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Potentiality of Epidermal Growth Factor or/and Cysteamine in Maturation Medium on *In vitro* Rabbit Embryo Production and Apoptosis

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Abstract: This study was conducted to determine the effect of adding epidermal growth factor (EGF, 10 ng/ml) or/and cysteamine (CYST, 100 µM/ml) to maturation medium on *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of rabbit oocytes. Oocytes were harvested by slicing from ovaries of 24 slaughtered mature does. Compact-cumulus oocytes (COCs) were *in vitro* matured in (TCM-199) containing 0, EGF (10 ng/ml), CYST (100 µM/ml) or their combination at 37.5°C for 20 h. Percentage of full expanded oocytes (PFEO) and maturation rate of metaphase II oocytes (NMR), fertilization rate of COCs matured with different TCM types was determined. Fertilized ova were cultured for 5 days at 37.5°C for determining morula (MPR) and blastocyst (BPR) production rates. Results showed that PFEO was the highest (56.67%, P<0.05) EGF+CYST medium, followed by 44% in EGF medium and the lowest (35.91and 30.83%, P<0.05) in free or CYST media, respectively. The NMR was the highest (88.33%, P<0.05) in EGF+CYST medium, 80.44% in EGF and 75.52 and 72.5% in CYST and free media. IVM of oocytes in EGF+CYST medium improved (P<0.05) FR than in EGF, CYST or free media (62.06 *vs*. 50.63, 50.00 and 45.23%). The MPR, BPR, total cell number (TC)/blastocyst and inner cell mass (ICM) was the highest (29.14%, 25.63%, 136 and 38) of IVM in EGF+CYST (P<0.05). In conclusion, supplementing TCM-199 with 10 ng/ml EGF plus 100 µM/ml CYST improved IVM, cleavage rate and cellular count/blastocyst, while decreased apoptotic cells per embryo at blastocyst stage.

Key words: Rabbit · Oocytes · Epidermal Growth Factor · Cysteamine · *In vitro* Embryo Production

INTRODUCTION

The high reproductive efficiency of rabbits allowed their usage as a model in basic and applied reproductive technologies. Improving the genetic selection and reproductive management increased commercial rabbit breeder profitability [1]. Oocyte IVM is an essential step for *in vitro* production of embryos [2].

The IVM plays a vital role and is considered as a basic tool for technology of reproduction [3]. Maturation of oocytes includes cytoplasmic maturation (CMR), in term of some changes for oocyte development to be fertilized and pre-implant; and NMR to the resumption of meiosis and development to metaphase II stage [4]. Mammalian oocyte development *in vitro* was reported to be negatively affected by increasing oxidative damage [5].

Oxidative stress induces dysfunction of mitochondria, damage of Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA) and protein, inhibition of fertilization and embryo apoptosis, particularly at the blastocyst stage [6].

Epidermal growth factor is a mitogenic polypeptide consisting of 53 amino acid residues and three intra-molecular disulfide bridges that is involved in regulating cell proliferation in many cell types, including granulosa cells of pre-antral follicles [7, 8]. EGF stimulates NMR and CMR in different species including cat [9], mouse and pig [10] oocytes. Also, EGF may help in oocyte maturation by acting on cumulus cells of the ovine oocytes [2] and through the mitogen activated protein kinase pathway during IVM of goat cumulus cells [11] which was found to express receptors of EGF [12].

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In addition, cysteamine (CYST) is a low molecular weight compound that may decrease cystine to cysteine ratio, leading to enhancing synthesis of glutathione (GSH) in the oocytes [13]. The GSH is a tri-peptide thiol compound that has many important functions in intracellular physiology and metabolism such as maintaining the redox state in cells, improving formation of male pronucleus, protein and synthesis of DNA [14]. During *in vivo* maturation, GSH content increases in the oocytes to use for cell protection in later steps [15]. Several authors reported improvement in the developmental competence of mammalian oocytes and embryos by supplementation of different types of antioxidants and growth factors to IVC media [16, 17].

Rate of IVM, blastocyst formation, TC/blastocyst, cell ratio of the ICM and the developmental competence of the oocytes were improved by addition of EGF to the maturation medium [18, 19]. Also, EGF stimulates oocyte maturation by destroying communications between oocyte and cumulus cells or signaling pathways promoting oocyte maturation observed by Abkenar *et al.* [20]. However, adding CYST to the IVM media was demonstrated to improve the maturation rate and blastocyst yield by protecting the oocytes from oxidative stress [21, 22].

Apoptosis crucially involving in the development and differentiation of embryos [23] can be prevented by nerve growth factor [24]. Also, EGF and bovine serum albumin synergistically increased a net cell number in porcine presumptive diploid parthenotes developing *in vitro* [23].

The current study aimed to evaluate the effect of supplementing maturation medium with EGF (10 ng/ml), CYST (100 µM/ml) or their combination on maturation, fertilization and subsequent development *in vitro* of rabbit oocytes.

MATERIALS AND METHODS

This study was conducted at the International Livestock Management Training Center (ILMTC), IVF laboratory, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture.

Animals: Total of 24 mature New Zealand white (NZW) rabbit does and 6 NZW rabbit bucks were taken from the flock of rabbit branch of Sakha Experimental Station, Agricultural Research Center, Egypt. All chemicals used in this study are produced by Sigma Co., St. Louis, Mo, USA.

Harvesting the Oocytes: Animals were slaughtered and ovaries were immediately removed, washed by saline (0.9% NaCl) . Oocytes were collected by slicing technique into tissue culture dishes contained Dulbecco's phosphate buffer saline (DPBS) supplemented with fetal bovine serum (10%), sodium pyruvate (0.03%), penicillin (100 IU) and streptomycin (100µg/ml). Oocytes were examined and evaluated under stereomicroscopy, according to cumulus layer into compact (COCs), expanded, denuded and partial denuded oocytes. Only COCs were selected for IVM.

***In vitro* Maturation (IVM):** Tissue culture medium (TCM-199) supplemented with 10% bovine serum albumin (BSA, w/v), hormones including 10 IU of PMSG, 10 IU of hCG, 1 µg estradiol/ml, 0.03% sodium pyruvate and 50 µg/ml gentamycin sulfates was used as IVM medium. Four types of IVM medium were used involving free TCM and TCM supplemented with 10 ng/ml of EGF, 100 µM/ml of CYST or their combination.

Adjustment of IVM medium filtered by 0.22-µm millipore was achieved at 7.2-7.4 pH and 280-300 mOsmol/kg osmolarity. IVM medium was placed in Petri dishes, covered by sterile mineral oil and incubated at 5% CO_2 and 37.5°C with saturated humidity for 60 minutes. COCs were washed 3 times with IVM medium and incubated under the same conditions for 20 h as maturation time. The degree of cumulus expansion in *in- vitro* matured oocytes was classified according to Sreenivas *et al*. [25] into full expanded oocytes with homogenously spread cumulus cells; partial expanded oocytes with non-homogenously spread cumulus cells and no expanded oocytes with cumulus cells tightly adherent to the zona pellucide. Full expanded oocyte percentages were considered as cytoplasmic maturation rate (CMR).

Cytological study begins after determining cumulus expansion. COCs were washed by DPBS containing 1 mg/ml hyaluronidase and two times with PBS plus 2% BSA. Then, oocytes were loaded into Farmer's fluid (3 ethanol: 1 glacial acetic acid) for one day and stained with in a mixture of orcein and acetic acid (1:45%, respectively). The matured oocytes at different stages were examined and percentage of oocytes having emission of 1st polar body (Metaphase II`(MII) was considered as nuclear maturation rate (NMR).

Sperm Preparation: Semen from 10 buck ejaculates was capacitated in DPBS medium supplemented with BSA (3 mg/ml), gentamycin sulfates (50 µg/ml) and heparin (35 µg /ml). Oocytes matured by different types of TCM

were IVF by 50 µl of fertilization medium under sterile liquid paraffin oil and incubated at 37.5°C for 2 h in 5% Co_2 and higher humidity. About 50 µl washing medium was added to each droplet with 10-15 oocytes and then 2 µl of prepared semen was added and incubated together at 37.5°C for 1day in 5% Co_2.

***In vitro* Fertilization and Production of Rabbit Embryos:** Spermatozoa and mature oocytes were co-incubated for 24 h and then cumulus cells were separated from presumptive zygotes by pipetting and washed twice in culture medium comprising solve modified fertilization (SOF) medium containing BSA (3mg), Na-pyruvate (20µg) and gentamycin sulfates (50 µg /ml). Total of 10 presumptive embryos after cleavage were placed in each four-well Petri dish containing 100 µl of culture medium under mineral oil and incubated at 37.5°C under 5% CO_2 in humidified air for 5 days to determine the formation rate of morulae and/or blastocysts.

Differential Staining: Five embryos at blastocyst stage in each group (EGF or CYST and combination EGF with CYST were randomly selected and stained to determine a count of TC and ICM with fluorescence microscope as described earlier by Chrenek and Makarevich [26] with some modifications. The blastocysts were incubated for 20 seconds in freshly prepared Triton X-100 (0.2%) in PBS supplemented with 2 mg/ml of BSA and immediately washed two times in PBS-BSA medium. Blastocysts were transferred into PBS-BSA containing 30µg/ml of propidium iodide and incubated at 37°C for 5 minutes in a dark warm chamber. Then blastocysts were washed twice in PBS-BSA medium, incubated in 4% (*w/v*) paraformaldehyde supplemented with 10µg/ml bisBenzimide for 30 minutes at 25°C and washed twice in PBS-BSA medium, thereafter. The blastocysts were incubated for 5 minutes in a freshly prepared ice-cold solution of Triton X-100 (0.1%) plus sodium citrate buffer (0.1%) and then washed two times in PBS-BSA medium. Finally, the blastocysts were covered with 5 µl of Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), mounted on to glass slides with coverslip.

Analysis of Apoptosis (TUNEL): The blastocysts in each group were removed from culture medium, washed three times in PBS containing 4 mg/ml polyvinylpyrrolidone for 5 min, then fixed for 5 min in formalin (3.7%) and for 10 min in ethanol (70%). The blastocysts were incubated for 15 min in Triton X-100(0.5 %) in PBS for membrane permeabilization. The blastocysts were processed for TUNEL using a MEBSTAIN Direct Apoptosis Detection Kit (Immunotech, Marseilles, France). Then, blastocysts counter stained with 1 µg/ml of propidium iodide in PBS, washed 3 times, covered with Vectashield mounting medium (5 µl, Vector Laboratories, Inc., Burlingame, CA94010, USA) and attached to micro slide using small columns of nailpolish [27].

Statistical Analysis: Data were analyzed by Analysis of Variance (ANOVA) using computer program of SAS [28] after arcsine transformation of original data expressed as percentages. Duncan Multiple Range Test [29] was used to set the significant differences among means at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of EGF and CYST Supplements to Maturation Medium On

Cumulus Expansion: According to degree of cumulus expansion, maturation rate in term of the percentage of expanded oocyte was significantly ($P<0.05$) the highest (56.67%) in maturation medium supplemented with EGF and CYST combination, followed by EGF (44%), while free medium or that supplemented with CYST showed significantly ($P<0.05$) the lowest values, (35.91and 30.83%, respectively). However, the effect of supplements on partial expansion was not significant. Yet, percentage of non-expanded oocyte showed an opposite trend to those of full expanded oocyte. It is of interest to note that effect of EGF or CYST was nearly similar on cumulus expansion, but there was a synergetic effect of both on cumulus expansion (Table 1).

In accordance with the present results, Sadeesh *et al.* [30] reported higher ($P<0.05$) expansion rate of bovine oocytes in maturation medium including EGF + β-mercaptoethanol (β-ME) as compared to free medium.

In sheep oocyte, Wani *et al*. [19] recorded higher ($P<0.05$) expansion of oocyte matured with EGF while those matured with CYST medium did not differ significantly from free medium. Similar trend was obtained by Sofi *et al*. [2] who found significantly ($P<0.05$) higher ovine oocyte expansion in maturation medium containing EGF than with CYST and control medium. Moreover, it was reported that cumulus expansion was significantly ($P<0.05$) higher for medium containing EGF than free medium in goats [31] and porcine oocytes [32].

During IVM, EGF seems to change proteins synthesized the pattern by increasing the activity of histone and mitogen-activated protein kinase during the early stages of IVM, leading to oocyte meiotic division acceleration [17]. Although, CYST supplementation to

Table 1: Effect of supplementation of maturation medium on cumulus expansion rate (%) of rabbit oocytes

		Cumulus expansion (%)		
Maturation medium	No. of oocytes	Full expansion	Partial expansion	No expansion
Control	120	30.83±2.2[c]	36.67±3.60	32.50±5.8[a]
EGF	130	44.00±2.31[b]	40.89±3.69	15.11 ±1.5[bc]
CYST	135	35.91±4.2[bc]	44.09±4.20	20.00±0.0[b]
EGF+CYST	120	56.67±0.8[a]	35.00±1.40	8.33±0.80[c]

[a, b and c:] Means denoted within the same column with different superscripts are significantly different at $P \leq 0.05$.

Table 2: Effect of supplementation of maturation medium on different nuclear maturation stages of rabbit oocytes

		Stage of nuclear maturation (%)				
Maturation medium	No. of oocytes	GV	GVBD	MI	MII	DEG
Control	120	1.67±0.8	2.50±0.0	3.33±0.8	72.50±1.4[c]	20.00±2.5[a]
EGF	130	1.33±0.6	2.44±0.4	6.44±1.9	80.44±1.6[b]	9.33±0.7[bc]
CYST	135	3.74±0.8	3.07±0.9	5.29±1.9	75.52±0.3[c]	12.37±1.8[b]
EGF+CYST	120	1.67±0.8	1.66±0.8	2.50±0.0	88.33±0.8[a]	5.83±0.8[c]

[a, b and c:] Means denoted within the same column with different superscripts are significantly different at $P \leq 0.05$.

maturation medium was reported to increase the cytoplasmic glutathione content and subsequent embryo development [33] the present poor results of CYST for oocyte cumulus expansion may be related to variation in composition of the media and animal species of oocyte donors [19].

Nuclear Maturation (NMR): The NMR of the oocytes, based on percentage of oocyte at MII (Table 2) showed that the NMR of oocyte was significantly ($P<0.05$) the highest (88.33%) in maturation medium supplemented with EGF and CYST combination, moderate with EGF (80.44%) and the lowest in CYST (75.52%) and free medium (72.50%). However, the differences in percentage of oocytes at GV, GVBD and MI stages were not significant. On the other hand, percentage of degenerated oocytes showed an opposite trend to those at MII stage.

In agreement with the present results, Sadeesh *et al.* [30] found that the proportion of oocytes with extruded first polar body was the highest in maturation medium with EGF + β-ME (82.5%) as compared to control medium (57.5%). Also, combined EGF, hyaluronic acid and CYST in maturation medium could be used to improve oocyte meiotic competence [34]. The observed improvement in NMR in medium with EGF as compared to control medium (Table 2) agreed with the results of Kandil *et al.* [35] who mentioned that EGF addition to maturation medium increased the maturation rate. Also, EGF supplementation to IVM medium may promote NMR and CMR [16].

The EGF play important role in the subtle coordination of proliferation and differentiation of the cells [30] and enhancing synthesis of proteoglycan and tissue plasminogen activator and urokinase plasminogen activator produced by cumulus cell layer. Also, it may be due to DNA synthesis stimulation in cumulus cells by EGF and FSH [35].

The recorded insignificant effect of CYST on CMR and NMR (Tables 1 and 2) was proved by several authors, indicating insignificant effect on maturation rate of oocytes matured in CYST and control media in ovine [2, 19] and mice [21]. Contrary, addition of CYST in maturation medium of oocytes improved maturation rate and increased significantly intracytoplasmic level of GSH in buffalo oocytes. Interestingly to observe similar synergetic effect of EGF or CYST on NMR as found on cumulus expansion, indicating beneficial effect of both supplements meiosis division of oocytes during IVM.

***In vitro* Fertilization:** Cleavage rate was significantly ($P<0.05$) higher (62.06%) for oocytes matured with EGF and CYST combination than that matured with EGF or CYST alone (50.63 and 50.00%) and even than that matured with the control medium (45.23%, Table 3).

Concerning the embryonic stage, oocyte matured with EGF and CYST combination showed significantly ($P<0.05$) the highest rate of morula (29.14%) and blastocyst (25.63%) formation and the lowest (26.04%) rate of embryos at 2-8 cell stage. However, embryos at 8-16 cell stage were not affected by medium supplementation. In comparison with all medium supplements, the free medium showed the lowest values (Table 3). Such findings indicated the positive effect of medium supplemented with EGF and CYST combination on rate of fertilization and morulae/blastocyts formation,

Table 3: Effect of supplementation of maturation medium on cleavage rate (%) and developmental competence of rabbit oocytes cultured *in vitro*

			Embryonic stage (%)			
Maturation medium	No. of oocytes	Cleavage rate (%)	2-8 cell	8-16 cell	Morula	Blastocyst
Control	105	45.23±2.9[b]	40.42±1.8[a]	27.64±1.9	17.08±2.3[b]	14.86±1.9 [b]
EGF	95	50.63±1.4[b]	29.25±2.4[b]	28.85±4.6	23.10±2.8[ab]	18.80±0.7 [b]
CYST	100	50.00±0.8[b]	41.50±4.8[a]	20.07±1.9	22.18±2.6[ab]	16.25±2.7 [b]
EGF+CYST	100	62.06±1.0[a]	26.04±2.79[b]	19.19±2.0	29.14±1.3[a]	25.63±2.3 [a]

[a and b:] Means denoted within the same column with different superscripts are significantly different at $P \leq 0.05$.

Table 4: Effect of supplementation of maturation medium on total cell number/blastocyst, number of inner cell mass (ICM) and apoptotic cells (%) of rabbit oocytes

Maturation medium	Total cell number/blastocyst	Number of ICM cells	Apoptotic cells (%)
Control	114±2.9[c]	28±0.9[c]	4.2±0.4[a]
EGF	126±2.9[b]	33±2.0[b]	3.0±0.5[ab]
CYST	120±0.0[bc]	27±2.0[c]	3.6±0.4[ab]
EGF+CYST	136±1.8[a]	38±1.2[a]	2.4±0.2[b]

[a, b and c:] Means denoted within the same column with different superscripts are significantly different at $P \leq 0.05$.

while medium supplemented with EGF or CYST alone had beneficial effect on these traits but the differences were not significant as compared to control medium.

The noted improvement significantly in cleavage rate and morulae/ blastocysts formation with EGF and CYST combination was observed when oocytes were matured in maturation medium with EGF+β-ME as reported by Sadeesh *et al.* [30]. In agreement with the present result, Wani *et al*. [19] showed that both EGF and CYST supplementation to maturation medium enhanced the IVP rates in sheep. However, the differences in cleavage rate were not significant between ovine oocytes matured by media containing CYST, EFG and control (52.7, 55.5 and 49.5%, respectively. Also, fertilization rate and blastocyst stage was not significantly higher in the treatment groups by EGF than control group (79.7, 79.5, 43.9and 43.8, respectively) as obtained in our study [16].

The impact of EGF and CYST on IVM may be the main reason for improving cleavage and developmental competence of rabbit oocytes, because fertilization needs the conditions that support the viability of the oocytes to be fertilized.

Cellular Count and Apoptosis: Mean of TC/blastocyst and ICM was significantly (P<0.05) the highest for embryos produced from oocytes matured by EGF and CYST combination (136 and 38) and those matured with EGF medium significantly (P<0.05) ranked the second, while those matured with CYST or free medium showed significantly (P<0.05) the lowest number (114 and 28, Table 4). It is worthy noting that the observed increases in the total blastocyst cell numbers was mainly due to increases in the numbers of ICM cells. These results may be attributed to that EGF stimulates proliferation and differentiation of many somatic cells [36] and increases the cellular number in blastocysts derived from IVF in bovine [37] in particular, total yak blastocyst cell number [38]. Data on the effect of CYST in maturation medium to regulate the molecular mechanisms and subsequently improving quality of mammalian blastocysts is scar. Moreover, Karadjole *et al.* [39] demonstrated that the supplementation of CYST to maturation media significantly improved the rate of blastocyst development and the quality of blastocyst by increasing inner cell mass cell number.

It is of interest to note that the recorded improvement in cumulus expansion, NMR and developmental competence of rabbit oocytes as affected by IVM medium with EGF and CYST combination was associated with enhancing cellular number of blastocyst and ICM as well as the lowest percentage of appoptic cells/blastocyst, being significantly (P<0.05) the lowest as compared to medium supplemented with EGF, CYST or free medium (Table 4).

It was reported that, the presence of growth factors in maturation medium may lead to improving growth, chromosome quality, unchanged gene expression, good metabolism and decreasing level of apoptosis during IVC [40, 41]. As reported in majority of mammalian blastocysts, the current study may suggest that apoptosis could be detected also in rabbits as found in bovine [42] and porcine [43]. In this way, surviving protects embryos from apoptosis via inhibition of the apoptotic pathway [44].

CONCLUSION

Supplementation of maturation medium with a combination of EGF and CYST at levels of 10 ng and 100µM/ml, respectively, lead to improving embryo production *in vitro* in term of increasing cumulus expansion, NMR, cleavage rate and cellular count and decreasing apoptotic cells per embryo at blastocyst stage.

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