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Supplementation of antioxidants for in Vitro embryo production of buffaloes

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Biological hazards associated with oxidative stress resulted in defective in vitro embryo production "IVEP". Reactive oxygen species "ROS" may originate from embryo metabolism and/or embryo surroundings. In an attempt to overcome such challenge, the present study correlates between the influence of 3 antioxidants and buffalo oocyte development up to morula and blastocyst stages. It was found that taurine addition (either alone or concomitant with ascorbic acid "AA" or mannitol) to media during in vitro maturation "IVM" has the potency to enhance IVEP in buffaloes. On the other side, AA and mannitol either alone or in combination are invalid to improve developmental rate of mature buffalo oocytes to reach morula and blastocyst stages.

In a second experiment, the best treatment (taurine and AA) was added to both in vitro fertilization "IVF" and culture "IVC" media. This treatment resulted in significant elevation of the cleavage rate and morula development without influencing blastocyst rate as compared with their corresponding values following the addition to IVM medium alone. Thus, it could be concluded that addition of taurine with AA into both IVM and IVF media enhances maturation of buffalo oocytes and activates the cells to undergo cleavage and morula development.

Among livestock, water buffalo is a principal source of milk, meat and ideal for fattening. However, the reproductive activity of such animal suffers from a series of disturbances leading to subfunctional ovarian performance and elongation of calving interval. Moreover, ovulations are highly unpredictable, thus frustrating breeding management.

Various methods have been sought to overcome infertility among buffaloes; IVF and embryo transfer have become indispensable. In this concern, efforts have been directed to use various additives to the maturation medium to improve the yield of IVF (Fukui, 1989 in cattle and Gasparrini, 2002 and Atef, 2005 in buffaloes). Moreover, studies have been implemented to apply various antioxidants to the maturation medium (Ealy *et al.,* 1995 and Fujitani *et al.,* 1997). In this respect, it was documented that oxygen concentration within the reproductive tract of mammals is lower than that in air (Kessler, 1974). Furthermore, the cleaving embryos has an optimal oxygen requirement in vitro approximately equivalent to 5 % and blastocysts that developed under 20 % O_2 had fewer cells than those developed under 5 % (Quinn and Harlow, 1978 and Fujitani *et al.,* 1997). Manipulation of oocytes and embryos in an in vitro environment carries the risk of cell exposure to higher levels of ROS. This condition of increased ROS levels without concomitant rise in antioxidant defense leads to oxidative stress upon developing embryos resulting in lipid, protein and even DNA damage responsible consequently for defective embryo development (Fujitani *et al.,* 1997; Sikka, 2004 and Agarwal *et al.,* 2006).

Therefore, the current study is an endeavor to clarify the influence of a variety of antioxidants (mannitol, taurine and AA) added to hormone - enriched media on IVEP in buffalo cows.

Materials and methods

This study was done during October 2007-March 2008. All chemicals and media were purchased from Sigma Chemical Co., St. Louis, MO, USA unless otherwise indicated. Media and

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buffers were freshly prepared and sterilized by passing them through 0.22 µm millipore membrane filter while the mineral oil was sterilized by 0.45 µm diameter filter (Sterivex-GV, Bedford, MA, USA). The solutions were allowed to equilibrate for 1 hr. in CO_2 incubator before being applied to the oocytes (Leibfried and First, 1979). Presterilized disposable plastic tools were used once.

Cyclic buffalo ovaries (n=1658) were collected from El-Hawamdya abattoir and preserved in a thermos containing presterilized warm (25°C) saline (Gordon, 1994) supplemented with 100 µg/ml streptomycin sulfate and 100 i.u. penicillin G (Pavasuthipaisit *et al.,* 1992) then transported within 2 hours (Leibfried-Rutledge *et al.,* 1987) to the laboratory where they were washed thrice with presterilized warm saline. Retrieval of immature primary oocytes (cumulus oocytes complexes "COCs") was carried out using the aspiration method (Suzuki *et al.,* 1992). The collected COCs were washed thrice in PBS containing 10 mg/ml BSA and also thrice with Ham's F-10 medium supplemented with 50 µg/ml gentamycin sulfate and 5 µl/ml L-glutamine. The cells were examined under microscope and classified into 3 categories : oocytes with evenly granulated cytoplasm and completely surrounded by multiple layers of cumulus cells (grade I), oocytes surrounded by scanty layers of cumulus cells (grade II) and nude oocytes (grade III) which were excluded from the study. In vitro maturation of oocytes was done in digitally adjustable CO_2 incubator favors 5.0 % CO_2, 39°C and 95 % relative humidity and Ham's F-10 medium with 10 % fetal calf serum "FCS ", 50 µg/ml gentamycin sulfate and 5 µl/ml L-glutamine was used for maturation (Totey *et al.,* 1993). The oocytes were subjected to 2 separate experiments; in the first the oocytes were exposed to the following treatments:

Group I (Control).The maturation medium was devoid of antioxidants but contained 0.2 i.u FSH (Gonal-F, Laboratoires Serono S.A., Switzerland), 2.0 i.u. hCG (Pregnyl, Nile Co. for Pharm., Cairo, Egypt) and 1.0 µg E_2/ml medium (Atef, 2005). This control medium was used as a basal maturation medium (BMM) applied in the following groups.

Group II (BMM with 0.3M mannitol). (Okada *et al.,* 2006).

Group III. BMM was supplemented with 10mM taurine **(Takahashi and Kanagawa, 1998)**.

Group IV. BMM + 250µM AA (Tao *et al.,* 2004).

Group V. 0.3M mannitol + 10mM taurine were added to BMM.

Group VI. The BMM contained 0.3M mannitol + 250µM AA.

Group VII. BMM with 10mM taurine + 250 µM AA.

Each treatment included a number of grade I and II oocytes contained in droplets of the maturation medium each of 50 µl (10 COCs/droplet).

In Experiment II, a treatment included a mixture of taurine and AA was applied to oocyte media during IVF and IVC as from Experiment I, this combination gave the best results.

The oocytes were incubated for maturation in CO_2 incubator for 24 hrs. (Fukui, 1990) then examined under stereomicroscope for evaluation of cumulus mass expansion. Accordingly, the cells were classified into 3 classes : excellent, good and unexpanded (Schellander *et al.,* 1989). In vitro capacitation of buffalo spermatozoa (frozen buffalo semen received from Artificial Insemination Centre, Beni Suef) was adopted according to Parrish *et al.,* (1988). IVF and culturing was done as described by Atef (2005). The presence of cleaved cells (2-16) as well as morula and blastocyst stages was recorded and the number of embryos which cleaved at least once was calculated. Data were subjected to statistical analysis as outlined by SAS Program (1994).

Results

Table 1 displays that oocyte recovery rate reached 1.11±0.03%. Grade I and grade II represented 33.73±0.54 and 39.33±0.72%, respectively while nude oocytes % reached 27.34 ±0.61; a value which differs significantly ($P \leq 0.001$) than the corresponding grade I and II oocytes. In addition, the recovery rate of grade I and II oocytes, undergoing subsequent maturation, was 0.72/ovary.

It appears from table 2 that the best maturation rate % was after a combination between taurine and AA followed by taurine alone then addition of taurine to mannitol. Also, addition of AA alone increased significantly the maturation rate while addition of either mannitol alone or in combination with AA did not induce any significant improvement compared with the corresponding

control values. Cleavage rate, as indicated by number of oocytes underwent cleavage (up to 16 cells) / total number of excellent and good mature oocytes, showed that the ideal treatment was the combination between taurine and AA followed by either taurine alone or with mannitol while AA treatment and either mannitol alone or concomitant with AA did not enhance cleavage rate than that of the control group.

Table 3 disclosed that the best treatment induced highest morula development was the mixture between taurine and AA followed by either taurine alone or accompanied with mannitol while AA and either mannitol alone or concomitant with AA did not stimulate morula development as compared with the corresponding control. In addition, combination between taurine and AA induced the highest rate for transformation of morulae into blastocysts followed by either taurine alone or accompanied with mannitol as compared with control value. On the contrary, treatments including AA and either mannitol alone or accompanied with AA did not give better blastocyst developmental rate than that of the control.

In experiment II (Table 4), addition of taurine and AA mixture to IVM and IVF media resulted in significant elevation of the cleavage rate and morula development compared with their corresponding values following addition of this treatment to IVM medium alone without influencing blastocyst developmental rate. This improvement was not further increased when the antioxidants were added into the IVC medium.

Table 1 : Classification of the recovered buffalo oocytes (Mean ± SE).

Number of ovaries	Number of recovered oocytes	Recovery rate (%)	Qualitative classification of buffalo oocytes (%)		
			Grade I	Grade II	Grade III
1658	1842	1.11 ± 0.03	33.73 ± 0.54*	39.33 ± 0.72*	27.34 ± 0.61*

SE : Standard error.
In the same row, values having * superscript differ significantly from each others at P < 0.001.

Table 2 : Maturation and cleavage rates % of buffalo oocytes following addition of different antioxidants (Mean ± SE).

Treatment	No. of oocytes	Maturation rate %	Cleavage rate %
Control	207	84.54±0.34 ADG	50.45±0.75 ABC
Mannitol	211	84.42±0.79 BE	50.50±0.51 DK
Taurine	212	90.28±0.92 ABC	55.17±0.59 ADEF
Ascorbic acid (AA)	205	86.73±0.13 ABE	48.70±1.67 EGH
Mannitol + Taurine	209	88.90±0.56 EFG	55.80±0.32 BGIK
Mannito + AA	212	85.97±0.48 CF	50.65±1.18 FIJ
Taurine + AA	214	93.25±0.49 ABFG	56.95±0.72 CDHJ

In the same column, values sharing one similar letter differ significantly from each others (at least P≤0.05).

Table 3 : Rate of morula development / cleaved oocytes and blastocyst development / morulae following addition of different antioxidants to maturation medium (Mean ± SE).

Treatment	N_1	Morula Development (%)	N_2	Blastocyst Development (%)
Control	44	43.78±0.56 ^{AB}	20	38.30±1.46 ^{ABC}
Mannitol	46	43.72±0.46 ^{CD}	20	37.86±1.55 ^{DEF}
Taurine	50	47.68±0.42 ^{ACE}	23	41.80±1.01 ^{ADGH}
Ascorbic acid (AA)	41	42.90±1.13 ^{EF}	18	38.41±1.07 ^{GIJ}
Mannitol + Taurine	45	47.97±0.61 ^{BDF}	22	42.06±1.17 ^{BEIK}
Mannitol + AA	44	45.33±1.71 ^{G}	20	36.05±2.07 ^{HKL}
Taurine + AA	43	52.14±0.60 ^{ADFG}	23	43.03±1.56 ^{CFJL}

SE : Standard error.
N_1 : No. of cleaved oocytes. N_2 : No. of morulae.
In the same column, values sharing one similar letter differ significantly from each others (at least $P \leq 0.05$).

Table 4 : Cleavage rate as well as morula and blastocyst development % of buffalo embryos following addition of taurine and ascorbic acid to both IVF and IVC media (Mean ± SE).

Type of media	Cleavage rate %	Morula %	Blastocyst %
IVM	56.95 ± 0.72 ^{AB}	52.14 ±0.60 ^{AB}	43.03 ± 1.56
IVF	64.85 ± 1.74 ^{A}	57.92 ± 2.11 ^{A}	45.25 ± 3.21
IVC	62.76 ± 2.07 ^{B}	56.09 ± 2.03 ^{B}	44.19 ± 2.97

SE : Standard error.
In the same column, values sharing one similar letter differ significantly from each others (at least $P \leq 0.05$).

Discussion

In the present study, ovaries of apparently healthy cyclic buffaloes were collected from abattoir in order to retrieve oocytes. This method is considered an easy, available and inexpensive to obtain oocytes as well as it provides a source to collect a large number of cells during the shortest period (Abd Allah, 2003 and Atef, 2005). In addition, the ovaries yield a higher distribution percentage of primary follicles than the decreased value detected in the pregnant buffaloes (Goswami, 1985). Moreover, Abd Allah (2003) recorded in buffalo that cyclic ovaries gave a higher number of oocytes/ ovary than that obtained from animals with ovarian defects. The collected buffalo ovaries were transferred to the laboratory preserved in presterilized warm (35^{o}C) saline within 2 hours. This time interval is critical for successful IVEP as an inverse relation was recorded between storage time and percentages of cleavage and blastocyst formation (Lonergan *et al.,* 1992). Moreover, Pollard *et al.,* (1996) in cows reported that the blastocyst formation was 33.70 % higher among oocytes recovered from ovaries preserved at 35^{o}C than those at either 25 or 30^{o}C; a result that contradicts the study of Yang *et al.,* (1990) who recorded that cleavage and blastocyst rates were higher when ovaries were preserved at 24^{o}C than those at 37^{o}C.

Many techniques were outlined for oocyte recovery including ovarian dissection, slicing and aspiration of vesicular follicles. The current study used the aspiration one. Leibfried and First (1979) recommended this way because of its advantage in speed of collection which is important for commercial IVEP. Also, Datta and Goswami (1998) in buffaloes recorded lower oocytes recovery by slicing method than that after aspiration; a finding that motivated Abd Allah (2003) and Atef (2005) in buffaloes to use aspiration procedure.

From table 1, the recovery rate was 1.11±0.03 %. Grade I and II oocytes was 33.73±0.54 and 39.33±0.72 %, respectively with 27.34±0.61 nude

cells. In buffaloes, variable rates were previously reported (Suzuki *et al.,* 1992; Hammam *et al.,* 1997 and Atef, 2005). This variation is due to age, follicle size, ovarian status, genetic factor and health condition (Ahmed *et al.,* 1999; Hagemann, 1999 and Abd-Allah, 2003). Also, oocytes convenient to undergo maturation was 0.73 which is > 0.20 (Jain *et al.,* 1995) and < 1.77 (Abd Allah, 2003).

The BMM used for IVM of buffalo oocytes was Ham's F-10 medium enriched with hormones (Atef, 2005). Totey *et al.,* (1993) and Abass (1998) found that Ham's F-10 medium supplemented with FCS and hormones was most suitable for buffaloes. Furthermore, Badr (2001) observed excellent cumulus expansion with high in vitro nuclear maturation, sperm penetration, cleavage rate and embryo development when buffalo oocytes were cultured in Ham's F-10 and TCM-199. Hormonal addition during IVM improved maturation and subsequent embryonic development (Atef, 2005). These findings coincide with the results of Younis *et al.,* (1989) and Nakagawa and Leibo (1997) who revealed that addition of even a single hormone (LH) or in combination with FSH and E_2 significantly enhanced the rate of bovine oocytes maturation. FSH and LH receptors are present on cumulus cell (Gasparrini, 2002) thus they elicit their effect via cumulus mediated interactions. Estradiol addition to IVM medium improved the completion of maturation, including the synthesis of the male pronucleus growth factor in the nucleus and cytoplasm of mammalian oocytes (Fukui *et al.,* 1982).

Regarding addition of antioxidants to IVM medium, it appears from table 2 that the best maturation rate was after application of a combination between taurine and AA followed by taurine. Also, addition of AA alone increased significantly the maturation rate while mannitol alone or in combination with AA did not induce any significant improvement. These findings coincide with previous studies that revealed the importance of taurine supplementation to enhance in vitro development of rabbit (Li *et al.,* 1993), pig (Reed *et al.,* 1992) and cattle embryos (Liu *et al.,* 1995). Furthermore, Ali *et al.,* (2003) recorded that addition of taurine or its derivatives improved bovine embryo development in contrast to extracellular antioxidants like catalase and superoxide dimutase "SOD" that caused no improvement. Gardner and Lane (1997) emphasized that taurine also serves as an osmolyte that buffers the intracellular pH of maturing oocytes. Regarding AA, Olson and Seidel (2000) and Tatemoto *et al.,* (2001) reported that AA reduced the threat of cellular apoptosis, enhanced synergistically the antioxidant effect of vitamin E and supported the cytoplasmic maturation during IVEP. Also, Tao *et al.,* (2004) found that AA prevented cumulus cell DNA fragmentation.

Table 2 also showed that combination between taurine and AA was optimal to enhance cleavage rate followed by either taurine alone or accompanied with mannitol as compared with the corresponding control value while AA and either mannitol alone or concomitant with AA did not enhance cleavage rate. These findings emphasize the role of taurine to stimulate IVF and cleavage in buffaloes. Dumoulin *et al.,* (1992) and Gardner and Lane (1997) clarified that taurine had a beneficial regulating effect on phospholipids and membrane protein receptor interactions as well as protects newly - formed embryo from impairment due to high potassium concentrations. Furthermore**,** Tatemoto *et al.,* (2001) recorded that AA enhanced transformation of the sperm nucleus into the male pronucleus (MPN) after IVF. However, in the present study AA alone or combined with mannitol did not show potential influence upon IVF; a finding which comes in agreement with that reported by Dalvit *et al.,* (2005) in bovine.

Data presented in table 3 clarify that combination between taurine and AA in maturation medium is the option to induce highest morula and blastocyst development followed by either taurine alone or accompanied with mannitol as compared with the corresponding control values. On the other side, AA and either mannitol alone or concomitant with AA did not show any effect to enhance the development. Supplementation of maturation media with taurine has been demonstrated to enhance in vitro development of embyos (Li *et al.,* 1993 and Liu *et al.,* 1995). In addition, Fujitani *et al.,* (1997) and Takahashi and Kanagawa (1998) detected that taurine improved the development of 4-8 cell bovine embryos to the blastocyst stage after 6 days of culture. Ali *et al.,* (2003) observed significant improvement in the proportion of oocytes undergoing morula and blastocyst development

when taurine was added to the maturation medium compared with the medium devoid of antioxidants.

Therefore, it could be concluded that taurine addition (either alone or concomitant with AA or mannitol) to media during IVM has the potency to enhance IVEP in buffaloes. On the other side, AA and mannitol either alone or in combination are invalid to improve developmental rate of mature buffalo oocytes to reach morula and blastocyst stages.

In the second experiment, the best treatment (taurine and AA) was added to both IVF and IVC media. Table 4 reveals that addition of this treatment resulted in significant elevation of the cleavage rate and morula development without influencing blastocyst developmental rate as compared with their corresponding values following addition of this treatment to IVM medium alone. Thus, it could be concluded that addition of taurine and AA into both IVM and IVF media is the best formula to achieve beneficial enhancement for maturation of buffalo oocytes as well as their activity to undergo cleavage and morula development while supplementation of IVC medium does not lead to further increment in these rates. In addition, it seems that the combination between taurine and AA has the potential activity for reducing the oxidative stress during IVM and IVF which favors better environment for the developing embryo to undergo further development up to morula and blastocyst stages. From the present study, it is worth mentioning that the application of the proposed combination between the aforementioned antioxidants during IVM-IVF is not cited in the literature for IVEP in cattle and buffalo (Fujitani *et al.,* 1997; Takahashi and Kanagawa, 1998; Ali *et al.,* 2003 and Dalvit *et al.,* 2005); a finding that opens the gates for better mechanisms that allow inhibition of ROS during IVEP in buffaloes.

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إضافة مضادات التأكسد لانتاج أجنة الجاموس فى المعمل

الأضرار البيولوجية الناتجة عن ضغوط الأكسدة تؤثر سلبا على انتاج أجنة الجاموس فى المعمل. تنشأ جزيئات الأكسجين النشطة من الأيض الغذائى للأجنة والبيئة المحيطة بها. فى محاولة للتغلب على هذة التأثيرات الضارة، تهدف الدراسة الحالية للربط بين تأثير ثلاثة مضادات للتأكسد ونمو البويضات فى المعمل حتى تصل الى الطور التوتى ومرحلة البلاستيولا. وجد أن اضافة التورين بمفردة أو مجتمعا مع حمض الأسكوربيك أو المانيتول الى البيئة الخاصة بنمو البويضات كان لة القدرة على تحسين انتاج أجنة الجاموس فى المعمل. من ناحية أخرى، وجد أن اضافة حمض الأسكوربيك بمفردة أو المانيتول أو كلاهما لا يؤدى الى تحسين معدل نمو بويضات الجاموس فى المعمل حتى تصل الى الطور التوتى أو مرحلة البلاستيولا.

فى التجربة الثانية، ثبت أن أفضل المعاملات هى اضافة التورين وحمض الأسكوربيك الى البيئة الخاصة بالاخصاب ونمو الأجنة. أدت هذة المعاملة الى زيادة فعلية فى معدل الانقسام والطور التوتى دون التأثير على نسبة البلاستيولا مقارنة بالمجموعة الضابطة. بالتالى يمكن استخلاص أن اضافة التورين مع حمض الأسكوربيك الى بيئة النمو والاخصاب تؤدى الى زيادة نسبة نمو بويضات الجاموس وتحفز الخلايا للانقسام وانتاج الطور التوتى.