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—Full Paper—

Effects of Iron and Copper in Culture Medium on Bovine Oocyte Maturation, Preimplantation Embryo Development, and Apoptosis of Blastocysts *In Vitro*

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Abstract. The aim of this study was to investigate the effects of iron and copper on bovine oocyte maturation, preimplantation embryo development and apoptosis of blastocysts. The concentrations of iron in the culture media were 0 (control), 0.45, 0.81, 1.96 and 3.26 mg/l, and the concentrations of copper were 0 (control), 0.093, 0.27, 0.46 and 0.68 mg/l. The changes in the iron (1.96 mg/l) and copper concentrations (0.46 mg/l) in the culture media were measured after oocyte maturation for 22 h and after zygote culture for 48, 96, 144 and 192 h. The results showed that there were no significant differences in oocyte maturation and cleavage between media containing iron and the control, but the media containing iron had higher (P>0.05) rates of 8-cell embryos, morulae, and blastocysts than the control, and addition of 1.96 mg/l of iron increased the blastocyst rate (P>0.05). The effects of copper on oocyte maturation and cleavage were similar to iron, and addition of 0.46 and 0.68 mg/l of copper increased the rates of morulae and blastocysts (P>0.05). Addition of iron or copper significantly decreased the number of apoptotic blastomeres compared with the control (P>0.05). After oocyte maturation for 22 h and zygote culture for 48 h, the iron concentrations decreased by 3.6 and 9.2%, respectively, and the copper concentrations decreased by 6.5 and 10.9%, respectively. After zygote culture for 96, 144 and 192 h, the iron concentrations decreased by 21.4, 25.5 and 27.0%, respectively, the copper concentrations decreased by 23.9, 28.3 and 30.4%, respectively. In conclusion, iron and copper played an important role in the success of culture of 8-cell embryos, morulae, and blastocysts, and long-term lack of iron or copper increased the number of apoptotic blastomeres. Furthermore, transition of primary demand for trace amounts of iron or copper from the cytoplast to culture medium for utilization by zygotes may occur after *in vitro* zygote culture for 48 h.

Key words: Copper, Embryos, *In vitro* culture (IVC), Iron, Oocytes

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The techniques of producing bovine embryos by *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) have shown remarkable progress in recent years. Several systems supporting IVM, IVF, and embryo culture have been established. Bovine *in vitro* embryo culture (IVC) techniques are increasingly being refined to the point that blastocyst development is successful in various media formulations and under different culture conditions [1, 2]. At the same time, only 30–40% of the zygotes obtained after IVM and IVF will reach the blastocyst stage in culture [3–5].

There are various factors that affect oocyte maturation and fertilization, and many studies have attempted to improve IVM of oocytes and IVC

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of embryos. Medium with or without serum is commonly used for IVM of bovine oocytes and IVC. Bovine oocytes grown in serum-free medium can be fertilized, but acquire insufficient embryonic development [6]. Serum has many undefined and variable components such as amino acids, carbohydrates, lipids, inorganic salts, vitamins, hormones, and growth factors, that may stimulate and/or inhibit oocyte maturation and embryonic development [7], which makes it difficult to determine the precise role of a substance in culture medium during IVM, IVF and IVC. It would be helpful to determine the precise role of a specific substance in IVM, IVF and IVC through addition of the substance into media that does not contain it.

Trace elements function was the activators of enzyme systems or as constituents of organic compounds. Many complex interactions have been identified among and between trace elements and between trace elements and vitamins [8–11]. In recent years, major efforts have been made to determine the effects of trace element on the procreation of mammals, but little work has been conducted in the bovine, especially on oocyte maturation and subsequent zygote development *in vitro*.

Apoptosis is cell self-destruction under physiological control and plays an important role in normal embryo development by eliminating both unnecessary and abnormal cells [12–14]. Apoptosis is a highly conserved and regulated program by which cells commit suicide under a variety of internal and external controls. However, whether the components of culture medium, such as trace elements, have an effect on the occurrence of apoptosis in blastocysts is unclear.

The objectives of the present study were to evaluate the impact of trace elements on bovine oocyte maturation, preimplantational embryo development, and apoptosis of blastocysts using iron and/or copper. In the following experiments, different concentrations of iron and copper were used separately with culture media that did not contain the respective element. Apoptosis of blastocysts was assessed by terminal deoxynucleotidyl transferase-mediated d-UTP nick-end labeling (TUNEL). In addition, changes in the iron and/or copper concentrations of culture media were determined during the period of oocyte maturation to blastocyst formation using plasma atomic spectrometric techniques. The concentrations of iron and copper in the culture media were consistent with those of follicular fluid from different sized follicles.

Materials and Methods

Collection of ovaries

Ovaries were transported to the laboratory from a local slaughterhouse within 1–3 h at 30 C in Dulbecco's phosphate buffered saline (DPBS) containing 100 IU /ml streptomycin and 100 IU/ml penicillin.

Determination of iron and copper concentrations

In the laboratory, the ovaries were washed twice with DPBS. Follicles were separated according to their diameter into the following four groups: smallest sized follicles (<3 mm), small sized follicles (3–7 mm), medium sized follicles (7–10 mm), and large follicles (>10 mm). The follicular fluid of each group was collected with a 12-ml disposable syringe fitted with an 18-gauge needle. The concentrations of iron and copper were measured using plasma atomic spectrometric techniques. A total of 126 bovine ovaries were used for collection of follicular fluid. The concentrations of iron of the four groups were 3.26, 1.96, 0.81 and 0.45 mg/l, respectively, and those of copper were 0.68, 0.46, 0.27 and 0.093 mg/l, respectively.

Collection of oocytes and in vitro maturation

Cumulus-oocyte complexes (COCs) were recovered from follicles 3–8 mm in diameter with a 12-ml disposable syringe fitted with an 18-gauge needle. The COCs with evenly granulated ooplasm surrounded by several layers (three or more dense layers) of compact cumulus cells were selected for use. Groups of 10–15 oocytes were placed in 100 μl droplets of IVM medium under mineral oil (Sigma, St. Louis, MO, USA). The basic maturation medium was TCM 199 with Earle's salts supplemented with 10% (v/v) fetal calf serum (FCS, Sigma), 25 mM Hepes, 0.25 mM sodium pyruvate, 10 μg/ml FSH, 10 μg/ml LH, 1 μg/ml estradiol, 100 IU/ml streptomycin, and 100 IU/ml penicillin. Trace amounts iron or copper were eliminated from the basic medium using analytical chemistry techniques. Various concentrations of iron chloride were added to maturation medium that did not contain iron, and various

concentrations of copper chloride were added to maturation medium that did not contain copper. The COCs were matured for 22 h at 38.5 C in an atmosphere of 5% CO_2.

In vitro fertilization

After maturation, morphologically abnormal oocytes were excluded and most cumulus cells were removed mechanically by gentle pipetting in DPBS to improve sperm penetration and pronuclear formation. All experiments were carried out using frozen semen shown to have good fertility in an IVF system. The semen was thawed in a 36 C waterbath for 30 sec. A swim-up method was used to prepare the sperm for IVF using TALP medium [15]. The capacitation and fertilization media consisted of modified TALP medium supplemented with 10 mg/ml heparin-sodium salt and 5 mM caffeine. Each fertilization droplet contained 10–15 oocytes in a 100 μl droplet of fertilization medium to which sperm were added to produce a final concentration of approximately 1.0×10^6 sperm/ml. Incubation was carried out under mineral oil for 10 h at 38.5 C in an atmosphere of 5% CO_2.

Embryo culture

After fertilization, the presumptive zygotes were washed three times in embryo culture medium before transfer to culture droplets (100 μl) in groups of 10–15 embryos. The basic embryo culture medium was TCM 199 with Earle's salts supplemented with 10% (v/v) FCS, 25 mM Hepes, 0.25 mM sodium pyruvate, 100 IU /ml streptomycin and 100 IU/ml penicillin. Trace amounts of iron or copper were eliminated from the basic embryo culture medium using analytical chemistry techniques. Various concentrations of iron chloride were added to embryo culture medium that did not contain iron, and various concentrations of copper chloride were added to embryo culture medium that did not contain copper. The presumptive zygotes were subsequently transferred to culture medium and co-cultured with granulosa cells at 38.5 C in 5% CO_2 in air with 100% humidity for 7 days.

Assessment of embryo development

Embryo development to the 2-cell, 8-cell, morulae and blastocyst stage was observed. The numbers of 2-cell embryos, 8-cell embryos, morulae and blastocysts were calculated based on the number of oocytes at the onset of culture.

Detection of apoptosis by the TUNEL assay

Apoptosis of blastocysts was detected by TUNEL assay using a In situ Apoptosis Dectection Kit (Takara Bio, Otsu, Japan) [16]. Blastocysts were fixed in PBS containing 4% (v/v) paraformaldehyde for 30 min at 39 C and then placed in the permeabilization buffer contained in the kit for 10 min at 4 C. After washing in PBS containing 1 mg/ml BSA, the blastocysts were treated with a terminal deoxynucleotidyl transferase-labeling buffer for 2 h at 39 C. The blastocystes were then placed in 70 μl fluorescein isothiocyanate solution for 60 min at 39 C and treated with 50 μg/ml propidium iodide for 30 min at room temperature. The blastocysts were mounted onto glass slides after treatment with 0.2 M diazabicyclo-octane in PBS supplemented with 50% (v/v) glycerol and were examined under a fluorescence microscope. Apoptotic blastomeres appeared as yellow, fragmented, and condensed nuclei, while normal blastomeres were red.

Mensuration of concentrations of iron and copper in culture medium

The concentrations of iron and copper in culture media were measured using plasma atomic spectrometric techniques. After oocyte maturation culture for 22 h, oocytes were transferred to IVF and then all oocyte maturation media was collected for measurement of the concentrations of iron and copper. The concentrations of iron and copper in zygote culture media were measured after culture for 48, 96, 144 and 192 h. A 10 μl sample of each 100 μl zygote culture medium droplet under mineral oil was collected after IVF for 48, 96, 144 and 192 h for measurement of the iron and copper concentrations.

Experimental design

Experiment 1: The objective of this experiment was to investigate the effects of iron in culture medium on oocyte maturation and subsequent embryo development. The concentrations of iron in culture media were 0 mg/l (control), 0.45, 0.81, 1.96 and 3.26 mg/l. In this experiment, 1,209 oocytes were randomly divided into five groups. 0 (control), 0.45, 0.81, 1.96 and 3.26 mg/l iron treatments were conducted with 201, 240, 271, 243 and 254 oocytes, respectively. Oocyte maturation and the numbers of cleavage embryos, 8-cell

Table 1. The effects of different concentrations of iron in culture media on bovine oocyte maturation and subsequent embryo development

Iron concentration (mg/l)	Number of collected oocytes	Number (%) of matured oocytes	Number (%) of embryos developed			
			2-cell	8-cell	morula	blastocyst
3.26	254	208 (81.9)	122 (58.7)	108 (51.9)[a]	101 (48.6)[a]	65 (31.2)[a]
1.96	243	185 (76.1)	123 (66.5)	114 (61.6)[a]	94 (50.8)[a]	78 (42.2)[b]
0.81	271	209 (77.1)	128 (61.2)	118 (56.5)[a]	73 (34.9)[b]	55 (26.3)[a]
0.45	240	193 (80.4)	122 (63.4)	102 (52.3)[a]	65 (33.7)[b]	47 (24.3)[a]
Control	201	158 (78.6)	101 (63.9)	60 (38.0)[b]	35 (22.2)[c]	22 (13.9)[c]

Values with different superscripts (a–c) within the same column are significantly different ($P<0.05$).

embryos, morulae and blastocysts were assessed.

Experiment 2: The objective of this experiment was to investigate the effects of copper in culture medium on oocyte maturation and subsequent embryo development. The concentrations of copper in culture media were 0 (control), 0.093, 0.27, 0.46 and 0.68 mg/l. In this experiment, 939 oocytes were randomly divided into five groups. The 0 (control), 0.093, 0.27, 0.46 and 0.68 mg/l copper treatments were conducted with 195, 188, 184, 191 and 181 oocytes, respectively. Oocytes maturation and the numbers of cleavage embryos, 8-cell embryos, morulae, and blastocysts were assessed.

Experiment 3: The objective of this experiment was to investigate the effects of iron in culture medium on apoptosis of blastomeres. Each treatment was investigated using 12 blastocysts.

Experiment 4: The objective of this experiment was to investigate the effects of copper in culture medium on apoptosis of blastomeres. Each treatment was investigated using 12 blastocysts.

Experiment 5: The objective of this experiment was to investigate the changes in the iron concentrations of culture media after different periods of culture. The concentration of iron in fresh culture medium was 1.96 mg/l. The concentrations of iron in the culture media were measured after oocyte maturation for 22 h and after IVF for 48, 96, 144 and 192 h.

Experiment 6: The objective of this experiment was to investigate the changes in the copper concentrations in culture media after different periods of culture. The concentration of copper in fresh culture medium was 0.46 mg/l. The concentrations of copper were measured after oocyte maturation for 22 h and after IVF for 48, 96, 144 and 192 h.

Statistical analysis

The results were pooled and then tested by Chi-square analysis for experiment 1, 2, 3 and 4. Differences with a probability of $P<0.05$ were considered significant. Each experiment was repeated three or four times.

Results

Experiment 1

The effects of trace amounts of iron on bovine oocyte maturation and subsequent embryo development are shown in Table 1. There were no significant differences in the rates of maturation and cleavage of oocytes between media containing iron and the control. However, the control had lower rates of development of oocytes to the 8-cell, morula, and blastocyst stages than the media containing iron. There were significant differences in the rates of development rates of oocytes to the 8-cell, morula, and blastocyst stages between the media containing iron and the control. Higher concentrations of iron (3.26 and 1.96 mg/l) produced significantly higher rates of development to the morula stage (48.6 and 50.8%, respectively) than lower concentrations of iron, and 1.96 mg/l produced a significantly higher rate of development to the blastocyst stage (42.2%) than other concentrations.

Experiment 2

The effects of trace amounts copper on bovine oocyte maturation and subsequent embryo development are shown in Table 2. There were no significant differences between media containing copper and the control in the rates of maturation and cleavage of oocytes. In zygote culture media supplemented with copper, the rates of

Table 2. The effects of different concentrations of copper in culture media on bovine oocyte maturation and subsequent embryo development

Copper concentration (mg/l)	Number of collected oocytes	Number (%) of matured oocytes	Number (%) of embryos developed			
			2-cell	8-cell	morula	blastocyst
0.68	181	142 (78.5)	88 (62.0)	76 (53.5)[a]	67 (47.2)[a]	48 (33.8)[a]
0.46	191	154 (80.6)	92 (59.7)	84 (54.5)[a]	67 (43.5)[a]	57 (37.0)[a]
0.27	184	138 (75.0)	77 (55.8)	66 (47.8)[a]	48 (34.8)[b]	33 (23.9)[b]
0.093	188	133 (70.7)	74 (55.6)	67 (50.4)[a]	47 (35.3)[b]	34 (25.6)[b]
Control	195	148 (75.9)	77 (52.0)	53 (35.8)[b]	36 (24.3)[c]	17 (11.5)[c]

Values with different superscripts (a–c) within the same column are significantly different (P<0.05).

Table 3. The effects of iron in culture media on apoptosis of blastomeres

Iron concentration (mg/l)	Number of blastocysts	Total cell number of blastocysts	Number (%) of apoptotic blastomeres
3.26	12	1112	88 (7.9)[a]
1.96	12	1058	86 (8.1)[a]
0.81	12	1088	83 (7.6)[a]
0.45	12	1073	90 (8.4)[a]
control	12	1079	119 (11.0)[b]

Values with different superscripts (a, b) within the same column are significantly different (P<0.05).

Table 4. The effect of copper in culture media on apoptosis of blastomeres

Copper concentration (mg/l)	Number of blastocysts	Total cell number of blastocysts	Number (%) of apoptotic blastomeres
0.68	12	1086	84 (7.7)[a]
0.46	12	1072	72 (6.7)[a]
0.27	12	1120	85 (7.6)[a]
0.093	12	1087	86 (7.9)[a]
Control	12	1104	105 (9.5)[b]

Values with different superscripts (a, b) within the same column are significantly different (P<0.05).

development of oocytes to the 8-cell, morula, and blastocyst stages were significantly higher than in the control. Higher concentrations of copper (0.68 and 0.46 mg/l) produced significantly higher rates of development to the morula (47.2 and 43.5%, respectively) and blastocyst stages (33.8 and 37.0%, respectively).

Experiment 3

The effects of iron in culture media on apoptosis of blastomeres are shown in Table 3. There were no significant differences among different concentrations of iron in apoptosis of blastomeres, while the control medium produced a significant increase in apoptotic blastomeres compared with the different concentrations of iron (the control *vs.* 3.26, 1.96, 0.81 and 0.45 mg/l were 11.0 *vs.* 7.9, 8.1, 7.6 and 8.4%, respectively).

Experiment 4

The effects of copper in culture medium on apoptosis of blastomeres are shown in Table 4. The control group had a higher rate of apoptotic blastomeres than the other groups (the control *vs.* 0.68, 0.46, 0.27 and 0.093 mg/l were 9.5 *vs.* 7.7, 6.7, 7.6 and 7.9%, respectively).

Experiment 5

The changes in the iron concentrations of the culture media at different stages of culture are shown in Table 5. The iron concentration of the culture medium after oocyte maturation for 22 h

Table 5. The changes in the iron concentrations of the culture media after different periods of culture

Fresh media (mg/l)	After oocyte maturation for 22 h (mean mg/l; decrease %[a])	Period of zygotes development (mean mg/l; decrease %[a])			
		48 h[b]	96 h[b]	144 h[b]	192 h[b]
1.96	1.89 (3.6)	1.78 (9.2)	1.54 (21.4)	1.46 (25.5)	1.43 (27.0)

[a] Decrease %=(iron concentration of fresh media–iron concentration of culture medium) ÷ iron concentration of fresh media × 100. [b] 0 h=the onset of zygote culture.

Table 6. The changes in the copper concentrations of the culture media after different periods of culture

Fresh media (mg/l)	After oocyte maturation for 22 h (mean mg/l; decrease %[a])	Period of zygotes development (mean mg/l; decrease %[a])			
		48 h[c]	96 h[c]	144 h[c]	192 h[c]
0.46	0.43 (6.5)	0.41 (10.9)	0.35 (23.9)	0.33 (28.3)	0.32 (30.4)

[a] Decrease %=(copper concentration of fresh media–copper concentration of culture medium) ÷ copper concentration of fresh media × 100. [b] 0 h=the onset of zygote culture.

and zygote culture for 48 h fell by 3.6 and 9.2%, respectively, compared with the iron concentration of fresh medium (1.96 mg/l). However, the decreases in the iron concentrations were particularly high after zygote culture for 96 (21.4%), 144 (25.5%), and 192 h (27.0%).

Experiment 6

The changes in the concentrations of copper in the culture media at different stages of culture are shown in Table 6. The concentrations of copper in the culture media were 0.43, 0.41, 0.35, 0.33 and 0.32 mg/l after oocyte maturation for 22 and *in vitro* zygote culture for 48, 96, 144 and 192 h, respectively[0]. The copper concentrations fell by 6.5, 10.9, 23.9, 28.3 and 30.4%, respectively, compared with the copper concentrations of fresh medium (0.46 mg/l). The decreases in the copper concentration were higher after zygote culture for 96, 144 and 192 h than those after oocyte culture for 22 h and zygote culture for 48 h.

Discussion

In our present study, we added various concentrations of iron or copper to culture media from which we had previously removed the respective element in order to examine the effects of iron and copper on bovine oocyte maturation and preimplantation embryo development. Because the concentrations of iron and copper in the base medium prior to their removal were within the scope of five concentration levers in the present study, we did not examine the effects of base medium without removal of iron or copper.

Oocyte maturation can be initiated by induction with various agents, including FSH, LH, gonadotropin, and growth hormone [17, 18]. Resumption of meiosis from the germinal vesicle stage results from induction of chromosome condensation, disintegration of the nuclear envelope (germinal vesicle breakdown) and spindle formation. In the present study, the changes in the iron and copper concentrations in the maturation media were slight during the first 22 h of oocyte maturation, and little iron or copper was utilized by the maturing oocytes. There was no significant difference in oocyte maturation between the experiment groups (iron, copper and the control). These results suggest that the iron or copper in the oocyte maturation media had no effect on oocyte maturation and that little of the iron or copper in the oocyte maturation media was utilized by the oocytes during oocyte maturation.

Trace elements are intrinsic components of many enzymes including some involved in DNA synthesis [19]. Iron and copper are essential for animals, and all mammalian cells contain them. Iron is an integral constituent of hemoglobin and myoglobin and is a component of many enzymes, including both the heme and non-heme Fe enzymes involved in oxidation-reduction reactions [20]. Copper is an essential micronutrient required by all living organisms [21] and plays a vital role as a catalytic cofactor for a variety of metalloenzymes.

The present study showed that there were significant differences between the iron and control groups in the rates of 8-cell embryos, morulae, and blastocysts. Copper produced the same effects as iron on the rates of 8-cell embryos, morulae, and blastocysts. Based on these results, we believe that the presence of iron and copper in culture medium has an important effect on successful formation of 8-cell embryos, morulae, and blastocysts. Intake of trace elements below the standard requirement can lead to deficiency [22]. In our study, higher concentrations of iron (1.96 and 3.26 mg/l) and copper (0.46 and 0.68 mg/l) increased the rates of morulae and blastocysts compared with lower concentrations. At the same time, trace element dose over can lead to toxicity. A high concentration of iron together with unsaturated fatty acids may stimulate formation of highly reactive free radicals and hydroperoxides, which can cause oxidative damage [23]. The blastocyst rate of the 3.26 mg/l iron group was significantly lower than the 1.96 mg/l iron group. This suggests that the presence of iron or copper in the culture media accelerated formation of 8-cell embryos, morulae, and blastocysts and that higher concentrations of iron or copper decreased the rates of morulae and blastocysts compared with lower concentrations.

Another approach to evaluation of viable embryos is to observe apoptosis in blastocysts using the TUNEL assay [24]. Apoptosis depends on the development stage of the embryos and occurs with different frequencies in different strains. Apoptosis is also affected by a wide variety of non-physiological stimuli in relation to *in vitro* culture conditions, such as temperature, toxicants, and oxidative stress [25–27]. In the present study, trace amounts of iron or copper in the culture media decreased the rate of apoptosis of blastocysts, and experiment groups lacking iron or copper had higher rates of apoptotic blastomeres. These results suggest that long-term lack of trace amounts of iron or copper in culture medium produces low quality blastocysts, although there was no significant difference in the cell numbers of blastocysts in the present study.

Embryonic block in cattle mostly occurs during the fourth or between the fourth and fifth cell cycle transition [28]. This developmental block is observed in many species. It occurs concurrent with the maternal-embryo transition, the stage of developmental when embryos conclude the major genome activation [29] and must rely on the mRNAs transcribed from their own genome to continue development. Besides the maternal-embryo transition, whether there is transition of primary demand for some component (such as a trace element) utilized by zygotes from cytoplast to culture medium is unclear. Our study showed that matured oocytes and zygotes that were cultured for less than 96 h utilized little of the iron or copper in the culture media. There are two possible reasons for this. One is that zygotes have low demand for trace amounts of iron and copper. The other is that primary demand for these elements is satisfied from the cytoplast of oocytes and not from culture medium. However, the changes in the concentrations of iron and copper were higher and steadier after zygote culture for 96, 144 and 192 h. This indicates that the zygotes had different demands for iron and copper from the culture media during different periods of development *in vitro* and that they had high and steady demand for these elements from culture medium after zygote culture for 48 h.

In conclusion, the results of the present study suggest that iron and copper play an important role in successful culture of 8-cell embryos, morulae, and blastocysts and that long-term lack of iron or copper increases the rate of apoptotic blastomeres. Furthermore, transition of primary demand for trace amounts of iron or copper from the cytoplast to culture medium for utilization by zygotes may occur after *in vitro* zygote culture for 48 h.

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