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EFFECT OF TYPE OF OVARIAN ANTRAL FOLLICLES ON OOCYTE QUALITY AND *IN VITRO* OOCYTE MATURATION IN BUFFALOES

By

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ABSTRRACT

The present study aimed to investigate the effect of ovarian antral follicle type (AF) on oocyte quality and oocyte maturation in vitro (IVM) and its relationship to some components of follicular fluid in buffaloes. Buffalo ovaries were collected from slaughterhouse.

Ovarian follicles were classified into two types. Type (1) antral follicles of ≥3mm in diameter (AF) and type (2) small antral follicles of < 3mm in diameter (small AF). Oocytes were aspirated from each type of follicles then it were classified into good oocytes embedded within compact cumulus cells and having granulating ooplasm and fair oocytes showing only a few layers of cumulus cells and poor oocytes (denuded oocytes and those with a pyknotic ooplasm). Oocytes were matured in a defined basic maturation medium **(TCM 199).** The results indicated that, the percentage of good quality oocytes was higher with oocytes which aspirated from type 1 (61.68%) than those aspirated from type 2 (28.32%).

The percentages of fair and poor quality oocytes were higher in type 2 (33.63 % and 38.05 %) than those with type 1 (26.51% and11.81 %), respectively and the differences were significant (P≤0.001). The percentage of oocyte maturation (reaching metaphase II (M II) within 21-24 h culturing time was higher with oocytes obtained from type 1 (75.00%) than those (42.48%) that were obtained from type 2 and differences were significant (P≤0.001).Chemical analysis of some follicular fluid components (Glutathione, malondialdehyde (MDA), zinc and Selenium) were 4.5 mmol/L, 1.76 mmol/L, 0.27 mg/dl and 5.35 mg/dl in follicular fluid of AF and 1.52 mmol/L, 3.89 mmol/L, 0.16 mg/dl and 2.78 mg/dl in follicular fluid of small AF, respectively and the differences were significant(p≤ 0.05). In conclusion, AF has an important role in oocyte quality and oocyte maturation rate. Besides high concentration of follicular fluid of glutathione, zinc and selenium was related to AF and high concentration of follicular fluid MDA was related to small AF.

Keywords:

Buffalo oocytes, anral follicle, oocyte maturation, oocyte quality, metaphase II.

INTRODUCTION

With increasing of the world population and limiting of meat and milk production because of the limitation of number of offspring produced by each animal. So that using reproductive technology like induction of supper ovulation, *in vitro* fertilization and embryo transfer farmers will be able to increase the number of offspring produced by genetically superior parents. Oocyte maturation involves changes at the nuclear cytoplasmic level that render the oocyte capable of undergoing fertilization and embryo development. In *vitro* maturation of oocytes (IVM) is the first step in an *in vitro* embryo production (IVP) which is considered as one of the most important aspects of modern science **(Davachi *et al.,* 2011).** During culture period, oocytes undergo series of cytoplasmic changes before the resumption of nuclear maturation leading to a variable competence of the resulting embryo **(Moor *et al.,* 1990).** The synthesis and storage of certain forms of m RNA and protein during IVM and early embryonic development are necessary **(Thibault *et al.,* 1987).** Oocyte quality, alteration of basic maturation conditions can significantly affect oocytes competence as reflected by the morula and blastocyst yield after *in vitro* fertilization **(Rose and Bavister, 1992).** The present study aimed to investigate the effect of antral follicles and some component of follicular fluid on oocyte quality and in vitro oocyte maturation in buffaloes.

MATERIAL AND METHODS

Collection of ovaries and follicular fluid:

Ovaries of buffaloes were collected from the slaughterhouse in several times along one year. The collected ovaries were immediately transported to the laboratory preserved in thermos flask with saline solution (physiological solution- 0.9% NaCl) containing 100000 IU/L penicillin and 100 mg/L streptomycin at 30 - 35 ^{o}C. Ovarian follicles were classified into two types 1- antral follicles with ≥3mm in diameter (AF), type 2- small antral follicles < 3mm in diameter (small AF) **Palanisamy *et al* (2013).** Oocytes and follicular fluid were collected by aspiration method using a 10-ml syringe with 21- g needle.

Samples of follicular fluids were collected from different types of follicles (follicles' of ≥3mm in diameter and small ones with < 3mm in diameter), and centrifuged at 3000 rpm for 15 minutes and were kept frozen at - 20 ^{o}C until analysis.

Oocyte preparation and in vitro maturation:

The oocytes were aspirated from each type of follicles and classified by using stereomicroscope into 1) good oocytes which embedded within compact cumulus cells and

having granulating ooplasm, 2) fair oocytes with only a few layers of cumulus cells and 3) poor oocytes (denuded oocytes and those with a pyknotic ooplasm **(Wani *et al*.,2000).**

Oocytes were cultured in maturation media TCM 199 (Sigma- Aldrich), supplemented with 10 % heat inactivated fetal calf serum, 10 i.u /ml LH (Pregnyl- Nile Co. A. R. E.), 5i.u/ml FSH (Folligon, intervit), 1µg/ml Estradiol- 17β (E_2), sodium pyruvate. Oocytes were placed in a 50 µl droplet of maturation medium (TCM 199), each group of 10 oocytes was kept in one droplet under 9 ml mineral oil in petri dishes and were statically cultured for 21-24h at 38.5 ^oC under 5% CO_2 in air and 95% humidity.

Oocytes were transferred into micro centrifuge tube containing 500 µl of HEPES buffered tirade's medium (TLH) and vortex - agitated for 4 - 5 min to remove the cumulus cells. The denuded oocytes (without cumulus cells) were transferred to a glass slide in a small drop using a stereomicroscope.Mixture of Vaseline:paraffin wax was used for preventing coverslip to be in contact with the oocytes. The slides were placed in mixture of ethanol: acetic acid (3:1) for at least 24 h before staining with 1% aceto - orcein (Sigma- Aldrich) and were examined for nuclear morphology using a phase contrast microscope at 400-x magnification **(Abbas *et al.,* 1998).** After fixation and staining, oocytes were examined microscopically for the presence of metaphase II (M II) stage and percentage of oocytes which reaching M II represented the maturation rate.

Experimental design:

Experiment 1: to examined the effect of type of ovarian antral follicle on quality and development of buffalo oocytes. In the experiment, oocytes, which obtained from each type of follicles were counted and classified into three types of oocytes (good, fair and poor) as mentioned above. The oocytes from these two types of follicles were cultured in maturation media TCM 199 supplemented with 10 % v/v heat inactivated fetal calf serum, 10 I.U. /ml LH, 1µg/ml E_2 and sodium pyruvate.

Experiment 2: to study the relationship between some component of follicular fluid and ovarian antral follicles. Samples in the experiment of follicular fluid were collected from different types of follicles, and centrifuged at 3000 rpm for 15 minutes and were kept frozen at - 20^oC until analysis. Some biochemical components of follicular fluid were determined such as Glutathione (mmol/L), MDA (mmol/L), zinc (mg/d), and Selenium mg/dl in the follicular fluid samples.

Statistical analysis:

Data from five replicates were analyzed according to SAS (2002) by means of Chi- square. Data of chemical analysis were expressed as mean ± SEM. Data were analyzed by ANOVA using SAS program. Means differences were evaluated among groups by Duncan's multiple range test **(Duncan, 1955)** in the experiment 2.

RESULTS AND DISCUSSION

The results are summarized as following:

1) Effect of ovarian antral follicle on oocyte quality: in (Table 1), the results indicated that, the percentage of good quality oocytes was higher with oocytes that aspirated from type 1 (61.68%) than those aspirated from type 2 (28.32%). The percentages of fair and poor quality oocytes were higher with type 2 (33.63 % and 38.05 %) than those with type 1 (26.51 % and 11.81%),respectively and the differences were significant (P≤0.001).These results demonstrated that as the size of the follicles increases/follicle grows, the quality of the oocyte also increases which agree with the results of **Palanisamy *et al* (2013).** In human **Hsu *et al.,* (2010)** reported that oocyte quantity and quality measured by AF.

Table (1): Effect of type of ovarian antral follicles on oocyte quality.

Ovarian follicle type in diameter	Oocyte number	Oocyte quality					
		Good oocytes		Fair oocytes		Poor oocytes	
		(n)	(%)	(n)	(%)	(n)	(%)
Follicles ≥3mm	728	449	61.68	193	26.51	86	11.81
Small follicles <3mm	113	28	28.32	38	33.63	43	38.05

Table represents number (n) and percentage (%) of each type of oocyte quality. (P≤0.001).

(2) Effect of ovarian antral follicles (AF) on oocyte maturation rate in vitro is represented in (Table 2). The results revealed that the percentage of oocyte maturation rate was higher with the oocytes which aspirated from AF (75.00%) than those aspirated from small AF (42.48%), respectively and the differences in maturation rate among the two groups were significant (P≤0.001). In human **Alaina Vrontikis *et al.,* (2010)** reported that AFsignificantly correlated with total and MII oocyte. **Satheshkumar *et al.,* (2016) and Amer *et al.,*(2008)** demonstrated that a great majority of the oocytes from follicles (more than 3 mm in diameter) reached the MII stage and developed to blastocysts **i**ndicating perfect completion of cytoplasmic/nuclear

maturation. **Katsuhisa *et al.,* (2016)** suggested that, the mitochondrial activity of oocytes before maturation in the high- AF group was higher than that in the low- AF group.

Table (2): Effect of type of ovarian antral follicles on oocyte maturation.

Ovarian follicle type in diameter	Oocyte number	Oocyte maturation									
		GV		GVBD		MI		MII		DEG	
		(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)
Follicles ≥3mm	728	34	4.67	60	8.24	68	9.34	546	75.0	20	2.70
Small follicles <3mm	113	17	15.04	20	17.70	18	15.93	48	42.48	10	8.85

GV= Germinal vesicle, GVBD = Germinal vesicle brake down, M I = Metaphase I, M II = Metaphase II and DEG = Degeneration.

Table represents number (n) and percentage (%) of oocytes in each stage of maturation.

(P≤0.001).

(3) The results of follicular fluid samples are represented in (Table 3) Present results indicated that: (1) follicular fluid glutathione level with AF was higher than small AF, which represent 4.5 and 1.52 mmol/L, respectively and the differences in follicular fluid glutathione concentration was significant between AF and small AF ($P \leq 0.05$).**Pankaj A.Patel *et al.,* (2015)** reported that glutathione plays an important role in protecting the cell or embryos from oxidative damage. (2) Follicular fluid MDA concentration was lower in AF than small AF, which represent 1.76 and 3.89 mmol/L respectively and the differences of MDA level between these different types of follicles was significant ($P \leq 0.05$). In human, **Liu *et al.* (2010)** investigated that a significant positive correlation between follicular fluid MDA level and incidence of apoptosis in granulosa cells and subsequently lower oocyte quality and lead to poor outcome of IVF-ET. Beside that in cows lipid peroxidation is a well - established mechanism of oxidative damage caused by reactive oxygen species (ROS), and the measurement of the MDA provides a convenient index of lipid peroxidation **(Heidarpour *et al.,* 2012).** (3) Follicular fluid Zinc concentration was found in higher level with AF than with small AF, which represent 0.27 and 0.16 mg/dl respectively and the differences in zinc, concentration was significant between each other type of follicles ($P \leq 0.05$). The role of zinc andcopperindestruction of free radicals,the oxidative stress (decreased serum total antioxidant

status and increased serum MDA **(Heidarpour *et al.*, 2012).** (4) Follicular fluid Selenium level was higher with AF than with small AFwhich represent 5.35 and 2.78 mg/dl respectively and the differences of selenium concentration was significant ($P \leq 0.05$) between follicular fluid samples related to AF than small AF. Selenium along with Vitamin E function as preventive and chain breaking antioxidant, and inactivates peroxidase formed during cell metabolic process **(Sudhir Kumar *et al*., 2011).**

Table (3): Relationship between some components of follicular fluid and type of ovarian antral follicles

Follicular fluid component	Type of follicles in diameter	
	Follicles ≥3mm	Small follicles <3mm
Glutathione	4.508 ±0.659 [a]	1.526 ±0.229 [b]
MDA	1.766 ±0.106 [a]	3.898 ±0.317 [b]
zinc	0.278 ±0.014 [a]	0.162 ± 0.015 [b]
Selenium	5.352 ± 0.191 [a]	2.782 ± 0.099 [b]

MDA= malondialdehyde.

Mean values of groups for each item with different superscripts in the same raw are significantly different ($P \leq 0001$).

Among these results, it is clear that there is a positive relationship between AF and the quality of the buffalo oocytes, its maturation rate and levels of (glutathione, zinc and selenium).

On the other hand, there is a negative relationship between the level of the MDA and AF, this level was lower in AF follicular fluid (1.76 mmol/L) than in small AF (3.89 mmol). So that AF might be considered the test of first choice in the assessment of ovarian reserve prior to IVF.

CONCLUSION

Results of the present study suggested that good quality oocytes and high percentage of oocyte maturation rate *in vitro* were associated with AF, an increase of follicular fluid (glutathione - zinc -selenium) concentration and decrease of MDA concentration.

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تاثير نوع الحويصلات المبيضية على جودة وانضاج البويضات معمليا في الجاموس

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الملخص العربي

تهدف هذه الدراسة الى دراسة تاثير نوع الحويصلات المبيضية (AF) على جودة وانضاج البويضات معمليا (IVM) وعلاقتها ببعض مكونات السائل الحويصلي في الجاموس. تم جمع مبايض الجاموس من المجزر ونقلها الى المعمل ، كما تم تصنيف الحويصلات المبيضية إلى نوعين , النوع الاول (1) وهو الحويصلات المبيضية (AF) والتي قطرها ≤ 3 مم اما النوع الثاني فهو الحويصلات المبيضية (small AF) والتي قطرها < 3 مم. تم سحب البويضات من كلا النوعين من الحويصلات المبيضية وتصنيفها الى ثلاثة انواع من حيث الجودة ، 1- النوع الاول وهي البويضات العالية الجودة (الجيدة) وهي التي حولها كم كبير ومدمج من الخلايا الركامية ، 2- النوع الثاني وهي البويضات ذات الجودة المتوسطة (المتوسطة) والتي حولها طبقات قليلة من الخلايا الركامية ،3 - النوع الثالث وهي البويضات ذات الجودة المنخفضة (الفقيرة في الجودة) والمعراة حيث لا يوجد حولها خلايا ركامية. تم انضاج البويضات معمليا في بيئة الانضاج المعملي (TCM 199). واشارت النتائج الى ان نسبة البويضات ذات الجودة العالية كانت 61.68% في البويضات المسحوبة من النوع الاول من الحويصلات المبيضية(AF) وكانت هذه النسبة اكبر من نسبة البويضات المسحوبة من النوع الثاني من الحويصلات المبيضية(small AF) حيث كانت نسبتها 28.32%، بينما كانت نسب البويضات المتوسطة والفقيرة في الجودة اعلى في حالة البويضات المسحوبة من النوع الثاني من الحويصلات المبيضية (small AF) (33.63و 38.05%) واقل في البويضات المسحوبة من النوع الاول من الحويصلات المبيضية (AF) (26.51 , 11.81%) وكانت الفروق معنوية ($p <0.001$).معدل الانضاج المعملي للبويضات (الوصول الى طور metaphase II (M II) بعد التحضين في بيئة الانضاج لمدة من 21 -24 ساعة في حالة البويضات التي تم الحصول عليها من النوع الاول من الحويصلات المبيضية (AF) كان أعلى (75.00٪) من تلك التي تم الحصول عليها من النوع الثاني (small AF) (42.48%) وكان الفرق معنوياً ($p <0.001$) التحليل الكيميائي لبعض مكونات السائل الحويصلي (الجلوتاثيون و المالونديالديهيد (MDA) و الزنك والسيلينيوم) كانت 4.5 مل مول / لتر و 1.76 ملي مول / لتر و 0.27 ملحم / ديسيلتر و 5.35 ملجم / ديسيلتر في السائل الحويصلي الذي تم الحصول عليه من (AF) و 1.52 ملي مول / لتر ، 3.89 ملي مول / لتر ، 0.16 مجم / ديسيلتر , 2.78 ملجم / ديسيلتر في السائل الذي تم الحصول عليه من (small AF) على التوالي وكانت الفروق معنوية ($p\leq 0.05$).ونستخلص من هذا ان الحويصلات المبيضية لها دورا هاما في جودة وانضاج البويضات معمليا، هذا الى جانب ان النسبة الاكبرمن البويضات عالية الجودة والمعدل الاعلى لانضاج البويضات معمليا والتركيز المرتفع من كل من الجلوتاثيون والزنك و السيلينيوم في السائل الحويصلي متعلق بالنوع الاول من الحويصلات المبيضية (AF) بينما النسبة الاقل من البويضات عالية الجودة والمعدل الاقل لانضاج البويضات معمليا والتركيز المرتفع من المالونديالديهيد (MDA) كان متعلقا بالنوع الثاني من الحويصلات المبيضية (small AF).