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Vitamin C Status and Nitric Oxide in Buffalo Ovarian Follicular Fluid in Relation to Seasonal Heat Stress and Phase of Estrous Cycle

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Abstract**—**Heat stress is a recognized problem causing huge economic losses to the buffalo breeders as well as dairy industry. The aim of the present work was to study the pattern of vitamin C and nitric oxide in follicular fluid of buffalo during different seasons of the year considering phase of estrous cycle. This study was conducted on 208 cyclic buffaloes slaughtered at Al-Qaliobia governorate, Egypt, over one year. The obtained results revealed that vitamin C in follicular fluid was significantly lower in summer than winter and spring. On the other hand, nitric oxide (NO) was significantly higher in summer and autumn than winter and spring. Both vitamin C and NO did not differ significantly between follicular and luteal phases. In conclusion, the present study revealed that alterations in concentrations of follicular fluid vitamin C and NO that occur in summer could be related to low summer fertility in buffalo.

Keywords**—**Buffalo, follicular fluid, vitamin C, NO and heat stress.

I. Introduction

BUFFALO is one of the most important livestock animals which has a vital role in solving the world-wide problem of deficiency of animal proteins [1]. Improvement and increasing buffalo population has a significant role in mitigation of poverty in the developing countries, where it is raised. Buffalo enterprise has also a main role in employment of rural communities' population. Accordingly, increasing of buffalo meat and milk production is vital for the developmental strategy in the world particularly in developing countries [2]. Summer heat stress in tropical and subtropical countries is a major limitation on buffalo leading to impairment of both production and reproduction performance [3] and influences buffaloes to develop oxidative stress [4]. Egypt has very high temperatures during summer ranges from 23 to 35 ◦C and 74% mean temperature humidity index (THI%) [5]. For buffaloes, the optimum conditions are 13-18◦C and in terms of the mean THI; values of THI > 72 is stressful to this animal [6].

Ovarian function is central to all reproductive problems in buffalo including seasonal reproductive patterns [7]. As a result, studying composition of follicular fluid microenvironment could increase knowledge regarding follicular development, oocyte maturation, follicular atresia as well as the pathogenesis of some reproductive problems in buffalo [8]. In addition, follicular fluid analysis provides information on metabolic changes in blood serum, as the circulating biochemical milieu may be reflected in the composition of follicular fluid [9]. Ascorbic acid, a water-soluble vitamin, has attracted the attention of scientists as it relates to stress conditions including high environmental temperature in different species of animals [10]. The ovary has long been recognized as a site of ascorbic acid accumulation. Moreover, ascorbic acid has three main biological actions relevance to reproduction; it is required for the biosynthesis of collagen, for the biosynthesis of steroid and peptide hormones and to reduce or prevent the oxidation of biomolecules [11]. Although the reproductive demand of vitamin C is met endogenously in bovine and other mammals capable synthesizing ascorbate, sub-fertility may result from under-production [12].

NO is a short lived inorganic free radical gas that is produced by granulosa cells *in vivo* from l-arginine by the action of NO synthases [13]. The effects and the mechanism of action of NO depend strictly on its concentration and consequently, NO may exert dual effects on the same process in the same cell [14]. NO is an important messenger molecule recognized in female reproduction like ovarian follicular development [15], preovulatory LH surge [16] and oocyte meiotic maturation [17]. On the other hand, an excessive production of NO in follicular fluid microenvironment surrounding the oocyte could cause apoptosis within the follicle before fertilization and affect oocyte development. NO is hardly measurable with direct methods because it is unstable gas having very short half-life. However, it is rapidly converted into nitrites and nitrates which can be measured in biological fluids [18]. While many studies have been performed on hormonal and biochemical components of buffalo follicular fluid, data regarding the pattern of follicular fluid vitamin C and NO during different seasons of the year as well as during follicular and luteal phases in buffalo is limited. Therefore, this study was designed to examine the effect of summer heat stress and phase of estrous cycle on pattern of vitamin C as powerful antioxidant and NO as important signaling molecule and free radical involved in reproductive functions in follicular fluid of Egyptian buffalo.

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II. Materials and Methods

A. Animals

A total number of 208 cycling non-pregnant female buffaloes (*Bubalus bubalis*) in apparently good health and with normal reproductive tracts upon macroscopical examination were used for this study after slaughtering at a local slaughterhouse (Bahtim abattoir, Al-Qaliobia governorate, Egypt).

B. Experimental Design

Ovarian follicles were collected over one year during different seasons. Animals (n = 208) were divided into four groups; summer group (n = 52), autumn group (n = 52), winter group (n = 52) and spring group (n = 52). Buffaloes in each group were subdivided according to stage of estrous cycle into two groups; follicular phase group and luteal phase group.

C. Sampling

Immediately after slaughtering, both ovaries from each animal were collected in plastic bags containing 0.9% NaCl and transported in ice tank to the laboratory where the stage of estrous cycle either follicular or luteal was identified according to morphological features of the corpus luteum as previously established by [19] and [20]. Follicles > 20 mm in diameter with thick wall and containing red-colored fluid were assumed to be cystic and were not included in the study. The contents of the ovarian follicles of different size were aspirated using a 5 ml syringe attached to an 18 gauge needle and centrifuged at 3000 r.p.m. for 10 minutes for separation of the fluid from the cell fraction. Collected follicular fluid samples were kept at -20 ºC till used for biochemical and hormonal assays.

D. Ascorbic Acid Assay

Ascorbic acid level was determined according to the method of [21] using kits purchased from Biodiagnostic, Egypt.

E. NO Assay

Endogenous nitrite concentration (NO_2^-) as indicator of NO production in biological fluids was measured using nitrite assay kit provided by Biodiagnostic, Egypt according to [22].

F. Statistical Analysis

The differences among different seasons of the year were analyzed statistically by one-way ANOVA. The differences between follicular and luteal phases were analyzed by independent samples *t*-test using SPSS 16.0 for windows. Means were compared by the least significance difference at 5% level of probability.

III. Results

A. Effect of Seasonal Heat Stress and Stage of Estrous Cycle on Levels of Ascorbic Acid (mg/L) in Ovarian Follicular Fluid of Buffaloes

It is evident from values shown in Table I that the overall means of vitamin C levels in follicular fluid were significantly ($P < 0.05$) lower in summer (211.19±11.15 mg/L) than winter and spring (262.26±9.23 mg/L and 243.67±10.13 mg/L, respectively). Meanwhile, overall means of ascorbic acid concentrations were not significantly ($P < 0.05$) changed in both follicular and luteal phases (229.01±7.96 mg/L and 241.76±7.27 mg/L, respectively).

TABLE I
Effect of Seasonal Heat Stress and Stage of Estrous Cycle on Levels of Ascorbic Acid (mg/L) in Ovarian Follicular Fluid of Buffaloes

Groups		Season				Overall mean
		Summer	Autumn	Winter	Spring	
Phase	Follicular	213.13±14.02	235.99±15.55	236.39±10.65	239.78±18.30	229.01^{a}±7.96
	Luteal	209.25±17.68	252.56±12.56	271.57±11.49	230.56±12.72	241.76^{a}±7.27
Overall mean		211.19^{A}±11.15	243.67^{AB}±10.13	262.26^{B}±9.23	233.91^{B}±10.33	

-Data are presented as means ± SE.
-Means having different superscripts in the same column (a, b) differ significantly between follicular and luteal phases.
-Means having different superscripts in the same row (A, B, C) differ significantly between different seasons.
-N = 8 per group.

TABLE II
Effect of Seasonal Heat Stress and Stage of Estrous Cycle on Levels of NO (µmol / L) in Ovarian Follicular Fluid of Buffaloes

Groups		Season				Overall mean
		Summer	Autumn	Winter	Spring	
Phase	Follicular	375.83±9.18	353.57±4.99	346.25±9.43	331.19±4.13	353.54^{a}±4.02
	Luteal	365.81±7.67	362.58±4.28	339.95±3.12	332.54±4.38	349.53^{a}±3.07
Overall mean		372.12C±6.41	356.80B±3.58	341.13A±2.99	331.80A±2.96	

-Data are presented as means ± SE.
-Means having different superscripts in the same column (a, b) differ significantly between follicular and luteal phases.
-Means having different superscripts in the same row (A, B, C) differ significantly between different seasons.
-N = 8 per group.

B. Effect of Seasonal Heat Stress and Stage of Estrous Cycle on Levels of NO (µmol/L) in Ovarian Follicular Fluid of Buffaloes

It is clear from Table II that overall means of follicular fluid NO concentration were significantly ($P < 0.05$) higher in summer and autumn (372.12C±6.41 µmol/L and 356.80B±3.58 µmol/L, respectively) than winter and spring (341.13A±2.99 µmol/L and 331.80A±2.96 µmol/L, respectively). No significant ($P < 0.05$) changes were detected in the overall mean of NO levels during follicular phase as compared to that obtained during luteal phase (353.54^{a}±4.02 µmol/L and 349.53^{a}±3.07 µmol/L, respectively).

IV. Discussion

Summer heat stress is a major restriction in buffalo industry and can result in impairment of production and reproduction performance [1]. While summer heat stress has been demonstrated to be accompanied with elevated ROS markers in several studies [23]-[25], vitamin C is a part of the ovarian antioxidant system against ROS [26] which protects cells from the toxic effect of free radicals [27]. In the current work, summer heat stress significantly affected vitamin C levels in buffalo follicular fluid as in summer follicular fluid vitamin C was lower than winter and spring. On the other hand, the present study indicated that follicular fluid ascorbic acid did not differ significantly between follicular and luteal phases. Lower follicular fluid vitamin C in summer observed in this study could be attributed to the fact that heat stress during summer is one of the main reasons for lipid peroxidation which result from high production of free radicals and a decrease in antioxidant defense [28]. Ascorbic acid has been characterized as one of the anti-stress substances [29] and it is also known to ameliorate the oxidative stress generated during different physiological and pathological conditions [30]. Hussein et al. [27] linked the decrease in follicular fluid ascorbic acid concentration with increased free radicals and role of vitamin C in regeneration of oxidized α-tocopherol. Moreover, decreased ascorbic acid concentration during summer season than winter and spring could be related to the influence of cortisol on the synthesis of ascorbic acid [10]. The above mentioned findings coincided with those given by [12] who observed no statistically significant difference in follicular fluid vitamin C levels relative to the phases of the cycle. On the other hand, these results disagree with that recorded previously by [27] who reported that ascorbic acid concentration decreased significantly in follicles at the diestrus to help the corpus luteum function properly.

Heat stress can have deleterious effects on reproductive function in mammals as a result of either the associated hyperthermia or the physiological changes made by the heat-stressed animal trying to regulate body temperature [31]. In this study, follicular fluid NO concentration increased significantly in summer and autumn than winter and spring. Van Voorhis et al. [32] reported that summer anestrus may result from high levels of serum NO adversely affecting aromatase activity and consequently estradiol production in the growing follicles. NO is an important mediator involved in regulation of reproductive and immune functions in mammals [33]. However, it is important to be emphasized that physiological levels of NO supports the process of follicular development but pathologically higher levels disrupt the process [34]. According to [35], increase in level of NO to pathologic levels results in damage to cell structures leading to infertility. Khan and Das [36] proposed that anestrus and anovulation during reproductive acyclicity in buffalo are in part a result of reduced follicular estradiol due to inhibition of aromatase activity by greater NO levels and oxidative damage to follicles from imbalance of NO and ascorbic acid concentrations. Concerning the effect of estrous phase, there were non-significant changes in follicular fluid NO concentrations between both follicular and luteal phases. Quite the opposite, increased NO levels during the late luteal stage was reported in buffalo cow and this increase was related to the development and regression processes of the corpus luteum [37]. Dubey et al. [38] reported that higher concentrations of NO in buffalo primordial follicles donors exert cytotoxic effects whereas NO physiological levels are involved in their proper development. Quite the opposite, [13] found that NO concentration in follicular fluid of ewes was significantly higher in proestrus and estrus as compared to other stages of ovarian cycle. In conclusion, the present study indicated that a) The decrease of ascorbic acid during summer season could be related to that heat stress during summer is one of the main reasons for oxidative stress which result from free radicals increased production and a decrease in antioxidant defense, b) Physiological levels of NO are involved in proper follicular development, higher concentrations during summer might indicate adverse effects on ovarian follicles and c) Alterations in concentrations of follicular fluid vitamin C and NO that occur in summer could be related to reproductive seasonality in buffalo.

References

[1] S. H. Abdel-Aziem, L. M. Salem, M. S. Hassanane, K. F. Mahrous, "Genetic analysis between and within three Egyptian water buffalo populations using RAPD-PCR", J. Am. Sci., vol 6, 2010, pp 217-226.
[2] I. Soliman, H. Bassiony, "Role of buffalo in international trade", J. Agric. Sci. Technol., B1, 2011, pp 799-809.
[3] I. F. Marai, A. A. Habeeb, "Buffalo's biological functions as affected by heat stress", Livest. Sci., vol 12, 2010, pp 89-109.
[4] A. Kumar, S. Mehrotra, G. Singh, K. Narayanan, G. K. Das, Y. K. Soni, M. Singh, A. S. Mahla, N. Srivastava, M. R. Verma, "Sustained delivery of exogenous melatonin influences biomarkers of oxidative stress and total antioxidant capacity in summer-stressed anestrous water buffalo (*Bubalus bubalis*)", Theriogenology, vol 83, 2015, pp 1402–1407.
[5] M. M. Nour El-Din, "Proposed climate change adaptation strategy for the ministry of water resources & irrigation in Egypt". Joint programme for climate change risk management in Egypt.
[6] Payne, W.J.A., 1990. An Introduction to Animal Husbandry in the Tropics. Longman Scientific and Technical, England.
[7] R. S. Manik, P. Palta, S. K. Singla, V. Sharma, "Folliculogenesis in buffalo (*Bubalus bubalis*)". Reprod. Fertil. Dev., vol 14, 2002, pp 315-325.
[8] K. H. El-Shahat, M. Kandil, "Antioxidant capacity of follicular fluid in relation to follicular size and stage of estrous cycle in buffaloes", Theriogenology, vol 77, 2012, pp 1513-1518.
[9] J. L. Leroy, T. Vanholder, J. R. Delanghe, G. Opsomer, A. Van Soom, P. E. Bols, J. Dewulf, A. de Kruif, "Metabolic changes in follicular fluid of

the dominant follicle in high-yielding dairy cows early post partum", Theriogenology, vol 62, 2004, pp 1131-1143.
[10] H. H. Elkhidir, "Vitamin C status in Sudanese camels". PhD thesis. Utrecht Univ., 2002; 98 p.
[11] S. Nayyar, R. Jindal, "Essentiality of antioxidant vitamins for ruminants in relation to stress and reproduction", Iran. J. Vet. Res., vol 11, No. 1, Ser. No. 30, 2010.
[12] B. Serpek, N. Baspinar, S. Haliloglu, H. Erdem, "The relationship between ascorbic acid, oestradiol 17β and progesterone in plasma and in ovaries during the sexual cycle in cattle", Revue Méd. Vét., vol 152, 2001, pp 253-260.
[13] W. A. Razzaque, R. Ali, A. Hussain, M. Mutha Rao, S. Naafia, U. Sharma, K. Hussain, "Follicular fluid nitric oxide concentration in relation to stages of estrus cycle in ewes", Indian Vet. J., vol 91,2014, pp 35 – 38.
[14] G. O. Zamberlam, "The involvement of nitric oxide in bovine follicular development and ovulation", PhD Thesis, 2013, Université de Montréal.
[15] A. Jablonka-Shariff, L. M. Olson, "Nitric oxide is essential for optimal meiotic maturation of murine cumulus-oocyte complexes in vitro", Mol. Reprod. Dev., 2000, vol 55, pp 412-421.
[16] K. M. Dhandapani, D. W. Brener, "The role of glutamate and nitric oxide in the reproductive neuroendocrine system", Biochem. Cell Biol., 2000, vol 78, pp 165-179.
[17] K. R. L. Schwarz, R. L. Pedro, Pires, Paulo, R., Adona, T. H., Camara de Bem., Claudia, L. and Leal, V., "Influence of nitric oxide during maturation on bovine oocyte meiosis and embryo development in vitro", Reprod. Fertil. Dev., vol 20, 2008, pp 529-536.
[18] A. Revelli, L. D. Piane, S. Casano, E. Molinari, M. Massobrio, P. Rinaudo, "Follicular fluid content and oocyte quality: from single biochemical markers to metabolomic", Reprod. Biol. Endocrinol., 2009, 7:40.
[19] S. Mondal, V. Kumar, I.J. Reddy, S. Khub, "Progesterone and nucleic acid content of buffalo corpus luteum in relation to stages of estrus cycle". Indian J. Anim. Sci., 2004, vol 74, pp 710–712.
[20] P. Jaglan, G.K. Das, B.V. Sunil Kumar, R. Kumar, F.A. Khan, S.K. Meur, "Cyclical changes in collagen concentration in relation to growth and development of buffalo corpus luteum", Vet. Res. Commun., 2010, vol 34, pp 511–518.
[21] L. J. Harris, S. N. Ray, Lancet. 1935, vol 1, pp 71- 462
[22] H. A. C. Montgomery, J. F. Dymock, Analyst,1961, vol 86, pp 414.
[23] M. Tanaka, Y. Kamiya, M. Kamiya, Y. Nakai, "Effect of high environmental temperatures on ascorbic acid, sulfhydryl residue and oxidized lipid concentrations in plasma of dairy cows," Anim. Sci. J., 2007, vol. 78, no. 3, pp 301–306.
[24] G. A. Megahed, M. M. Anwar, S. I. Wasfy, M. E. Hammadeh, "Influence of heat stress on the cortisol and oxidant antioxidants balance during oestrous phase in buffalo-cows (Bubalus bubalis): thermo-protective role of antioxidant treatment", Reprod. Domest. Anim., 2008, vol. 43,no. 6, pp.672– 677.
[25] M. Sakatani, A. Z. Balboula, K. Yamanaka, and M. Takahashi, "Effect of summer heat environment on body temperature, estrous cycles and blood antioxidant levels in Japanese black cow", Anim. Sci. J., 2012, vol. 83, no. 5, pp. 394–402.
[26] F. H. Thomas, R. Leask, V. Srsen, S. C. Riley, N. Spears, E. E. Telfer, "Effect of ascorbic acid on health and morphology of bovine preantral follicles during long-term culture", Reproduction, vol 122, 2001, pp 487–495.
[27] H. A. Hussein, M. R. Abd Ellah, D. R. I. Derar, "Antioxidant concentrations in serum, follicular fluid, and corpus luteum of cyclic buffalo cows", Comp. Clin. Pathol., vol 22, 2013, pp 829–833.
[28] H. F. Hozyen, H. A. Hodallah, G. E. S. Essawy, S. I. A. Shalaby, "Seasonal changes in some oxidant and antioxidant parameters during folliculogenesis in Egyptian buffalo", Anim. Reprod. Sci., vol 151, 2014, pp 131-136.
[29] O. S. Afify, M. N. Makled, "Effect of ascorbic acid on productive and reproductive performance of Bouscat rabbits exposed to heat stress", First Egyptian-Hungarian Conference. Alexandria, Egypt. 17-19 September 1996, pp. 313.
[30] S. Nayyar, R. Jindal, "Essentiality of antioxidant vitamins for ruminants in relation to stress and reproduction", Iran. J. Vet. Res., 2010, vol. 11, no. 1, Ser. no. 30.
[31] P. J. Hansen, "Effects of heat stress on mammalian reproduction", Phil. Trans. R. Soc. B, vol 364, 2009, 3341-3350.
[32] B. I. Van Voorhis, M. S. Dunn, G. D. Synder, C. P. Weiner, "Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis", Endocrinology, vol 135, 1994, pp1799-806.
[33] S. Naafia, W. A. A. Razzaque, M. Mutha Rao, and K. Hussain, "Nitric oxide profiles in serum and follicular fluid of cyclic and acyclic sheep", J. Res. Dev., 2011, vol. 11, pp 199-128.
[34] M. Rosselli, P. J. Keller, R. K. Dubey, "Role of nitric oxide in the biology, physiology and pathophysiology of reproduction", Hum. Reprod. Update, 1998, vol 4, pp 3-24.
[35] H. S. Lucky, S. Gupta, Y. Kim, A. Agarwal, "Female infertility and antioxidants", Curr. Womens Health Rev., vol 6, 2010, 84-95.
[36] F. A. Khan, G. K. Das., "Follicular characteristics and intrafollicular concentrations of nitric oxide and ascorbic acid during ovarian acyclicity in water buffalo (Bubalus bubalis)". Trop. Anim. Health Prod., 2012, vol 44, pp 125-131.
[37] A. B. Motta, A. Estevez, M. F. de Gimeno, "The involvement of nitric oxide in corpus luteum regression in the rat: feedback mechanism between prostaglandin F(2alpha) and nitric oxide". Mol. Hum. Reprod., 1999, vol 5, no 11, pp 1011-1016.
[38] P. K. Dubey, V. Tripathi, R. P. Singh, G. T.Sharma, "Influence of nitric oxide on in vitro growth, survival, steroidogenesis, and apoptosis of follicle stimulating hormone stimulated buffalo (Bubalus bubalis) preantral follicles". J. Vet. Sci., 2011, vol 12, no 3, pp 257-265.