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EFFECT OF SEASONS ON OOCYTES QUALITY AND IN VITRO MATURATION RATE IN BUFFALO

By

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Short running title: Effect of season and antioxidants on buffalo oocyte.

ABSTRACT

Heat stress (HS) during summer was found to impair the reproductive potentials in buffalo. The present study was designed to evaluate the effect of seasons on: a) Number of the ovarian follicles and number and quality of their enclosing oocytes, b) Cumulus cell expansion and nuclear maturation rates of in vitro matured buffalo oocytes In experiment 1: During whole year, buffalo ovaries were collected and the number of ovarian follicles was counted, oocytes were recovered/ovary and classified according to their morphology into 4 Grades. In Experiment 2: Grade 1 and 2 cumulus-oocyte-complexes (COCs) were in vitro matured (IVM), cumulus-cell expansion and nuclear maturation were determined. In Experiment 3: Mitochondrial activities in non-matured and matured buffalo oocytes were assayed using Mito-Tracker Red stain. Results showed that the number of ovarian follicles and the quality of oocytes were diminished in summer. Also, summer season significantly ($P<0.05$) decreased cumulus-cell expansion and nuclear maturation of IVM buffalo oocytes. Matured buffalo expressed clustering homogenous distribution, while, non-matured ones showed polarized mitochondrial distribution.

Keywords:

Season, ovarian function, in vitro maturation, mitochondrial distribution.

INTRODUCTION

Nowadays, Global warming may be a common problem touching largely the livestock population and inflicting severe economic losses worldwide. Buffaloes play an important role in agriculture economy in several countries like Egypt. They are used for supplying meat and milk. However, reproductive performance in buffalo is low compared to cattle. Delayed puberty, silent estrus, low conception rate, long inter-calving interval are among the foremost factors affecting reproduction in buffalo. Moreover, buffaloes are more sensitive to heat stress (HS) throughout summer because of their specific peculiarities like the black coloration of the skin and also the low number of sweat glands. HS was reported to decrease number of natural follicles and also the number of good quality oocytes in buffalo ovaries **(Abdoon *et al.,* 2001**; **Abdoon 2014**; **Soliman 2016)**. HS during hot seasons induces a rise in the reactive oxygen species (ROS) levels that are induced harmful effects on the integrity and functions of gametes **(Zoheir *et al.,*2007**).Higher levels of ROS above the physiological level might result in oxidative stress (OS) and might resulted in deterioration in oocyte quality and thereby have an effect on reproductive outcomes **(Hansen *et al.,*2001)**. Most bovine oocytes that matured under heat-shock conditions remained at the germinal vesicle breakdown (GVBD) stage **(Chaube *et al.,*2014)** and showed aberrant chromatin configuration. OS caused by HS within the oviduct, may presumably be involved in the heat stress induced early embryonic death **(Gharibzadeh *et al.,* 2015).** Also, HS impaired the developmental competence of buffalo oocytes through the alteration of *HSP70* gene expression **(Al-Katanani *et al.,* 2002)**.

Therefore, the present study was carried out to investigate the effect of seasons on the ovarian function in buffalo. This includes: 1) The effect of season on number of ovarian follicles, oocytes and their quality; 2) The effect of season on oocytes cytoplasmic and nuclear maturation rates; and 3) Mitochondrial distribution in mature metaphase II or non-mature buffalo oocytes.

MATERIAL AND METHODS

Unless otherwise mentioned, all chemicals used in the present study were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Statement of Animal Rights:

All animal studies in the present work were conducted in accordance with the requirements of the Institutional Animal Care Committee and were reviewed and approved by the Animal Ethics Committee of the National Research Centre of Egypt (NRC: 12/1/7).

Experimental procedures:

The present work was conducted at the Department of Animal Reproduction and Artificial Insemination, Research Division, National Research Centre, Egypt.

Experiment 1: The experiment was conducted during the period extended from Jan., 2017 to Jan., 2018. Ovaries of buffaloes were collected at the local slaughterhouse of Giza province and transported in thermos contains warm normal saline to the laboratory within 2 hours. At the laboratory, the extra tissues were removed and the ovaries were washed several times in warm normal saline supplemented with 50 µg/ml gentamycin. The number of ovarian follicles per ovary was counted and then the cumulus-oocytes-complexes (COCs) were aspirated from follicles 3 to 8 mm in diameter using 18-gauge needle attached to 10 ml disposable syringe. According to their morphology (number of cumulus cell layers and the homogeneity of the cytoplasm) COC were classified into 4 Grades as adopted 1). Grade 1, COCs surrounded by more than 5 layers of cumulus cells and with homogenous cytoplasm Fig. (1A); Grade 2, oocytes surrounded by 2 to 4 layers of cumulus cells and with homogenous cytoplasm (Fig. 1B); Grade 3, oocytes surrounded by one layer of cumulus cells Fig. (1C); and Grade 4, denuded oocytes Fig. (1D). COCs with expanded cumulus cells were excluded from the study.

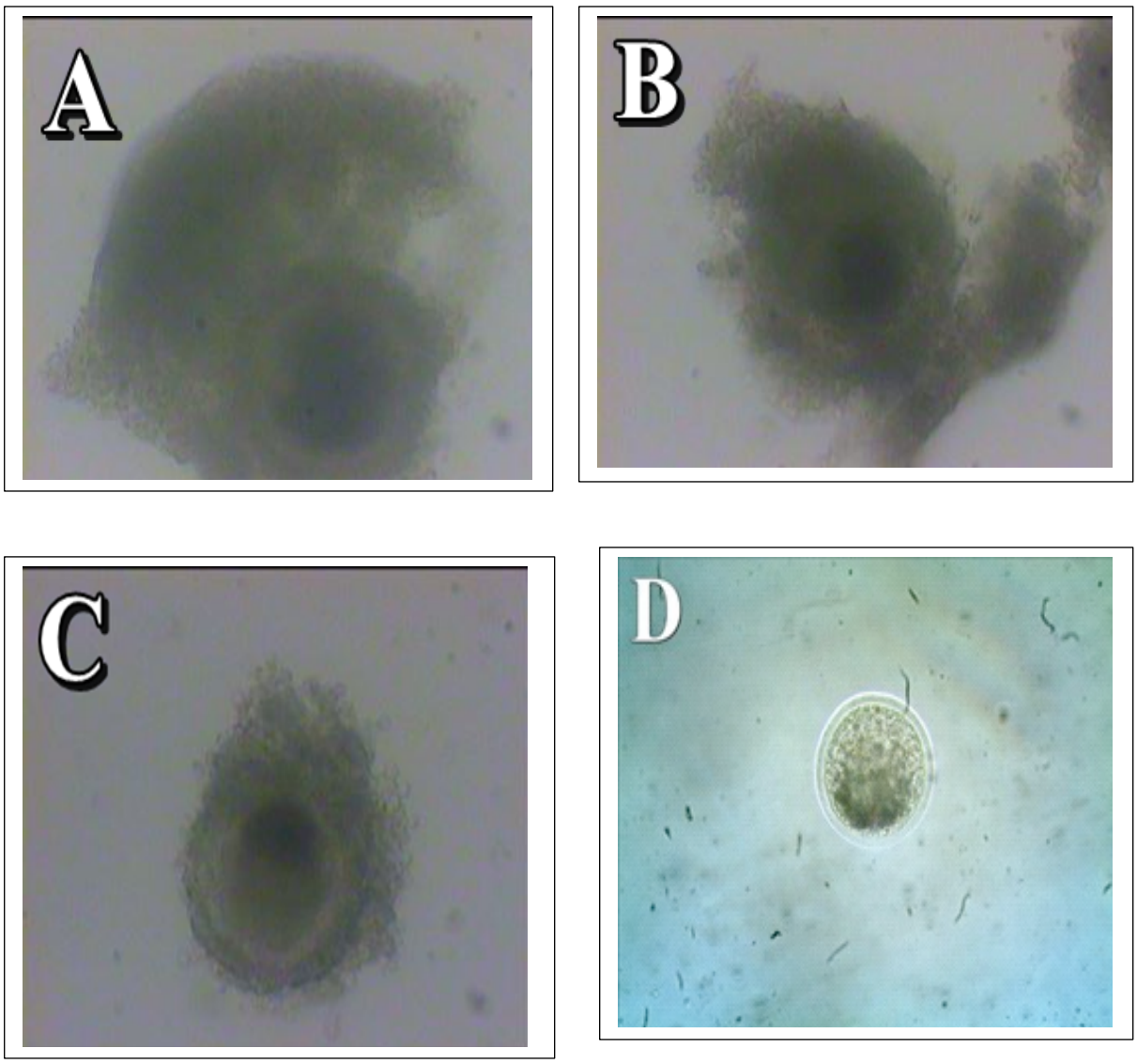

Fig.(1): Photomicrograph showing the different Grades of buffalo oocyte qulaity; A) Excellent quality COCs with multiple layers oc cumulus cells; B) Buffalo COCs with 2-4 - layers of cumulus cells; C) Buffalo COCs with single layer of cumulus cells; and D) Denuded oocyte (x 100).

Experiment 2:

Effect of season on buffalo oocytes cytoplasmic and nuclear maturation rates in vitro Buffalo's ovaries were collected and processed as in experiment (1) during the period from Feb 2018 to Feb 2019. After collection, COCs were washed at least 3 times in PBS supplemented with 4 mg/ml bovine serum albumin +50 µg/ml gentamycin then washed at least 2 times in IVM basic medium (BMM) which consists of TCM-199 medium supplemented with 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulating hormone (p-FSH) + 50 IU equine chorionic gonadotropin (eCG, Intervet, The Netherlands) + 50 µg/ml gentamycin (Control group).

Twenty-five to fifty COCs were cultured in 500 µl of IVN basic medium in 4-well culture plate (Nunc, Denmark). Oocytes were washed three times with PBS then one time in IVM medium and incubated for 22-24 hours under 5% CO_2 + 20% O_2 (Contherm CO_2 incubator, New Zealand).

Assessment of cytoplasmic maturation as indicated by cumulus cell expansion:

After maturation, the degree of cumulus cells expansion was recorded according to (**Abdoon *et al.*, 2014):** as follows:

Grade zero (G0): with no expansion; **Grade one (G1):** with slight expansion in the outer layer of cumulus-cells; **Grade two (G2):** with moderate expansion; and **Grade three (G3):** with full expansion.

Assessment of nuclear maturation by extrusion of first polar body according to Nandi *et al.*, (2002):

To assess the polar body of the metaphase II (MII) oocyte, the samples were collected after 44 h of maturation. The cumulus cells were removed by gently pipetting and examined with an inverted microscope (Axiom Vert1, Zeiss, Germany). The number of oocytes extruding the 1st polar body was considered MII oocytes and the oocytes without polar body were considered non-matured oocytes.

Experiment 3:

Evaluation of mitochondrial distribution in the IVM buffalo oocytes according to Gaviria *et al.*, (2019):

After in vitro maturation, about 30 oocytes were selected (immature cultured or M II oocytes) and incubated in TCM-199 supplemented with 12.5 µmol/L MitoTracker Red CMRox (Life Technologies, Grand Island, NY, USA) at 37 °C under 5% CO_2 in air for 30 min. The stained oocytes were washed twice in PBS, mounted on a slide and covered with a cover slip, and examined under confocal laser scanning microscope (Zeiss 710, Germany). The images of oocytes were captured with a digital camera. The mitochondrial distribution pattern of oocytes was characterized according to their labeled distribution within the ooplasm.

Statistical analysis:

Data were expressed as the Mean ± SEM. Statistical analyses were performed using Analysis of Variance (ANOVA) with the aid of SPSS 20.0 statistical software. $P < 0.05$ was considered highly significant.

RESULTS

Effect of season on the number of ovarian follicles:

The number of oocytes recovered per ovary and the quality of the recovered oocytes in buffalo are presented in (Table 1). The results revealed that summer season significantly (P<0.05) decreased the number of ovarian follicles in buffalo ovaries when compared with winter, autumn or spring seasons. Also, the percentage of excellent and good quality oocyte retrieved from buffalo ovaries during summer was significantly (P<0.05) lower than that retrieved in the other seasons. On the other hand, the percentage of fair quality and denuded oocytes was higher (P<0.05) during summer than winter, autumn or spring seasons. Total number of oocytes recovered per ovary was not affected by seasons.

Table (1): Effect of season on the number of ovarian follicles, number and quality of oocytes in buffalo ovaries (Mean ± SEM).

Season	No. of ovaries	No. of follicles	Follicle /ovary	No. of oocytes	Oocyte /ovary	Oocytes quality (%)			
						Excellent	Good	Fair	Denuded
Winter	257	1093	4.2±0.2^{a}	766	2.9±0.2	38.8±1.5^{a}	28.1±1.8^{a}	15.8±1.3^{b}	13.4±2.0^{b}
Autumn	245	912	3.8±0.2^{a}	656	2.7±0.1	36.7±2.8^{a}	23.6±1.6^{a}	21.9±1.5^{b}	17.8±1.1^{b}
Spring	234	852	3.7±0.1^{a}	594	2.5±0.2	40.4±2.1^{a}	25.2±2.0^{a}	17.8±1.6^{b}	16.6±0.9^{b}
Summer	279	931	3.4±0.1^{b}	684	2.5±0.2	18.0±2.3^{b}	17.6±2.9^{b}	24.8±3.5^{a}	40.4±5.1^{a}

a, b Differ significantly within the same column at P<0.05.

Table (2) represents the effect of season on cumulus cell expansion of the in vitro matured buffalo oocytes. The results indicated that G0 (no cumulus cell expansion) was significantly (P<0.05) higher for buffalo oocytes in vitro matured during summer season when compared with winter, spring or autumn. Buffalo oocytes with slight cumulus cell expansion (G1) was significantly lower (P<0.05) for buffalo oocytes IVM during spring season compared with the other seasons. G2 cumulus cell expansion was significantly (P<0.05) higher for buffalo oocytes IVM during spring and autumn compared with winter and summer. Moreover, full cumulus cell expansion (G3) was significantly (P <0.05) higher for buffalo oocytes IVM during spring compared to those oocytes matured during autumn, winter or summer. The lowest (P<0.05) percentage of G3 was recorded in buffalo oocytes IVM during summer. The effect of season on the percentage of the in vitro matured buffalo oocytes reaching the

MII stage is demonstrated in (Table 2). Data indicates that the lowest ($P<0.05$) percentage of oocytes reaching MII stage, and the highest ($P<0.05$) percentage of non-matured oocytes was recorded for buffalo oocytes IVM during summer when compared with winter, autumn or spring seasons.

Table (2): Effect of season on cumulus cell expansion and nuclear maturation rate of in vitro mature buffalo oocytes (Mean ± SEM).

Season	No. COCs	Degree of cumulus cell expansion (%)				Maturation rate (%)	
		G0	G1	G2	G3	M II	Non-matured
Winter	**274**	**21.3±1.9^{b}**	**20.3±1.7^{a}**	**19.9±1.6^{b}**	**38.5±2.0^{b}**	**56.2±3.7^{a}**	**43.8±3.7^{b}**
Autumn	**256**	**16.8±1.0^{b}**	**21.3±0.8^{a}**	**25.9±0.7^{a}**	**36.0±1.0^{b}**	**50.0±1.7^{a}**	**50.0±1.7^{b}**
Spring	**282**	**9.0±1.5^{b}**	**14.3±1.7^{b}**	**27.6±2.0^{a}**	**49.1±3.7^{a}**	**53.8±2.3^{a}**	**46.2±2.3^{b}**
Summer	**268**	**40.6±2.9^{a}**	**22.6±2.6^{a}**	**16.9±1.0^{b}**	**22.0±1.3^{b}**	**35.9±1.2^{b}**	**64.1±1.2^{a}**

a, b Differ significantly within the same column at $P<0.05$.

Evaluation of mitochondrial distribution in the MII and non-matured buffalo oocytes.

Mitochondrial distributions in non-matured or matured IVM buffalo oocytes are presented in Fig. (2). The results showed that, after in vitro maturation of buffalo oocytes, the n on-matured group showed polarized mitochondrial distribution at the periphery of the cytoplasm Fig.(2A). While, matured buffalo oocytes reaching MII stage showed homogenous mitochondrial distribution throughout the cytoplasm Fig. (2B).

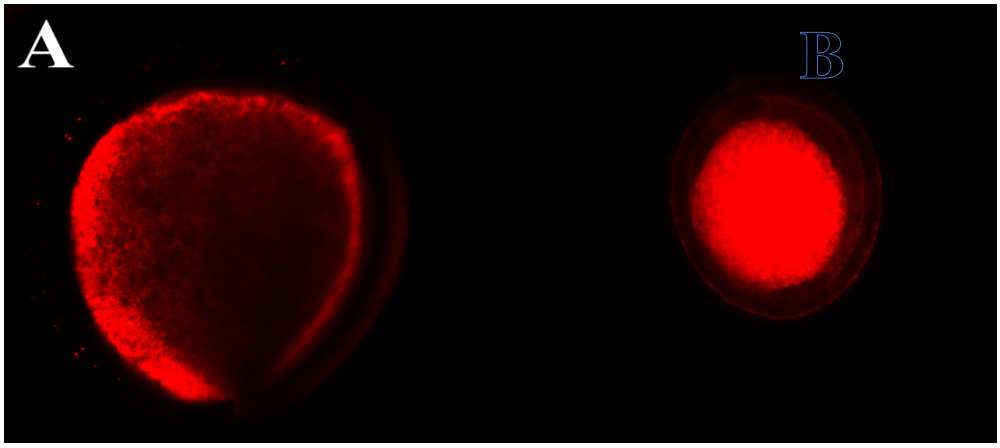

Fig. (2): A,B : Photomicrograph showing the pattern of mitochondrial distribution in IVM buffalo oocytes; A) non-matured buffalo oocytes with polarized mitochondrial distribution; B) matured buffalo ooctres with homogenous distribution throughout the cytoplasm.

DISCUSSION

In the present study HS during summer season was found to decrease the reproductive potentials in buffalo by altering their ovarian function. Number of ovarian follicles was significantly lower ($P<0.05$) in summer than in winter, autumn and spring seasons.

Those results are concurrent with that previously recorded in buffalos **(Abdoon *et al.,* 2001**; **Abdoon *et al.,* 2014**; **Gharibzadeh *et al.,* 2015**) and cattle **(Al-Katanani *et al.,* 2002**; **Roth *et al.,* 2000**). Early astral follicles of 0.5-1.0 mm in diameter were reported to be more sensitive to heat stress in bovine **(Roth *et al.,* 2000)**. HS during summer also was found to inhibit ovarian follicular development leading to diminished reproductive efficiency of dairy cows **(Li *et al.,* 2016).** In the goats, recruited of follicles during heat stress was regressed and never developed to large size or ovulate **(Paes *et al.,*2016)**. Heat stress was also found to induce changes, which include the number of ovarian follicles and the follicular environment, resulting in morphologically damaged oocytes **(Alves *et al.,* 2014, Pandey *et al.,* 2014)**. This could be attributed to the inhibition of the ovarian follicles development due to the hyper-prolactinemia produced as a result of heat stress which in turn inhibits the secretion of both FSH and LH at the hypophyseal level **(Pandey *et al.,* 2014)**. On the contrary, other studies reported that HS increased the number of bovine small ovarian follicles **(Trout *et al.,* 1988)**, while, HS had no effect on the number of bovine oocytes recovered per ovary **(Wilson *et al.,* 1998)**, and had no effect on the number of buffalo oocytes recovered per ovary

(Di Francesco *et al.,* 2010). These discrepancies could be related to the time and duration or severity of heat stress during summer, localities, or differences in genetic susceptibility to HS. In the current study, number of retrieved oocytes during summer was slightly lower but non- significantly differ than the other season. However, HS during summer had a deleterious effect on buffalo oocytes quality. In summer the percentages of excellent and good quality oocytes were significantly lower than in other seasons, while the percentages of fair and poor-quality oocytes were significantly higher during summer than those obtained during the other seasons. These findings are concomitant with previous reports in which HS during summer adversely affect the number of oocytes recovered in buffalo **(Abdoon*et al.,*2001**; **Gharibzadeh *et al.,* 2015**). Similarly, HS has a deleterious effect on follicular development and follicular fluid contents which was directly reflected on the bovine oocyte quality and affected its developmental competence **(Matsuzuka *et al.,* 2005)**.

Furthermore, in the present study, IVM of buffalo oocytes during summer season was significantly ($P<0.05$) decreased the cumulus cell expansion and their nuclear maturation as indicated from the percentages of matured oocytes reaching the MII stage. These results are in accordance with an earlier study, in which, IVM of bovine COCs at 41ºC for 24 h showed altered function, reduced cumulus-cell hyaluronic acid production, and impede meiosis resumption **(Lenz *et al.,* 1983)**. Moreover, HS during summer was found to impair both nuclear and cytoplasmic maturation of bovine oocytes and decrease the translocation of cortical granules to the lemma **(Payton *et al.,* 2004)**, cytoskeleton rearrangement **(Roth *et al.,* 2005]**, and spindle formation

(Ju *et al.,* 2005). Such alterations may potentially lead to incomplete nuclear maturation **(Payton *et al.,* 2004)**

The present results revealed that maturation of buffalo oocytes as indicated by the presence of 1st polar body was associated with clustering of mitochondrial distribution throughout the cytoplasm. Meanwhile, non-matured group showed low mitochondrial distribution and polarized mitochondrial distribution throughout the cytoplasm. HS increased the production of ROS and peripheral polarization of the mitochondria instead of homogeneous distribution which leads to reduced developmental competence of bovine oocytes **(Paes *et al.,* 2016**).

CONCLUSION

HS during summer season decreases the number ovarian follicle, number of excellent and good quality oocytes and retards cumulus cell expansion and nuclear maturation in vitro. Maturation of buffalo oocytes was associated with clustering and homogenous mitochondrial distribution throughout the cytoplasm.

Conflict of interest:

Authors declare that there is no conflict of interest

Acknowledgements:

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