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# Effect of Enrichment of *In Vitro* Fertilization Medium with Cysteine on Fertilization and Embryo Development in Buffaloes

Research Article

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## Abstract

The current study aimed to elucidate the effect of adding various concentrations of cysteine to the *in vitro* fertilization culture medium on the fertilization rate and following embryonic development of buffalo oocytes. *In vitro* matured oocytes were fertilized using different concentrations of cysteine (0.5, 1.3 mM) in the presence of 5.0 mM caffeine. After *in vitro* fertilization, fertilization rate, cleavage rate and developmental rates till the blastocyst stage have been evaluated. The present results revealed that supplementation of the fertilization media with 0.3 Mm cysteine enhanced significantly ($P<0.05$) the fertilization rate and the development rates till the morula and blastocyst stages (53.45% ± 6.61%, 19.18% ± 4.64% and 13.70% ± 4.05%, respectively) compared to those of the control groups (30.65% ± 5.90%, 5.56% ± 3.15% and 1.85% ± 1.85 %, respectively). The current results demonstrated that addition of 0.3 mM cysteine to a fertilization medium had positively improved *in vitro* fertilization rates and supported embryonic progress till the blastocyst stage of buffalo oocytes.

## Introduction

Animal production industry depends on the dissemination of the genetic properties of superior animals. Therefore, *In vitro* embryo production emerged as a promising strategy to produce embryos with high genetic properties. However, the overall success to improve the *in vitro* developmental rate of buffalo preimplantation embryos remained very low. Therefore, it is essential to find the suitable approach to improve the developmental competence of bubaline preimplantation embryos.

*In vitro* embryo development is passively compromised by the oxidative stress induced by suboptimal culture conditions. Oxidative stress perturbs the functional integrity of the cells through the release of reactive oxygen species (ROS) [1]. For example, oxidative stress-induced ROS elevation has the potential to react with the polyunsaturated fatty acids of lipid membranes and induces lipid peroxidation. Moreover, ROS oxidize free amino acid residue side chains resulting in protein aggregation with subsequent cell damage. Furthermore, ROS elevation was identified as one of the main pathways that induce DNA double strand breaks, the leading cause of cancer and apoptosis [2]. However, the cells respond to the oxidative stress by triggering the antioxidant machinery. Accordingly, catalase and superoxide dismutase, as well as the "thiol" components were elevated to act as metabolic lids to neutralize the harmful effect of the ROS [3].

Cysteine is the precursor of intracellular glutathione (GSH) biosynthesis. Therefore, cysteine enrichment has the ability to increase the GSH level, another potent antioxidant enzyme. Previous work revealed that the supplementation of cysteine to the bovine *in vitro* maturation medium improved the embryonic progress and quality [4] and [5]. However, the effect of cysteine supplementation during *in vitro* fertilization, the step that is critical for the subsequent embryonic development, has not been studied. This study aimed to elucidate the effect of cysteine supplementation to the *in vitro* fertilization medium on the fertilization rate and the subsequent developmental competence of buffalo preimplantation embryos.

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## Materials and Methods

### Cumulus Oocyte Complexes Collection

Ovaries were collected within thirty minutes after slaughter from mature Egyptian buffaloes in a local abattoir and transported within two hours to the laboratory in a sealed flask inclosing sterile PBS (pH 7.35) provided by with 100 IU penicillin G, as well as, 100 µg/mL of streptomycin in 30° [6]. Cumulus oocyte complexes (COCs) have been aspirated from medium size follicles (2-8 mm) using 18-gauge needle attached to a ten mL sterile syringe. Only COCs having multi-layers of cumulus cells, intact zona pellucida and homogeneous cytoplasm have been selected for further processing [7].

### *In vitro* Maturation of Oocytes

The selected COCs have been washed 3 times with Dulbecco's PBS. Maturation was performed using 10 to 15 COCs in 10×35 mm Petri dishes with 100 µL drops of modified synthetic oviduct fluid (SOF) media comprising Earle's salts (GibcoTM, Ref. 31-10-035, Invitrogen Corporation, USA) provided with 0.0225 mg/mL sodium pyruvate, 0.01IU r-hFSH/mL, 0.05mg/mL of sLH (Lutrophin-V, Bioniche Animal Health, Canada) and 10% of fetal calf serum (FCS). The selected COCs have been washed twice in the maturation medium and then incubated in 200 µL drops of SOF covered with sterile mineral oil (Sigma) and incubated in the $CO_2$ incubator in 39° and 5% $CO_2$ in air, and saturated humidity for 24 h. After 24 h, oocytes were examined for cumulus expansion under stereomicroscope. Cumulus cells were then removed mechanically from some COCs using 0.1 % hyaluronidase, and the denuded oocytes have been inspected for the formation of the first polar body extrusion (PBE). Both cumulus expansion and PBE were evaluated to assess the maturation rate [8].

### Sperm Preparation and *in vitro* Fertilization

Three straws of frozen buffalo semen have been thawed in a water bath at 38° for 30 seconds. After thawing, swim up technique was used to isolate the most motile spermatozoa have been separated within sperm-TALP medium containing 6 mg/mL BSA, for 30 min [9]. The uppermost layer of the medium in closing the motile spermatozoa was collected. The selected spermatozoa were washed two times by centrifugation (x500g/10 min). The sperm pellet has been reconstituted with the fertilization TALP (F-TALP) medium containing 5 mM caffeine. The prepared sperm was incubated in the $CO_2$ incubator in 38.5°, 5% $CO_2$ for two hours before further use. The matured COCs were washed with F-TALP medium and the prepared sperm was added into the droplets containing matured oocytes to achieve a final concentration of $2\times10^6$ sperm cells/mL. Gametes have been co-incubated in the fertilization drops under sterile mineral oil for 18 h. At the end of gametes co-incubation, some of inseminated oocytes have been freed of the attached cumulus cells, fixed in acetic acid-ethanol (1:3), stained with 1% aceto-orcein stain and examined under phase-contrast microscope (×400) for assessing the *in vitro* fertilization rate according to Totey SM, et al., [6].

### *In vitro* Culture

Putative zygotes were denuded from cumulus cells and the extra spermatozoa by gentle pipetting, and washed three times in modified SOF media. Immediately, 20 to 25 zygotes were randomly distributed in 100 µL drops of modified SOF medium according to [10], with 5% FCS, 20 µL/mL of essential amino acids and 10 µl/mL of non-essential amino acids, under mineral oil in 10×35 mm Petri dishes for 7 days at 38.5° in an atmosphere of 5% $CO_2$ in air with maximum humidity. Developmental competence was assessed by evaluating cleavage, morula and blastocyst rates on days 2, 5 and 7, respectively, according to Totey SM, et al., [6].

### Experimental Design

Matured COCs were *in vitro* fertilized in the absence (control) or the presence of different concentrations of cysteine (0.1 mM, 0.3 mM or 0.5 mM) followed by assessing the fertilization rate. The putative zygotes were then *in vitro* cultured for 7 days. Cleavage, morula and blastocyst rates were evaluated on days 2, 5 and 7, respectively.

### Statistical Analysis

Costat Computer Program, Version 3.03 copyright (1986) Cottort Software, and were compared using ANOVA followed by LSD as a post hoc test. Variant were considered significant when $P<0.05$. Inserting Graphics.

## Results

Data regarding the effect of replenishing of the *in vitro* fertilization medium with different concentrations of cysteine on the fertilization rate was shown in Table 1. Addition of 0.3 mM cysteine to the fertilization medium significantly improved ($P<0.05$) the fertilization rate (53.45% ± 6.61%) when compared to that of control group (30.65% ± 5.90%). Importantly, although cleavage rate did not vary significantly, supplementation of the fertilization medium with 0.3 mM cysteine increased significantly ($P<0.05$) morula and blastocyst rates (19.18% ± 4.64% and 13.70% ± 4.05%, respectively) in comparison to those of the control group (5.56% ± 3.15% and 1.85% ± 1.85 %, respectively; Table 2).

## Discussion

Elevation of ROS during *in vitro* fertilization perturbed the fertilization rate and subsequent embryonic development [11]. Oxidative damage to cellular components through the ROS release is one of the main pathways that cause damage to many cellular functions [3]. These observations may explain the results in the present study. The supplementation of different levels of cysteine improved the *in vitro* fertilization and the development rates of buffalo embryos. These findings were similar to results obtained by previous studies reporting that the addition of cysteine to the *in vitro* culture medium increased the proportion of fertilized oocytes that developed to morula and blastocyst stages [12-14].

The higher rates of fertilization and embryo progress in 0.3 mM cysteine-treated group may be through increasing GSH level. Previous studies demonstrated that, addition of cysteine during the culture medium stimulated the synthesis of GSH and, accordingly, improved the *in vitro* embryo production [15]. This improvement may be due to the beneficial antioxidant

**Table 1. Effect of Different Cysteine Concentrations on the *in vitro* Fertilization Rate of Buffalo Oocytes.**

| Treatment | No. Oocytes | Fertilization rate (%) |
|---|---|---|
| Control | 62 | 30.65 ± 5.90[a] |
| Cysteine 0.1 mM | 78 | 51.28 ± 5.70[b] |
| Cysteine 0.3 mM | 58 | 53.45 ± 6.61[b] |
| Cysteine 0.5 mM | 84 | 39.29 ± 5.361[ab] |

Values with dissimilar superscript letters in the same column are significantly different ( P<0.05 ).

**Table 2. Effect of Different Cysteine Concentrations on the *in vitro* Embryo Developmental Rate of Buffalo Oocytes.**

| Treatment | No. oocytes | Cleavage rate (%) | Morula stage (%) | Blastocyst stage (%) |
|---|---|---|---|---|
| Control | 54 | 25.93 ± 6.02[a] | 5.56 ± 3.15[a] | 1.85 ± 1.85[a] |
| Cysteine (0.1 mM) | 67 | 34.33 ± 5.84[a] | 17.91 ± 4.72[b] | 8.96 ± 3.52[ab] |
| Cysteine (0.3 mM) | 73 | 36.99 ± 5.69[a] | 19.18 ± 4.64[b] | 13.70 ± 4.05[b] |
| Cysteine (0.5 mM) | 52 | 32.69 ± 6.56[a] | 9.62 ± 4.13[ab] | 3.85 ± 2.69[a] |

Values with dissimilar superscript letters in the same column are significantly different ( P<0.05 ).

mechanism of cysteine in neutralizing the reactive oxygen species and their harmful effects. Moreover, It was proved that addition of low molecular weight thiol compounds such cysteine to the culture media or the use of a cysteine-rich medium (TCM 199), promotes the synthesis of GSH, increased its intracellular level and improved embryo development and quality in pig [16] and bovine [17].

There are different mechanisms for controlling cellular ROS levels such as GSH and superoxide dismutase. GSH is a non-protein sulphydryl compound in cattle cells and considered an important regulator of the ROS. GSH aids as a reservoir for cysteine and plays an essential role in guarding mammalian cells from oxidative stress, and its intracellular synthesis is an important factor in oocyte cytoplasmic maturation [19]. Moreover, GSH has a possible role in sperm nucleus decondensation and regulate spindle microtubule development in the ovum, and protect ova and embryo during *in vitro* fertilization, thus improving the outcome of pregnancy [20]. Recently, it was shown that GSH has a positive impact on *in vitro* embryo development [21] and [22].

## Conclusions

The current results inferred that cysteine has a positive effect on buffalo oocytes fertilization and subsequent embryo development in a dose dependent trend. Moreover, the addition of 0.3 mM cysteine to the fertilization medium is a promising approach to improve the blastocyst rate in buffaloes.

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