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Research Article

Effect of Cysteamine and 13-Cis-Retinoic Acid on Bovine *In Vitro* Embryo Production

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Abstract

One of the main factors decreasing the success of an *in vitro* embryo production is oxidative stress-mediated reactive oxygen species resulting in cellular damages. The present study was carried out to evaluate the effects of addition of different doses of cysteamine and 13-cis-retinoic acid during *in vitro* maturation on oocyte maturation and blastocyst formation. Five-hundred oocyte complexes were randomly collected from slaughterhouse and assigned to five groups; oocytes matured *in vitro* maturation (IVM) medium without any supplementation (control), IVM medium supplemented with 50 µM or 100 µM cysteamine, IVM supplemented with 5 nM 13-cis-retinoic acid and IVM supplemented with 10 nM 13-cis-retinoic acid. Matured oocytes were fertilized and cultured. Oocytes and embryos were evaluated for nuclear maturationand blastocyst formation, respectively. The highest numbers of oocytes in the cumulus expansion, cleavage, murola and blastocyst stages were seen in cows treated with 13-cis retinoic (5 nm) (92%), cysteamine (100 µL) (74%), cysteamine (50 and 100 µL) (48%), cysteamine (100 µL) and 13-cis retinoic (24%) and finally cysteamine (100 µL) (22%), respectively.Nuclear maturation of oocytes decreased significantly in the media supplemented with 10 nM 13-cis-retinoic acid ($P<0.05$). The results of the present study demonstrated that IVM supplementation with cysteamine and a low concentration of 13-cis-retinoic acid improved the efficacy of an *in vitro* production of bovine embryo.

Keywords: *Cysteamine, 13-cis-retinoic acid, Bovine, In vitro embryo production*

Sığır *İn Vitro* Embriyo Üretiminde Sistamin ve 13-Cis-Retinoik Asitin Etkisi

Öz

In vitro embriyo üretiminin başarısını azaltan en önemli faktörlerden biri oksidatif stres aracılı reaktif oksijen türleri ve bunun sonucu oluşan hücresel hasarlardır. Bu çalışma, *in vitro* olgunlaşma süresince farklı dozlarda sistamin ve 13-cis-retinoik asit ilavesinin oosit olgunlaşması ve blastosit oluşumu üzerine etkililerini araştırmak amacıyla yapılmıştır. Beş yüz oosit kompleksi kesimhaneden rastgele toplandı ve beş gruba ayrıldı; ilave katkısız *in vitro* olgunlaştırma medyumu (IVM)'nda olgunlaşan oositler (kontrol), 50 µM veya 100 µM sistamin katkılı IVM'de olgunlaşan oositler, 5 nM 13-cis-retinoik asit katkılı IVM'de olgunlaşan oositler ve 10 nM 13-cis-retinoik asit katkılı IVM'de olgunlaşan oositler. Olgunlaşan oositler fertilize ve kültüre edildi. Oositler ve embriyolar sırasıyla nükleer olgunlaşma ve blastosit oluşumu bakımından değerlendirildi. Kümülüs genişlemesi, çentiklenme, morula ve blastosit evrelerindeki en yüksek oosit sayıları sırasıyla 13-cis-retinoik (5 Nm) (%92), sistamin (100 µL) (%74), sistamin (50 ve 100 µL) (%48), sistamin (100 µL), 13-cis-retinoik (%24) ve son olarak sistamin (100 µL) (%22) uygulananlarda gözlemlendi. Oositlerin nükleer olgunlaşması 10 nM 13-cis-retinoik asit katkılı medyumda anlamlı oranda düştü ($P<0.05$). Bu çalışmanın sonuçları IVM içerisine sistamin ve düşük konsantrasyonda 13-cis-retinoik asit katkısının sığır embriyosunun *in vitro* üretimini iyileştirdiğini göstermiştir.

Anahtar sözcükler: *Sistamin, 13-cis-retinoik asit, Sığır, İn vitro embriyo üretimi*

INTRODUCTION

An *in vitro* production (IVP) of bovine embryos has become a popular technology which may solve part of reproductive issues in modern dairy farms. Achieving appropriate protocols and media which produce highly qualified embryos may improve the efficiency of IVP which is still as low as 30-40% [1]. The procedures of the IVP of embryo are consist of an *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of embryos. IVP is a sequential process and failure in each step may influence the final result [2-4]. One of the most important factors with detrimental effects on the quality of gametes and embryos under culture conditions is oxidative stress

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(OS) [2-4]. Oxidative stress induces mitochondrial, DNA, RNA, and protein damages [2-4], inhibition of sperm-oocyte fusion [2-4] and finally embryo cell apoptosis [2-4]. Free radicals such as reactive oxygen species (ROS) interact with oocytes and embryos and decrease blastulation rates in embryo development [2-4]. Therefore, it is important to decrease the levels of ROS formation during an IVP.

Progress toward the simplification and modification of a medium used for IVP, especially for the reduction of ROS, may increase the efficacy of embryo production in commercial aspects. *In vivo* mammalian cells possess efficient antioxidant systems such as catalase or superoxide dismutase as well as thiol compounds that act as metabolic buffers which scavenge active oxygen species. These systems are more critical for important processes such as the maturation, formation, and growth of gametes and embryos. In recent years, various materials with antioxidative properties have been examined to reduce the risk of ROS formation in IVP procedure [5].

Vitamin A is a fat-soluble unsaturated isoprenoid and is well known to regulate development, cellular growth, differentiation, and maintenance of tissue function. Vitamin A may influence bovine ovarian follicular development, steroidogenesis, oocyte maturation, embryo and conceptus development and uterine environments [6-8]. The effects of vitamin A and its metabolites including 9-cis-retinal forms photoactive isorhodopsin, 11-cis retinal forms which is present in the retina, and 13-cis-retinoic acid which is a metabolite of retinol found in many tissues of bovine reproduction is not only exerted as an antioxidant, but also acts as a local modulation for the development and differentiation of cells [6-8]. Xing and Sairam [9] showed that retinoid has direct effects on the modulation of the gonadotropin receptor promoter [9]. Another way by which vitamin A is postulated to affect reproduction is the induction of midkine (neurite outgrowth-promoting factor 2) by retinoic acid (RA) in cumulus-granulosa cells [10]. Moreover, some studies have suggested that RA may regulate nitric oxide synthesis in granulosa cells [11]. In the past two decades, a number of studies have examined different concentrations of cis-retinoic acid on the efficacy of bovine IVP media. Results showed that the addition of cis-retinoic acid into the IVM medium increased bovine blastocyst development and hatching rate [12]. Duque et al.[8] showed that the cytoplasmic competence of bovine oocytes improved in the presence of cis-retinoic acid during prematuration. Moreover, Livingston et al.[13] demonstrated that the addition of retinol to the IVM medium may improve the embryonic development and blastocyst rate of bovine oocytes.

Another way to scavenge active oxygen species is the synthesis of glutathione (GSH) which protects the cell from OS damages. The production of GSH depends on the existence of cysteine [14], and the synthesis of cysteine depends on the availability of cysteamine as a thiol compound that reduces cystine to cysteine [15]. The effect of cysteamine supplementation to IVM media on nuclear maturation rates, cleavage rates, and blastocyst development is controversial. Balasubramanian et al.[16] indicated that the supplementation of cysteamine during bovine IVM had no effect on the cleavage rate but enhanced embryo development. Furthermore, Oyamada et al.[17] concluded that the addition of cysteamine to IVM had no effect on the nuclear maturation of oocytes but improved the fertilization rate and developmental competence of bovine embryos.

Regarding uncertain roles of cysteamine and 13-cis-retinoic acid on bovine *in vitro* embryo production, the present research was planned to test the effects of different concentrations of cysteamine and 13-cis-retinoic acid on nuclear maturation rate, cumulus expansion, cleavage rate, and morula and blastocyst formation in an *in vitro* bovine embryo production.

MATERIAL and METHODS

Ethical Consideration

The study was approved by the Ethical Council of Research of the Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran. Iran (910950746). Verification of this research project and the licenses related to sampling process were approved by the Prof. Mohammad Amin Eslampour and Dr. Mehran Farhoodo Moghadam.

Chemical Reagents

All chemicals used in the present study were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

Collection of Oocytes

Bovine ovaries were obtained from slaughtered cows (Isfahan, Iran) and placed in a thermoflask containing 0.9% NaCl supplemented with 100 IU/mL penicillin and 100 µg/mL of streptomycin at 30-35°C and transported to the laboratory within 1 h. Ovaries were washed twice in distilled water and once in freshly prepared saline. Cumulus-oocyte complexes (COCs) were aspirated from the antral follicles of 2-8 mm using an 18-gauge needle attached to a 10 mL sterile disposable syringe, pooled in 50 mL conical tubes, and allowed to gravitate for 10 min. COCs were assessed morphologically and only the oocytes with three or more layers of compact and non-atretic cumulus with a homogeneous cytoplasm were selected.

Experimental Design

From June to August 2017, a total of 500 oocytes were divided in five maturation media, including Group (1):

without any supplemented material (control); Group (2): IVM supplemented with 50 µM cysteamine; Group (3): IVM supplemented with 100 µM cysteamine; Group (4): IVM supplemented with 5nM 13-cis-retinoic acid; and Group (5): IVM supplemented with 10 nM 13-cis-retinoic acid. The 13-cis-retinoic was solved in ethanol, aliquoted, and stored at -20°C in darkness. In each group, 50 oocytes were assessed for nuclear maturation and 50 oocytes were placed in the IVP protocol.

In Vitro Maturation (IVM)

All selected COCs were washed in triplicate in washing medium (90% H-Tissue culture medium-199 (TCM 199), 10% Fetal calf serum (FCS), 2.5 mg/mL $NaHCO_3$, 5 µL/mL GlutaMAX, 1 µg/mL penicillin/streptomycin). COCs were incubated in maturation media (using 5-10 COCs per 50-100 µL of maturation medium) in tissue culture dishes under mineral oil and matured for 22-24 h at 38.5°C in an atmosphere of 5% CO_2 in highly humidified air. The base of IVM medium was 25 mM HEPES-buffered TCM 199 supplemented with 2 mM sodium pyruvate, 1 mM l-glutamine, gentamicin (50 µg/mL), 10% steer serum, porcine FSH (1 µg/mL), and 17 beta-estradiol (1 µg/mL).

In Vitro Fertilization (IVF) and In Vitro Culture (IVC)

Sperm separation was performed using a swim-up procedure. The semen from a single bull which had been previously tested for IVF efficiency in the laboratory was used. A total of 1000 µL semen was used for swim up. The frozen-thawed semen was carefully added to button of tube of 1.5 mL Tyrode's albumin lactate pyruvate (TALP) and incubated for 1 h. After that, 500-700 µL of the upper layer of the supernatant containing motile spermatozoa was removed. The spermatozoa were centrifuged at 200 × g for 10 min provided pellet formation and supernatant had been removed then pellet was resuspended with 0.6-0.8 mL of HEPES-TALP medium. Spermatozoon concentration was determined with a haemocytometer, and IVF was accomplished by incubating oocytes and sperm cells together for 18-20 h at 39°C in 5% CO_2 and high humidity. Heparin was used as a capacitor agent in IVF medium. The concentration of spermatozoa was of 2×10^6 cells/mL in 200 Lof medium containing 25 COCs per well.

After 18-20 h, the presumptive zygotes were removed from the IVF medium, cumulus cells were denuded by repeated gentle pipetting, washed several times in a pre-warmed culture medium, and then transferred into IVC droplets made of modified synthetic oviductal fluid (mSOF) added with 1 mM glucose and 3 mg/mL of bovine serum albumin (BSA). The embryo culture was maintained at 39°C, 5% CO_2, 5% O_2, and 90% N_2 under mineral oil and examined under a stereomicroscope after 24 h for fertilization rate. On Day 2-3 of IVC, cleavage rate was evaluated, unfertilized oocytes were removed, and cleavaged embryos were transferred into fresh IVC medium. On Day 5-7, embryos were evaluated for morula and blastocyst formation.

For the morphological evaluation of oocytes, the cumulus cells were first stripped in a medium containing hyaluronidase (0.01% w/v). Then, denuded oocytes were fixed in 60% methanol and stained with 1 mg/mL of Hoechst 33342 (Sigma-Aldrich) to assess their nuclear maturation status. Finally, nuclear maturation rate was assessed by evaluating oocytes that reached the M II stage.

Statistical Analysis

Statistics were subjected to Microsoft office Excel (version 15; Microsoft Corp., Redmond, WA, USA). Statistical analysis was performed by means of the SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences among different groups were analyzed by One Way analysis of variance (One Way ANOVA). Moreover, differences among means were analyzed by Duncan's test. A probability of P value <0.05 was considered to be statistically significant.

RESULTS

Totally, two-hundred and fifty oocytes in different groups were evaluated for nuclear maturation (MII). Oocytes staining method with Hoechst (nuclear maturation) is shown in *Fig. 1*. *Fig. 2* represents the nuclear maturation rate (MII) in bovine oocytes matured in the control and other supplemented IVM groups. The proportion of oocytes that reached the metaphase II stage was similar in control, cysteamine, and RA (5 nm)-treated group. However, statistically significant difference was seen for the proportion of oocytes reached to Metaphase II stage between 10 nM RA and other studied groups (P<0.05).

A total of 250 oocytes were placed in five groups and

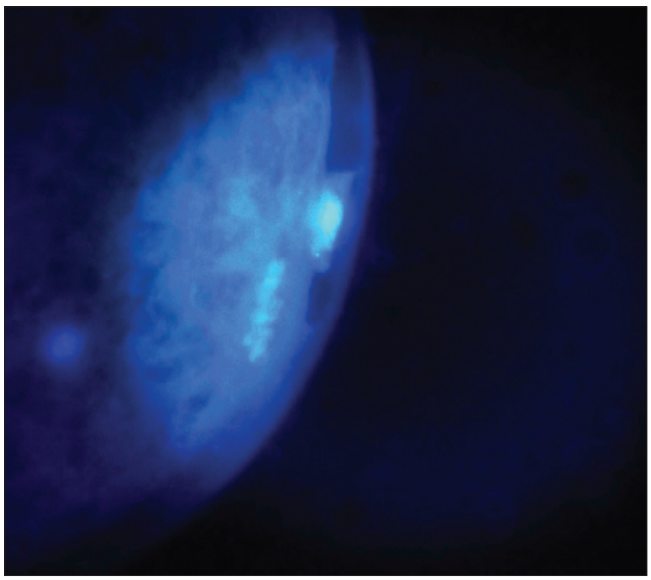

Fig 1. Oocytes appearance stained with Hoechst (nuclear maturation) staining method

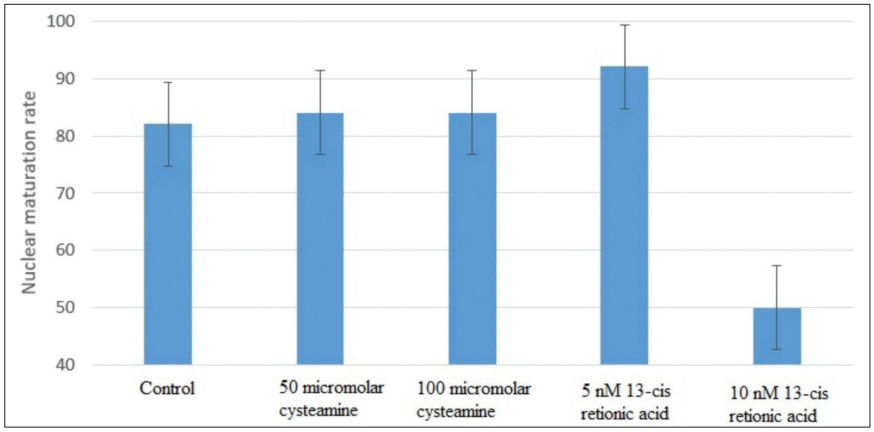

Fig 2. Nuclear maturation rate (MII) in bovine oocytes matured in the control and other supplemented IVM groups. Different superscripts show significant differences (P<0.05)

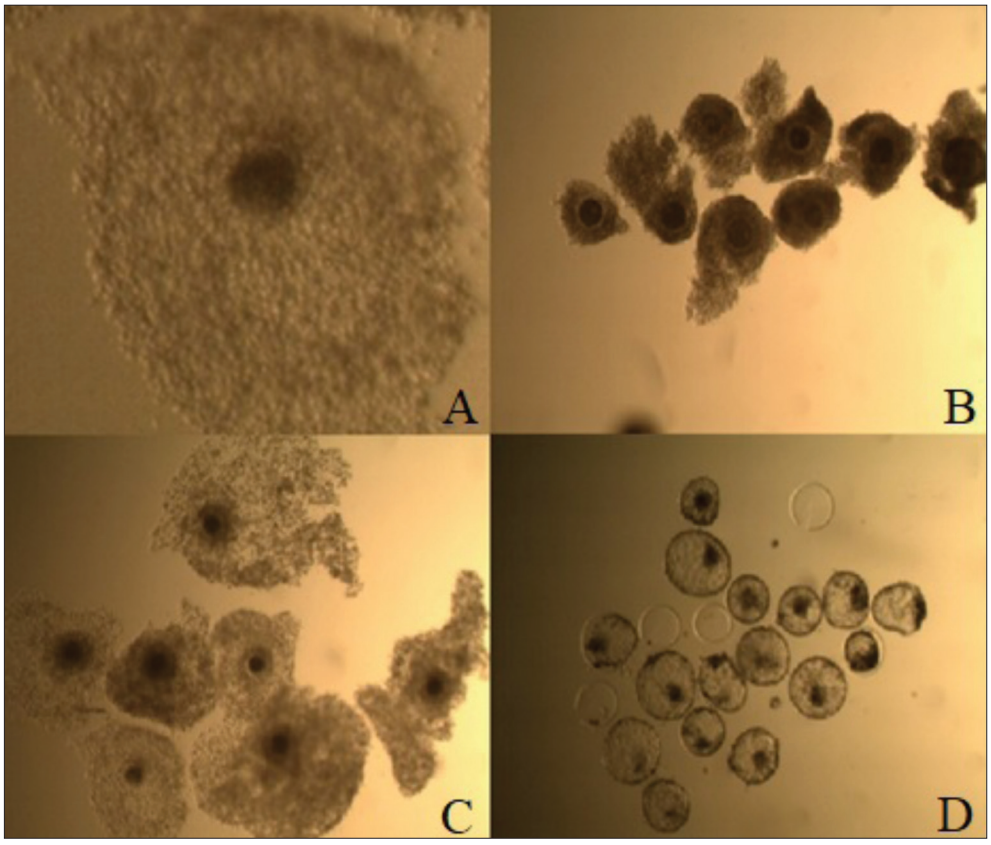

Fig 3. Cumulus expansion of COCs (A), immature oocytes (B), mature oocysts (C) and blastocyst (D)

evaluated for cumulus expansion, cleavage rate, morula, and blastocyst formation. *Fig. 3* represents the cumulus expansion of COCs (A), immature oocytes (B), mature oocysts (C) and blastocyst (D). *Fig. 4* represents the cleavage process 3 days after fertilization.

Table 1 represents the cumulus expansion, cleavage rate, morula and blastocyst formation in bovine oocytes matured in the control and other supplemented IVM groups. A total of 500 oocytes were studied. Each group contained 100 oocytes and a total number of 50 oocytes were studied for nuclear maturation and other 50 oocytes were entered to cumulus expansion, cleavage, murola and blastocyst stages. Cows treated with 13-cis retinoic (5 nm) had the highest numbers of oocytes in the nuclear maturation (92% (46/50). We found that the highest numbers of oocytes in the cumulus expansion, cleavage, murola and blastocyst stages were seen in cows treated with 13-cis retinoic (5 nm) (92%), cysteamine (100 µL) (74%), cysteamine (50 and 100 µL) (48%), cysteamine (100 µL) and 13-cis retinoic (24%) and finally cysteamine (100 µL) (22%), respectively. There were significant differences in cumulus expansion and cleavage rate between cows treated with 13-cis-retinoic acid (10 nm) and other studied groups. After cleavage, the percentage of embryos that reached morula and blastocyst stages were higher in oocytes matured in the medium supplemented with cysteamine and 13-cis-retinoic acid (5 nm) (P<0.05) than those matured in media supplemented with 13-cis-retinoic medium (10 nm).

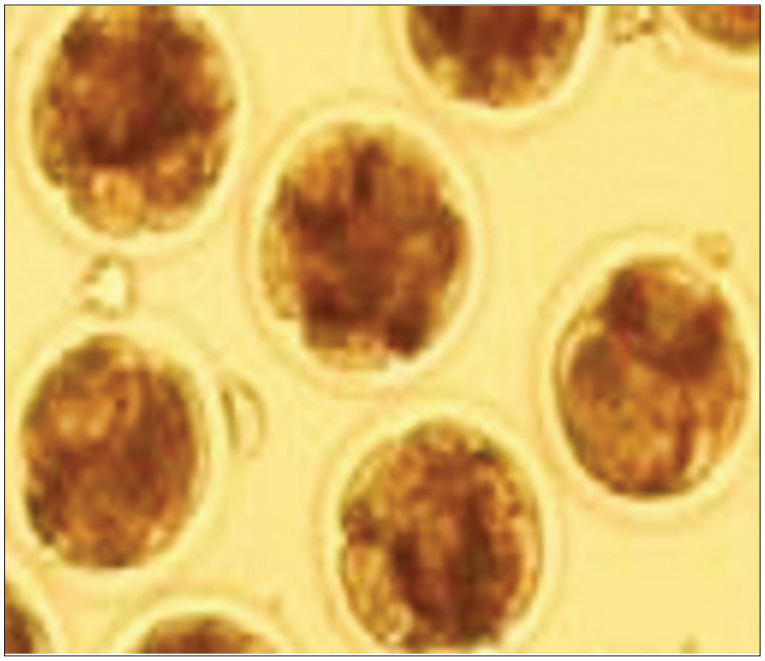

Fig 4. Cleavage process 3 days after fertilization

of bovine oocytes improved in the presence of 9-cis-retinal acid during pre-maturation. Moreover, Livingston et al.[13] demonstrated that the addition of retinol to the IVM medium may improve the embryonic development and blastocyst rate of bovine oocytes. Nasiri et al.[18] reported that the rate of oocytes that reached to the metaphase II stage of maturation significantly increased with 2 and 4 μM t-RA compared to the control and sham groups ($P<0.05$). Additionally, number of fertilized oocytes was significantly higher in 4 μM RA compared to the control ($P<0.05$) which was mainly similar to our findings. Rodríguez et al.[19] reported that retinoid prescription caused significant increase in the blastocyst development in bovine embryonic cells. Akçay et al.[1] reported similar findings about the maturation and fertilization of bovine oocytes under the effects of serum, steroid and gonadotropins. Similar findings about the effective activities of cis-retinal acids on proportion of oocytes that reached the meta-

Table 1. *Cumulus expansion, cleavage rate, morula and blastocyst formation in bovine oocytes matured in the control and other supplemented IVM groups*

Groups	Nuclear Maturation	Stages (%)*			
		Cumulus Expansion	Cleavage	Murola	Blastocyst
Control	41 (82)	35 (70)	23 (46)	8 (16)	6 (12)
Cysteamine (50 μL)	42 (84)	34 (68)	24 (48)	11 (22)	10 (20)
Cysteamine (100 μL)	42 (84)	37 (74)	24 (48)	12 (24)	11 (22)
13-cis retinoic (5 nm)	46 (92)	34 (68)	18 (36)	12 (24)	10 (20)
13-cis retinoic (10 nm)	25 (50)	20 (40)	12 (24)	6 (12)	4 (8)

** Numbers of oocytes in each group is 50*

DISCUSSION

The preservation, maturation, formation, and growth of gametes and embryos in an *in vitro* condition are inferior to those of *in vivo*, indicating that an *in vitro* condition induced various cellular and metabolic stress situations and, therefore, these cells need more attention and energy to adapt to the environment. In the past three decades, *attempts* have been made to achieve appropriate *in vitro* media for decreasing these stresses and increasing capabilities for creation of embryos with acceptable fertility.

The effects of retinol as an antioxidant in IVP on nuclear maturation rates, cleavage rates, and blastocyst development were examined by some researchers [1-4,8,13,18]. This study evaluated different concentration of 9-cis-retinal acid on the efficacy of bovine IVP media is examined in a study and results revealed that the addition of 9-cis-retinal acid into the IVM medium increased bovine blastocyst development and hatching rate [12] which were similar to our findings. Duque et al.[8] showed that the cytoplasmic competence

phase II stage and also *in vitro* embryo production were reported from Spain [20], India [21], Iran [22] and United States [23]. Öztürkler et al.[2] reported that the percentages of oocyte maturation, cleavage, 8-cell and morula stage embryos in L-ergothioneine supplemented group were significantly higher compared with the other groups. They showed that the percentages of oocyte maturation in L-ergothioneine group and L-ascorbic acid group were 2.67 and 1.22 times higher than control group, respectively. Similar reproductive effects have been reported for nitric oxide concentrations, estradiol-17β progesterone in cows by Pancarci et al.[3] and Pancarci et al.[4].

However, the possible mechanisms of the positive effects of RA on oocytes are hypotheses, but RA may promote cytoplasmic maturation of oocytes via its modulatory effects on the gene expression of gonadotropin receptors, midkine, cyclooxygenease-2 and nitric oxide syntheses in cumulus-granulosa cells [8,24]. Additionally, RA induced cortical granules prior to maturation. Also, the cortical granules distribution after RA exposure formed a uniform

monolayer beneath the oolemma with lesser clustering once RA pre-matured oocytes were allowed to mature in the absence of RA [8,24]. Moreover, treatment of cumulus-enclosed oocytes with cis RA during meiotic arrest was observed to improve cortical granule migration, increase subsequent blastocyst development and increase total cell number [8,24]. Gomez et al.[25] suggested that retinoid administration may improve mRNA quality based on the observation that 9-cis RA increased poly-(A) mRNA content in meiotically arrested oocytes. Poly-(A) mRNA content of oocytes treated with 9-cis RA or ethanol vehicle was greater in matured oocytes than in oocytes prematured in the presence of 9-cis RA and then matured. These results suggest a role for RA in the improvement of developmental competence of oocytes. However, the exact timing (and possibly also the concentration) of RA exposure is critical since it alters the normal RA migration and distribution.

The effect of cysteamine supplementation to IVM media on nuclear maturation rates, cleavage rates, and blastocyst development is controversial [16,17]. Balasubramanian et al.[16] evaluated the effect of cysteamine (100 µM) of the IVM medium on chilled and non-chilled embryos. Cysteamine supplementation during IVM had no significant effect on oocyte maturation or fertilization but increased the proportions of oocytes developing to the blastocyst stage ($P<0.05$). Furthermore, Oyamada et al.[17] reported that the addition of 100 µM cysteamine to the IVM medium of bovine oocytes significantly increased ($P<0.05$) the intracellular GSH concentration in the oocytes, improving capacity of fertilization and development competence following vitrification, but had no positive effect on nuclear maturation. Dematos et al.[26] were the first that examine the effects of 100 µM cysteamine in IVM on GSH synthesis, subsequent development of embryos and the ability of freezing of bovine embryos. They showed that the addition of cysteamine in IVM increases the GSH of *in vitro*-matured oocytes to protect the embryo until the blastocyst stage, thus increasing the efficiency of *in vitro* blastocyst production from immature oocytes [26]. The effect of cysteamine added during the IVM (0, 50, 100, and 200 µM) of sheep oocytes on GSH synthesis and embryo development was examined by Zullo et al.[3] which represented similar findings with those of cow. Dematos et al.[27] evaluated the effect of cysteamine in maturation and culture medium on the developmental rate and embryo quality of bovine oocytes. They showed that the percentage of embryos developed to the blastocyst stage was significantly higher ($P<0.05$) when 100 µM of cysteamine was added during IVM, and this was further improved when 100 and 50 µM of cysteamine were present during IVM and IVC, respectively ($P<0.05$). Cysteamine supplementation during IVM improves embryo development and better-quality embryos can be obtained if cysteamine is also added during the first stages of embryo development [27]. Lojkic et al.[28] cultured bovine embryos in different concentrations (0, 50, 100, and 200 µM) of cysteamine. The results of their study revealed that the supplementation of IVC media with 100 µM cysteamine increased the blastocyst yield ($P<0.05$) without affecting the hatching rate. Furthermore, the addition of 100 µM cysteamine to an IVC media improved embryo quality, which may lead to the improvement of the IVC system for bovine embryos [28]. Significant effects of cysteamine on an *in vitro* maturation and embryo development were also reported from Japan [29], Turkey [30], Italy [31], Belgium [15] and Netherlands [32].

The results of our study showed that cysteamine supplementation and a low concentration of 13-cis-retinoic acid in IVM had no positive effect on nuclear maturation in comparison to the control group but improved the embryo development of bovine oocyte. In the present study, although nuclear maturation rate and the first step of embryo development were similar among cysteamine, low concentration of 13-cis-retinoic acid, and control groups, the cumulative effect of supplementation was detected in the blastulation formation of embryos. These results suggest that, when cysteamine stimulates GSH synthesis during IVM, increased sources of GSH help embryos develop faster and reach the blastocysts stage. In addition, 13-cis-retinoic acid in the first step of IVP may improve the maturation, development, and differentiation of embryonic cells [33,34].

In conclusion, we have demonstrated that enriching the IVM medium with cysteamine and a low concentration of 13-cis-retinoic acid improves IVEP efficiency in cattle. It is likely that the presence of cysteamine and a low concentration of 13-cis-retinoic acid should promote early embryo development by facilitating the completion of oocyte cytoplasmic maturation, ensuring a normal pro-nuclear development and hence improving cleavage rate and the overall embryo yield. In future, these results may be of great help for obtaining a culture medium most suitable for oocyte nuclear and cytoplasmic development.

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