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EFFECT OF GLUTATHION AND α- TOCOPHEROL SUPPLEMENTATION ON THE RATE OF IN VITRO MATURATION OF BUFFALO OOCYTES

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ABSTRACT: *The aim of this study was to determine effect of the addition of glutathione (GSH) or α-tocopherol (V.E) to the maturation medium (MM) on buffalo oocytes in vitro maturation (IVM). Maturation is evaluated by the cumulus cells expansion rate. The cumulus oocyte complexs (COCs) were recovered and classified according to their cumulus morphology and ooplasm homogeneity into grades (A, B) as good quality oocytes and grades (C, D) as poor quality oocytes. The COCs were incubated for 24h in MM with GSH (100, 150 or 200 µm) or with V.E (100, 200 or 300 µM) or without addition (control).*

Results illustrate that supplementation of maturation media with 100, 150 and 200µm glutathione (GSH) resulted in increasing the cumulus cell expansion of buffalo good quality oocytes to 96.95, 89.83 and, 99.23 %, respectively as compared to oocytes cultured with GSH-free medium (88.33%) The improvement in the fully expansion rate of the oocytes was only significant with 200µm GSH supplementation and insignificant with either 100µm or 150µm GSH. However, addition of GSH with 100, 150 and 200 µm increased the cumulus expansion and maturation ratesof poor quality buffalo oocytes (grade C+D)to be 69.59, 72.12 and 82.89% of culturing compared to the oocytes cultured without GSH supplementation (69.01%). Utilization of V.E significantly ($p < 0.05$) affected the cumulus expansion rate of both good or poor quality buffalo oocytes. Supplementation MM with either 100 or 200µM (VE) resulted in decreasing the total cumulus cell expansion rates; however, utilization of 300µM VE improved the total expansion rate (99.17%) as compared to oocytes cultured with VE-free medium (86.62%). Whenever the expansion rates of the poor quality oocytes cultured in MM supplemented with 100, 200 and 300µM were 61.02, 68.02 and 85.89. It could be concluded that the additives of GSH in the MM improved cumulus expansion rate of buffalo oocytes more than that happened in the additives of V.E.

Key words: *Buffaloes, glutathione , α-tocopherol , oocytes maturation.*

INTRODUCTION

Buffaloes are known as the important domestic ruminant in more than 40 countries, mostly in tropical and subtropical region. In Egypt, buffaloes represent the main source of both milk and meat production. However, their productive and reproductive performances are still delayed. Efforts have been initiated to improve the reproduction potential of these animals using modern biotechnologies (Madan *et al.,* 1996 and Libertado, 2000).

Oocyte in vitro maturation (IVM), an important process of the IVF system, is intended to yield oocytes that normally could complete their first meiotic division (Downs, 1993; Royere 2006) to undergo normal fertilization, and would result in a zygote capable of full-term development after embryo transfer. Optimal expansion of the cumulus mass appears to be essential for cytoplasmic maturation (Testart *et al.* 1983, Chen *et al,*1993). In . bovine, the induction of cumulus expansion prior to fertilization increased the incidence of oocyte penetration (Ball *et al*. 1983).

It has been also speculated that buffalo oocytes / embryos, due to their high lipid

content (Boni *et al*, 1992), are particularly sensitive to the increased oxidative stress that occurs under in vitro conditions (Gasparrini *et al*, 2003), causing damage effects on cellular structure such as mitochondria and microtubles and could disturbe normal cell function (De Matos and Furnus 2000; Khatir *et al.* 2005). To protect oocytes and embryos from oxidative stress during in vitro culture, various antioxidants could be added to culture media. In mammalian cells, there is an enzymatic antioxidant system (superoxide dismutase, glutathione peroxidase and catalase), which acts as an reactive oxygen species (ROS) scavenger, controlling their production to prevent cell damage (Gasparrini *et al.,* 2006).

De Matos *et al*. (2003) illustratd that addition of antioxidants compounds to culture media could have different effects depending on the concentration used, the species and types of oocytes and medium in study. Glutathione (GSH) is a tripeptide thiol compound has many functions, such as, protecting the cell from oxidative damage (De Matos and Furnus. 2000; De Matos *et al.* 2003; Gasparrini *et al*. 2003; Downs and Verhoeven 2003; Urdaneta *et al.* 2004), transporting amino acids, synthesis of DNA and protein and reduction of disulfides (Funahashi 2005). Also, it has been observed that alpha-tocopherol, the most active form of vitamin E, acts as a protective liposoluble agent against lipoperoxidation by removing peroxyl and alcoxyl radicals, generating the poorly reactive tocopheryl radical (Chow 1991; Liebler 1993). In human spermatozoa, membrane and motility alterations caused by lipid peroxidation are partially inhibited by alpha-tocopherol (Aitken *et al*. 1989).

The aim of the present study was to determine the effect of the addition of glutathione and alpha-tocopherol to the maturation medium on the expansion maturation rate of buffalo oocyte in vitro.

MATERIALS AND METHODS

Ovaries collection:

Buffalo ovaries were collected from Shebin El Kom slaughterhouse and transported to our laboratory within 0.5-1 h from slaughter in phosphate buffer saline (PBS) containing 50 µg/ml gentamycin at 30-34°C. Thereafter, ovaries were washed twice in phosphate buffer saline to remove any adhering clotted blood.

Oocyte recovery:

The cumulus oocyte complexs (COCs) were recovered by aspiration of antral Follicles of 2-8 mm diameter using an 18-gauge needle attached to 10 ml syringe.The (COCs) were examined under a stereomicroscope. The quality of the oocytes were assessed according to their cumulus morphology and ooplasm homogeneity as described by De Loos *et al.* (1989) and classified into four grades as follow:

1- **Grade (A):** characterized by dark homogenous ooplasm and more than four compact layers of cumulus cells.
2- **Grade (B):** characterized by dark homogenous cytoplasm and more than four layers of less compact cumulus cells starting to expand peripherally.
3- **Grade (C):** characterized with heterogeneous granulated ooplasm;
4- **Grade (D):** oocytes completely or partially denuded of cumulus cells;

The oocytes of grade (A) and (B) were considered as good quality oocytes, while those of grade (C) and (D) were considered as poor quality oocytes (De Loos *et al.* 1989, Fouladi-Nashta *et al.* 2007).

Experimental Treatments:

The maturation media consisted of TCM 199 supplemented with 10% estrous buffalo serum, 10 µg/ml FSH, and 5 µg/ml LH, 1 µg /ml estradiol l7 β and, 50µg/ml gentamicine. The maturation medium was supplemented with different concentration of either glutathione (GSH) or α- tochopherol (V.E) as the following:

1. Maturation media without any supplementation and served as a control.
2. Maturation media was supplemented with either 100, 150 or 200 µm GSH
3. Maturation media supplemented with either 100, 200 or 300 µM V.E.

All chemical and supplementations were purchased from sigma chemical Co.

In vitro maturation (IVM):

The oocytes of each grade were washed two times in one of the intended maturation media before culturing in that medium, and then the oocytes of each grade were incubated in groups of 20-25 oocytes in a 100 µl droplet of each culture maturation medium. The droplets were covered with mineral oil in 35 mm dish and incubated for 24h at 38.5°C in an atmosphere of 5% CO2 in humidified air.

After 24 h in culture, the expansion degree of the cumulus-oocyte complex (COCs) in each treatment of maturation medium was determined to evaluate the efficiency of each medium which was classified as follow:

- Fully expanded (all cumulus cells were loosened)
- Partially expanded (the outer layer of cells was loosened) or
- Not expanded.

The degree of expansion of cumulus cell mass has been routinely considered in evaluating buffalo oocyte maturation in vitro (Palta and Chauhan, 1998 & Nandi *et al*., 2002), and for predicting subsequent in vitro fertilization success (Herrler *et al*., 1992). In addition, the maximum cumulus expansion and extrusion of first polar body is expected to occur during 22-24h of culture (Nandi *et al.,* 2002).

Statistical analysis:

The obtained data were statistically analyzed using SPSS program (v19, 2010) while differences among the treatment means were performed using Duncan's Multiple Range Test (Duncan's, 1955).

RESULTS AND DISCUSSION

1-Effect of glutathione supplementation on the maturation rate (expansion) of buffalo oocytes:

Results in Table (1) demonstrate that supplementation of maturation media with 100, 150 and 200µm glutathione (GSH) resulted in increasing the cumulus cell expansion of buffalo good quality oocytes to 96.95, 89.83 and, 99.23 %, respectively, as compared to oocytes cultured with GSH-free medium (88.33%).The respective differences were significant ($P< 0.05$).

Data in Table 1 also reveal that the improvement in the fully expansion rate of the oocytes was only significant with 200µm GSH and insignificant with either 100µm or 150µm GSH. The obtained data further reveal that supplementation of the maturation media with 200µm GSH has the greatest effect on the expansion rate compared with 100 or 150 µm GSH. Whenever, the supplementation of 200µM GSH to the maturation medium insignificantly decreased the partially expansion rate (17.82%) of good quality oocytes compared to those oocytes cultured in GSH–free culture medium, with 100µM or with 150µM.

The obtained maturation rate in the present study characterized by expansion of COCs is higher than that reported by Totey *et al.* (1991, 1992 and 1993) (40, 63.6 and 80.0 %, respectively), Jainudeen *et al.* (1993) 47.0%, Chauhan *et al.* (1998) 82.0 % and Nandi *et al.* (2003) 83.0 % in buffaloes. In cattle, Im, and Park, (1995) reported maturation rate of 86.4 % by using the medium TCM-199 with FSH, LH and E2, on the contrary, Kobayashi *et al.* (1994) recorded higher expansion of 99-100 % in cattle COCs. On the other hand, Harper and Brackett (1993), % and Rieger *et al.* (1998) reported comparatively lower values in cattle (66.7% and 31.1%, respectively). It could be concluded that the maturation rate of COCs in buffaloes depend on the type of cultured media and it's supplementation.

Table (1): Effect of glutathione (GHS) supplementation on expansion rate of good quality (grade A+B) buffalo oocytes.

GSH Conc. (µM)	Total tested oocytes	Cumulus expansion							
		Full expanded		Partially expanded		Expansion Rate		Non expanded	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
0	151	74	50.58[a]	59	37.75	133	88.33[a]	18	11.67[a]
100	144	105	72.60[ab]	35	24.34	140	96.95[ab]	4	3.39[ab]
150	124	81	64.16[ab]	31	25.67	112	89.83[a]	12	10.17[b]
200	126	108	81.41[b]	17	17.82	125	99.23[b]	1	0.77[b]
p<	-	-	0.03	-	NS	-	0.03	-	0.04

[a,b,] Values having different superscript within the same column differ (P<0.05).

With regard to the effect of GSH supplementations on the expansion rate of buffaloes poor quality oocytes (grade C+D). Results in Table (2) illustrate that the expansion rate of buffaloes poor quality oocytes (grade C+D) significantly (P<0.05) affected by GSH treatment. In this regared, addition of GSH (200µM) during culturing poor quality buffaloe oocytes increased the full expanded and the total of expansion rate (70.11 and 82.89%, respectively). It also decreased the rate of partially expanded oocytes (12.78%) as well as the immature oocytes (17.11%). In general, GHS supplementation tended to decrease the rate of partially extended oocytes as the GHS concentration increased, so that the rate of the control group was (35.58%) versus (12.78%) for the (200µM) GHS supplemented group.

The majority of the respective improvement in the expansion rate of oocytes was observed in the rate of fully expanded oocytes, which ranged from 8.51 to 36.68% as compared to those cultured in GSH-free medium (33.43%), and the differences in this concept were significant. On the contrary, the rate of immature (unexpanded) oocytes tended to insignificantly decrease as the concentration of GSH supplementation was increased (Table, 2).

Generally, data in Tables (1 and 2) indicate that supplementing the TCM maturation medium with GSH improved the expansion rate of buffaloes oocytes either being of good quality (grade A,B) or of poor quality (grade C). The greatest improvement in this concept took place when the maturation medium was supplemented with 200µM GSH, and this improvement ratio was comparatively higher in the poor quality oocytes (13.9%) than that recorded for the good quality ones (10.9%) as compared to the control groups. The cumulus cells surrounding oocytes are structurally and metabolically linked with the gametes and involved in the process of glutathione synthesis in either buffalo (Mori *et al.,* 2000), cow (De Matos *et al.,* 1997) or pig (Yaumachi and Nagai, 1999).

Glutathione has been shown to play an important role in oocyte maturation. The process of oocytes cytoplasmic maturation involves numerous molecular events, including synthesis of biochemical compounds, protein phosphrylation and activation of particular metabolic pathways (Eppig, 1996). On other hand, Tatemoto *et al.* (2000) suggested that the quality of immature oocytes and the composition of culture medium were the critical factors for in vitro maturation of oocytes and that the presence of cumulus cells during in vitro maturation was of prime importance in protecting oocytes from apoptosis induced by oxidative stress.

Table (2): Effect of glutathione (GHS) supplementation on maturation rate (expansion) of poor quality (grade C+D) buffalo oocytes.

GSH Conc. (μM)	Total tested oocytes	Cumulus cells expansion							
		Full expanded		Partially expanded		Maturation rate		Non expanded	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
0	50	19	33.43[a]	16	35.58	35	69.01	15	33.22
100	88	40	41.94[a]	22	27.65	62	69.59	26	30.41
150	69	32	42.81[a]	18	29.31	50	72.12	19	27.88
200	68	49	70.11[b]	10	12.78	59	82.89	9	17.11
P<	-	-	0.02	-	Ns.		Ns.	-	Ns.

[a,b,] Values having different superscript within the same column differ (P<0.05).

2-Effect of α – tocopherol (Vitamin E) supplementation on the maturation rate (expansion) of buffalo oocytes:

As shown in Table (3) the utilization of α-tocopherol (VE) as an antioxidant supplement insignificantly affected the total cumulus expansion rate of good quality buffalo oocytes (grade A,B). In this regard, supplementing of the maturation media with either 100 or 200μM α–tocopherol (VE) resulted in decreasing the total cumulus cell expansion rates; however, utilization of 300μM VE improved the total expansion rate (99.17%) as compared to oocytes cultured with VE-free medium (86.62%). These results are in agreement with those obtained by Marques *et al.* (2008) who used α-tocopherol as an anioxidant to the bovine cumulus-oocyte complexes with concentrations (25,50,100,200 μm) and found negative effect as oncentration increased. ,

Also, Natarajan *et al.* (2010) indicated that α-tocopherol supplementation to the oocyte maturation medium did not cause any significant change with respect to the rate of sheep oocyte maturation. Similar observation was also made in bovine in vitro studies, wherein it was reported that the active form of VE in maturation medium did not have any effect on developmental competence of oocytes and embryos by Dalvita *et al.,* 2005, who reported that during IVM, α-tocopherol content naturally in COC membranes diminished 50%, indicating the partial loss of antioxidant activity during the period of culture in vitro. The obtained results in the present study (Table 3) illustrate that supplementing maturation medium with 300μM α-tocopherol increased cumulus cell expansion of buffalo good quality oocytes to 99.17% comparing with control group (86.62%). This could suggest that addition of 300μM VE has a positive physiological effect in improvement of maturation rate of good quality buffalo oocytes.

Furthermore, results in Table (3) indicate that fully expansion rate is 64.82% for oocytes cultured in VE-free medium, and this was insignificantly decreased to 48.95% and 46.53% when the maturation medium supplemented with either 100 or 200μM, thereafter, it increases to 69.36% with supplementation of 300μM VE.

Table (3): Effect of α - tocopherol (V.E) supplementation on maturation rate (expansion) of good quality (grade A+B) buffalo oocytes.

VE Conc. (µM)	Total tested oocytes	Cumulus expansion							
		Full expanded		Partially expanded		expansion rate		Non expanded	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
0	132	86	64.82	30	21.80	116	86.62	16	13.38
100	130	75	48.95	35	29.68	110	78.63	20	21.37
200	111	61	46.53	37	33.22	98	79.75	13	20.25
300	144	101	69.36	42	29.80	143	99.17	1	0.83
P<	-	-	n.s	-	n.s		n.s	-	n.s

[a,b,] Values having different superscript within the same column differ (P<0.05).

It could be concluded that supplementing the maturation media with 300µM VE slightly improved the fully expanded oocytes and consequently total cumulus expansion and nuclear maturation rate of good quality buffalo oocytes (grade A+B).

With regard to the effect of α - tocopherol (V.E) addition on the expansion rate of buffaloes poor quality oocytes (grade C+D). Results in Table (4) indicate that supplementation of VE to the maturation medium with either 100 or 200µM decreased the cumulus expansion rate to be 61.02% and 68.02%, respectively of buffaloes poor quality oocytes as compared to the oocytes cultured without VE (77.75%), Table (4) further shows that adding 300µM VE in the maturation medium increased the maturation and expansion rate to 85.89%. but the differences in this concept were insignificant.

On the contrary, as the concentration of VE supplemented to the maturation medium increases from 100 to 200µM VE, the rate of unexpanded (immatured) oocytes of poor quality (grade C) was insignificantly increased to be 38.98% and 31.98%, respectively as compared to (22.25%) for those oocytes cultured in VE-free medium, meanwhile, the supplementation with 300µM VE resulted in decreasing the rate of unexpanded (immature) oocytes (14.11%) compared to that obtained for control group (22.5%) (Table 4).

The addition of vitamin E to the culture medium of bovine embryos, improved the developmental competence up to the blastocyst stage. Olson and Seidel (2000) suggested that the supplementation of the culture medium with vitamin E increased bovine embryo development and blastocyst formation due to the inhibition of NADPH oxidase, protecting cell membranes. Studies of Tao *et al.* (2004) showed that α-tocopherol promotes the meiotic maturation of denuded oocytes, especially from MI to MII; however, this effect was not observed in cumulus enclosed oocytes. However, Sudano *et al* (2010) reported that the use of vitamin E significantly reduced the number of blastocysts produced. De Matos *et al.* (2003) and Tatemoto *et al.* (2000) concluded that the addition of antioxidants compounds to culture media could have different effects depending on the concentration used, the species and types of oocytes and medium.

Table (4): Effect of α - tocopherol (V.E) supplementation on maturation rate (expansion) of poor quality (grade C+D) buffalo oocytes.

VE Conc. (µM)	Total tested oocytes	Cumulus expansion							
		Full expanded		Partially expanded		Maturation rate		Non expanded	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
Con.	105	47	46.76	36	30.98	83	77.75	22	22.25
100	71	28	30.52	23	30.49	51	61.02	20	38.98
200	51	28	52.30	11	15.71	39	68.02	12	31.98
300	58	25	42.40	26	43.49	51	85.89	7	14.11
P<	-	-	n.s	-	n.s	-	n.s	-	n.s

[a,b,c] Values having different superscript within the same column differ (P<0.05).

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تأثير اضافة الجلوتاثيون والفا توكوفيرول على معدل انضاج بويضات الجاموس معمليا

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الملخص العربى

تم دراسة تأثير اضافة الجلوتاثيون و والفا توكوفيرول (فيتامين E) على معدل انضاج بويضات الجاموس معمليا. تم جمع وتصنيف البويضات وتصنيفها حسب طبقة الخلايا المحيطة بالبويضة والشكل الظاهرى للسيتوبلازم الى بويضات عالية الجودة واخرى منخفضة الجودة. تم تحضين البويضات 24 ساعة فى بيئة انضاج تحتوى على الجلوتاثيون بتركيزات مختلفة (100, 150 او 200µm) أو فيتامين E بتركيزات (100, 200 أو 300µm) او فى بيئة لاتحتوى على اى اضافات من الجلوتاثيون أو فيتامين E .

وقد أوضحت النتائج أن اضافة الجلوتاثيون الى بيئة الانضاج عند مستوى 100, 150 او 200µm قد أدى الى ارتفاع معدل انضاج البويضات الى96,95% و 89,83 و 99,23%, على التوالى مقارنة فى حالة عدم وجود اى اضافات فى بيئة الانضاج(88,33%). وكان مستوى تحسين معدل انضاج البويضات معنويا عند اضافة 200 µm وغير معنويا عند اضافة 100 أو 150 µm . بينما اضافة الجلوتاثيون الى بيئة الانضاج عند مستوى 100, 150 او 200µm قد أدى الى ارتفاع معدل انضاج البويضات منخفضة الجودة الى 69,59 و 72,12% و 82,89% مقارنة فى حالة عدم وجود اى اضافات فى بيئة الانضاج (69,01%).

كما أوضحت النتائج أن اضافة الفا توكوفيرول (فيتامينE) قد اثر تأثيرا معنويا بدرجة 5% على معدل انضاج البويضات العالية أو المنخفضة الجودة. حيث أدت اضافة 100 او 200µm من فيتامينE الى انخفاض معدل الانضاج الكلى للبويضات بينما اضافة 300 µm من فيتامين E ادى الى ارتفاع معدل انضاج البويضات (99,17%) مقارنة فى حالة عدم وجود اى اضافات فى بيئة الانضاج (86,62%). بينما كان معدل انضاج البويضات المنخفضة الجودة عند اضافة الفيتامين الى بيئة الانضاج عند مستوى 100, و 200 و 300 µm هى 61,02% و 68,02% و 85,89%, على التوالى.

ويستخلص من هذه الدراسة أن اضافة الجلوتاثيون الى بيئة الانضاج أدى الى تحسين معدل انضاج بويضات الجاموس بنسبة اعلى عنه مما حدث عند اضافة الفا توكوفيرول (فيتامين E).