderson, K.A. & P.T. Cupps, 1990. Acid and alkaline phosphatase in bovine antral follicles. *J. Anim. Sci.*, 68(5): 1363-9.

16. Sinha, K., J. Das, P.B. Pal & P.C. Sil, 2013. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Arch. Toxicol.*, 87: 1157-80.

17. Gupta, R.K., K.P. Miller, J.K. Babus & J.A. Flaws, 2006. Methoxychlor inhibits growth and induces atresia of antral follicles through an oxidative stress pathway. *Toxicol. Sci.*, 93: 382-9.

18. Sharma, R.K. & J.K. Bhardwaj, 2009. *In situ* evaluation of granulosa cells during apoptosis in caprine ovary. *Int. J. Integ. Biol.*, 5: 58-61.

19. Yang, M.Y. & R. Rajamahendran, 2000. Involvement of Apoptosis in the Atresia of Non-ovulatory Dominant Follicle during the Bovine Estrous Cycle. *Biol. Reprod.*, 62: 1209-17.

20. Linhardt, K. & K. Walte, 1965. 'Phosphatase' In methods of Enzymatic Analysis, Ed. by Bergmeyer, H.U. Academic press, New York, London. p783.

21. Marklund, S. & G. Marklund, 1974. Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *Eur. J. Biochem.*, 47: 469-74.

22. Aebi, H. 1984. Catalase in vitro. *Methods. Enzymol.*, 105: 121-126.

23. Cohen, D., D. Dembiec & J. Marcus, 1970. Measurement of catalase activity in tissue extracts. *Annals. Biochemistry.*, 34: 30–8.

24. Lowry, O.H., N.J. Rosenbrough, A.L. Farr & R.I. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265-75.

25. Rotruck, J.T., A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman & W.G. Hoekstra, 1973. Selenium: biochemical role as a component of glutathione peroxidase. *Science.,* 179(4073): 588-90.

26. Sangha, G.K. & S.S. Guraya, 1989. Histochemical changes in acid and alkaline phosphatase activities in the growing follicles and corpora lutea of the rat ovary. *Acta. Morphol. Neerl. Scand.,* 26: 43-9.

27. Pangas, S.A., 2007. Growth factors in ovarian development. *Semin. Reprod. Med.,* 25: 225-234.

28. Sharma, R.K., 2010. Follicular atresia in goat: A review. *Ind. J. Anim. Sci.,* 70: 1035-46.

29. Gilchrist, R.B., L.J. Ritter & D.T. Armstrong, 2004. Oocyte-somatic cell interactions during follicle development in mammals. *Anim. Reprod. Sci.,* 82-83**:** 431-46.

30. Tatone, C., F. Amicarelli, C. Carbone, P. Monteleone, D. Caserta, R. Marci, R.G. Artini, P. Piomboni & R. Focarelli, 2007. Cellular and molecular aspects of ovarian follicle ageing. *Health. Hum. Reprod.,* 14(2): 131-42.

31. Cooke, M.S., M.D. Evans, M. Dizdaroglu & J. Lunec, 2003. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB. J.,* 17: 1195-1214.

32. Escobar, M.L., O.M. Echeverría, A.S. Casasa, G. García, S.J. Aguilar, & G.H. Vázquez-Nin, 2013. Involvement of pro-apoptotic and pro-autophagic proteins in granulosa cell death. *Cell. Biol.,* 1(1): 9-17.

33. Kohen, R. & A. Nyska, 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions and methods for their quantification. *Toxicol. Pathol.,* 30: 620-50.

34. Mahfouz, R., R. Sharma, A. Thiyagarajan, V. Kale, S. Gupta, E. Sabanegh, & A. Agarwal, 2010. Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species. *Fertil. Steril.,* 94(6): 2141-6.

35. Devine, P.J., S.D. Perreault, & U. Luderer, 2012. Roles of Reactive Oxygen Species and Antioxidants in Ovarian Toxicity. *Biol. Reprod.,* 86(2): 1-10.

36. Tatone, C., M.C. Carbone, R. Gallo, S. Delle Monache, M. Di Cola, E. Alesse & F. Amicarelli, 2006. Age-associated changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and survival factor BCL2. *Biol. Reprod.,* 74: 395-402.

37. Waggiallah, H. & M. Alzohairy, 2011. The effect of oxidative stress on human red cells glutathione peroxidase, glutathione reductase level, and prevalence of anemia among diabetics. *N. Am. J. Med. Sci.,* 3(7): 344-7.

38. Jancar, N., A.N. Kopitar, A. Ihan, I. Virant Klun & E.V. Bokal, 2007. Effect of apoptosis and reactive oxygen species production in human granolusa cells on oocyte fertilization and blastocyst development. *J. Assist. Reprod. Genet.,* 24: 91-7.

39. Gbotolorun, S.C., A.A.A. Osinubi, A.A. Oremosu, & C.C. Noronha, 2011. The effect of amodiaquine on oestrus cycle, ovarian histology and oxidative stress makers in regular cyclic sprague-dawley rats. *Agric. Biol. J. N. Am.,* 2(4): 630-7.