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Effect of cysteamine supplementation during *in vitro* culture of early stage bovine embryos on blastocyst rate and quality

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Abstract

The aim of this study was to evaluate whether the addition of cysteamine to the *in vitro* culture media enhances the yield, hatching rate, total cell number and inner cell mass/total cell number ratio of bovine embryos. A total of 933 bovine oocytes collected from ovaries of 60 slaughtered donors were subjected to *in vitro* maturation and *in vitro* fertilization. Following fertilization, embryos were cultured in synthetic oviductal fluid without glucose. After 24 h embryos were transferred into synthetic oviductal fluid with 1.5 mM glucose and 0 (control), 50, 100 and 200 μM of cysteamine. After 48 h, the embryos were transferred into synthetic oviductal fluid with glucose but without cysteamine and cultured until Day 9. The number of cleaved embryos on Day 2, the total number of blastocysts on Day 7 and the number of hatched blastocysts on Day 9 were calculated. Differential staining of inner cell mass and trophectoderm cells of blastocysts were performed on Day 7 and Day 9 of *in vitro* culture. Supplementation of *in vitro* culture media with 100 μM cysteamine increased the blastocyst yield ($P < 0.05$) without affecting the hatching rate. Furthermore, the embryos cultured in the presence of 100 μM cysteamine had significantly higher number of inner cell mass cells ($P < 0.05$) and the proportion of inner cell mass cells ($P < 0.05$) compared with the controls. The results of the present study demonstrated that the addition of 100 μM cysteamine to the *in vitro* culture media improved blastocyst production rate and enhance embryo quality, which could lead to the improvement of the *in vitro* culture system for bovine embryos.

Oxidative stress, antioxidants, embryo yield, differential staining, total cell number

Oocytes and embryos are protected against oxidative stress by oxygen scavengers that are normally present in follicular and oviductal fluids. In an *in vitro* production system, oocytes and embryos are removed from their natural environment and this defense system is lost (Wang et al. 2002). Oxidative stress induces mitochondrial dysfunction, DNA, RNA and protein damage (Comporti 1989), inhibition of sperm-oocyte fusion (Aitken et al. 1993), embryo cell block and apoptosis, especially at the blastocyst stage (Parchment 1991). In order to improve *in vitro* embryo production, oxidative stress must be controlled during *in vitro* culture. The addition of various antioxidants to the *in vitro* culture media may protect oocytes and embryos from the detrimental effect of oxygen-derived free radicals (Del Corso et al. 1994).

Glutathione (GSH) is a major non-protein sulphhydril compound in mammalian cells which protects the cell from oxidative damage. Synthesis of glutathione depends on the availability of cysteine in the media (Furnus and De Matos 1999). Cysteamine is a low molecular-weight thiol compound that may reduce cystine to cysteine and enhance oocyte glutathione synthesis (Issels et al. 1988). It was demonstrated that the addition of cysteamine to the *in vitro* maturation media improves the rate of embryo development by increasing glutathione synthesis in cow and sheep (de Matos et al. 1996; 2002a). The benefits of supplementing the *in vitro* maturation medium (IVM) with

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cysteamine are reviewed by Deleuze and Goudet (2010). However, it is still unclear if supplementation with antioxidants is required during *in vitro* culture of embryos due to low oxygen concentration. It was reported that the addition of antioxidants to the *in vitro* culture improved blastocyst production (Takahashi et al. 1993; Olson and Seidel 2000; de Matos et al. 2002b). Furthermore, as the lowest glutathione content was found in 2- to 8-cell embryos, the early cleavage stage embryos are, therefore, probably more sensitive to oxidative stress than the other stages.

The aim of this study was to evaluate the effect of cysteamine supplementation to the *in vitro* culture media during early stages of development on the embryo developmental rate and quality by means of differential staining.

Materials and Methods

Reagents and media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (Sigma-Aldrich Chemie GMBH, Germany). The oocyte maturation medium was TCM 199 bicarbonate medium supplemented with 10% foetal calf serum (FCS) (GIBCO Invitrogen Corporation, Auckland, New Zealand), follicle stimulating hormone and luteinizing hormone (Pergonal, 75/75 IU, Serono), 1 µg/ml estradiol-17ß and 100 µM cysteamine. The *in vitro* fertilization medium (IVF) was modified by Tyrode's bicarbonate buffered solution supplemented with 10 µg/ml heparin, 5 µg/ml hypotaurine, 5 µg/ml epinephrine and 6 mg/ml bovine serum albumine (BSA). The embryo culture medium was synthetic oviductal fluid (SOF) with amino acids, 1 µM glutamine, 8 mg/ml BSA and 0 (SOF0) or 1.5 mM glucose (SOFg). All media were allowed to equilibrate at 39 °C in the atmosphere of 5% CO_2.

In vitro embryo production

A total of 60 bovine ovaries were collected at the abattoir and transported to the laboratory in saline with antibiotics (100 IU penicillin and 100 µg streptomycin/ml) at 37 °C within 3 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from 2 to 8 mm diameter follicles using 18G needle attached to a vacuum pump. Oocytes with intact cumulus investment and evenly granulated cytoplasm were matured *in vitro* in groups of 10 in 50 µl droplets of maturation medium under mineral oil at 39 °C with 5% CO_2 in air for 24 h. The expanded COCs were washed in HEPES-TALP medium supplemented with 6 mg/ml BSA and transferred into 40 µl droplets of IVF medium under mineral oil. In all experiments, frozen semen from the same bull was used. Sperm preparation for IVF on BoviPure® (Nidacon Laboratories AB, Gothenberg, Sweden) gradient was accomplished according to Samardzija et al. (2006). Final concentration was adjusted to 1×10^6 spermatozoa/ml. Incubations were carried out at 39 °C in 5% CO_2 in air for 24 h. After the sperm-oocytes co-incubation (day 0 = day of fertilization), the presumptive zygotes were denuded from cumulus cells and spermatozoa and then washed in HEPES-TALP media and SOF0 media. Fertilized oocytes were cultured *in vitro* in SOF 0 media for 24 h and then transferred into SOFg with 0, 50, 100 and 200 µM cysteamine. Cysteamine was added at Day 2 of *in vitro* culture (IVC) and the medium was replaced 48 h later with SOFg without cysteamine. The embryos were cultured *in vitro* until Day 9 at 39 °C in 5% CO_2, 7% O_2 in 88% N_2. The endpoints for evaluation were Day 2 cleavage rates; Day 7 blastocyst rates; Day 9 hatching rates, total cell number (TCN) and differentiation of the embryonic cells into inner cell mass (ICM) and trophectoderm (TE) cells in Day 7 and Day 9 embryos.

Differential staining of blastocysts

Hatched blastocysts were used as such and the zona of intact blastocysts was removed by treatment with 0.5% pronase. Zona-free embryos were washed × 5 in phosphate buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA). Embryos were then incubated in a 30 : 70 dilution of rabbit anti-bovine whole serum in TCM 199 bicarbonate at 39 °C for 1 h. After washing in PBS 0.1% PVA, the embryos were incubated in a 1 : 4 dilution of a guinea pig complement in TCM 199 bicarbonate supplemented with 10 µg/ml propidium iodide (PI) for 1 h. Embryos were then briefly washed in ice-cold TCM 199 Hepes supplemented with 10 µg/ml PI and fixed in ice-cold absolute ethanol. After fixation, the embryos were transferred for 3–5 min to 10 µg/ml bisbenzimide (Hoechst 33343) in absolute alcohol at room temperature. Presumptive stained blastocysts were transferred to a drop of glycerol on a microscopic slide and covered with a cover slip. Embryos were examined under a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a UV filter. Bisbenzimide-stained ICM nuclei appeared blue and TE nuclei labelled with both bisbenzimide and PI appeared red or pink. The ICM and TE nuclei were counted under the microscope.

Statistical analysis

The statistical analyses among treatments were done by ANOVA (StatSoft, Statistica, 7.1 version 2005) using the arcsine transformation ($\arcsin\sqrt{P}/100$) of the percent values and comparisons by Tukey's post hoc test analysis. $P < 0.05$ was considered significant.

Results

Effect of cysteamine supplementation during IVC on embryo development

A total of 933 oocytes in 7 independent replicates were submitted to IVM, IVF and IVC to evaluate the effect of cysteamine supplementation to the *in vitro* culture medium on subsequent embryo development. The blastocyst yields at Day 7 of *in vitro* culture were significantly higher than the corresponding value for controls following supplementation of *in vitro* culture medium with 100 μM cysteamine ($P < 0.05$), whereas the concentration of 50 and 200 μM had no effect (Table 1).

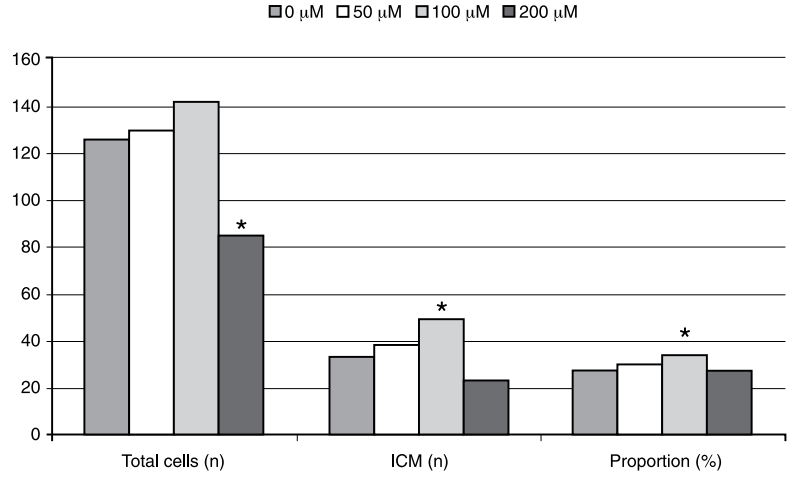
Table 1. Effect of supplementation of *in vitro* culture media with cysteamine on blastocyst development (mean ± S.E.M.)

Cysteamine (μM)	Oocytes (n)	Day 2 cleavage rate (%)	Day 7 blastocyst rate (%)	Day 9 hatching rate (%)
0	234	82.49 ± 3.3	21.94 ± 3.2	8.89 ± 2.7
50	229	83.21 ± 3.4	29.17 ± 2.7	15.12 ± 3.2*
100	221	82.15 ± 3.7	33.26 ± 2.2*	13.98 ± 1.3
200	249	82.44 ± 3.8	21.38 ± 4.3	7.31 ± 2.0

*values within the same column differ significantly from the controls ($P < 0.05$), controls = 0 μM cysteamine

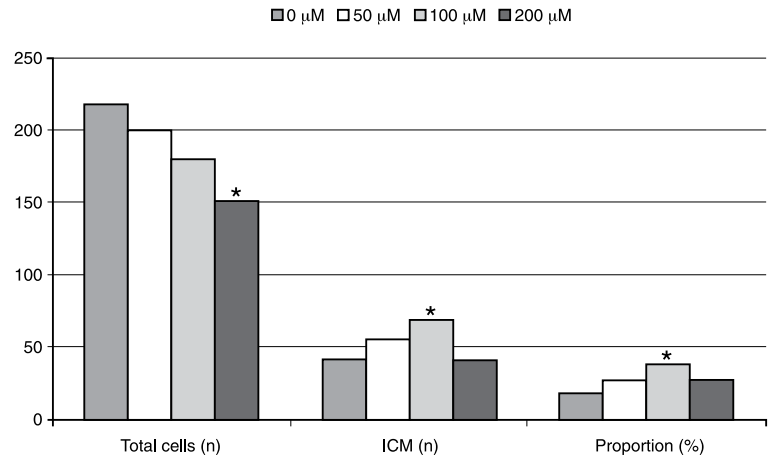
Effect of cysteamine supplementation during IVC on embryo quality

A random sample of grade 1 blastocysts on day 7 (n = 42) (Fig. 2) and all grade 1 hatched blastocyst (n = 59) on Day 9 of *in vitro* culture (Fig. 3) were selected for differential staining. The total number of cells was significantly affected when 200 μM cysteamine was added to *in vitro* culture both in Day 7 or Day 9 embryos. This concentration reduced the total cell number ($P < 0.05$) compared to controls. However, ICM cell number was



*values differ significantly from the controls ($P < 0.05$), controls = 0 μM cysteamine

Fig.1. Mean total cell number, number of inner cell mass cells (ICM) and proportion of inner cell mass in total cell number on Day 7 blastocysts following supplementation of *in vitro* culture media with 50, 100 and 200 μM cysteamine



*values differ significantly from the controls ($P < 0.05$), controls = 0 μM cysteamine

Fig. 2. Mean total cell number, number of inner cell mass cells (ICM) and proportion of inner cell mass in total cell number in Day 9 hatched blastocysts following supplementation of *in vitro* culture media with 50, 100 and 200 μM cysteamine

the highest in 100 μM cysteamine group in both Day 7 blastocysts and Day 9 hatched blastocyst ($P < 0.05$). The same group also displayed significantly higher proportion of ICM cells ($P < 0.05$).

Discussion

Oxidative stress mediated by reactive oxygen species results in an imbalance of the intracellular redox potential (Balaban et al. 2005). Intracellular glutathione plays a critical role in maintaining the redox state of the oocytes and embryos by protecting cells against oxidative damage. Glutathione is synthesized by γ-glutamyl cycle, and its biosynthesis depends on the availability of cysteine, a highly unstable aminoacid that is readily oxidized to cystine (Furnus and de Matos 1999). Intracellular glutathione concentration after IVM is a good biological indicator of the cytoplasmic maturation and viability of the oocyte. After the addition of exogenous cysteine during oocyte maturation *in vitro*, GSH content increases, providing a pool of GSH which are necessary for sperm decondensation, oocyte activation and transformation of fertilizing sperm head into the male pronucleus (Yoshida et al. 1993; Furnus and de Matos 1999). The addition of cysteamine in the maturation media of bovine and sheep oocytes resulted in an increase in GSH levels by reducing oxidized cystine to cysteine in media and an improvement in embryo developmental rate up to the blastocyst stage (de Matos et al. 1996; 2002a). This fact suggests that the beneficial effects of cysteamine on *in vitro* maturation and subsequent embryo development were mediated by GSH (Isles et al. 1988). This was in agreement with our previous study (Karadjole et al. 2006) which demonstrated that the addition of cysteamine to the *in vitro* maturation media significantly improved the rate of blastocyst development and the quality of blastocyst by increasing inner cell mass cell number. The intracytoplasmic glutathione concentration varies during preimplatation development of *in vitro* produced bovine embryos. The lowest level of GSH was found in 2–8 cell embryos as the *de novo* synthesis of GSH begins to increase at the 9–16-cell stage embryos (Van Langendonckt et al. 1998) following the highest level found in hatched blastocyst (Lim

et al. 1996). The fact that early stage embryos are probably more sensitive to oxidative stress because of exhausted GSH pool synthesized during IVM inspired us to supplement the *in vitro* culture media with cysteamine during the first stages of embryo development. The results demonstrated that the addition of 100 μM cysteamine during early *in vitro* development of the embryos significantly improved the development of the blastocyst on Day 7 of *in vitro* culture compared to controls ($P < 0.05$). This was in agreement with de Matos et al. (2002b) who demonstrated that the addition of cysteamine to maturation media and 2–8-cell bovine embryos results in an increase of embryo quality and blastocyst production. Similarly, the supplementation of cysteamine to both IVM and IVC media improved the blastocyst production rate in buffalo, without compromising their health (Anand et al. 2008). The beneficial effect of cysteamine present during the early stages of embryo development is probably acquired through increased glutathione concentration, which could help overcome the developmental block at 8- to 16-cell stage embryos (Lee et al. 2000) when the GSH stores produced during *in vitro* maturation are nearly exhausted. Although reactive oxygen species are deleterious to the oocyte and embryo quality, an excess of antioxidant supplementation adversely affects blastocyst development (Guerin at al. 2001; Anand et al. 2008). In our study, supplementation of *in vitro* culture media with 100 μM cysteamine gave the best results, followed by 50 μM cysteamine, while 200 μM cysteamine was found ineffective regarding the blastocyst yield, but significantly reduced the total cell number in developing embryos ($P < 0.05$). The addition of 100 μM cysteamine to the *in vitro* culture media resulted in a significantly higher ICM cell number and ICM/TCN proportion in Day 7 blastocyst ($P < 0.05$), without affecting the total cell number compared to the control group. Blastocoel fluid contains hydrogen peroxide which is cytotoxic and can induce apoptosis in inner cell mass cells. Treatment with hydrogen peroxide applied to oocytes or embryos decreased the blastocyst development and increased the induction of permanent embryo arrest or apoptosis in a stage-dependent manner (Bain et al. 2011). The increase of inner cell mass cells could, therefore, be explained with the fact that cysteamine addition to the IVC media increases intracellular glutathione content and since glutathione is involved in the removal of hydrogen peroxide, inner cell mass cells would not become apoptotic.

In conclusion, the inclusion of low-molecular-weight thiol compound such as cysteamine to *in vitro* culture system controls the oxidative stress, improves the blastocyst production rates and enhances embryo quality, which could lead to the improvement of the *in vitro* culture system.

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