Attempts to improve the yield of bovine blastocysts by incorporating insulin, selenium and transferrin in the *in vitro* system

C.M. Bowles and A.W. Lishman

Department of Animal Science and Poultry Science,University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, 3209 Republic of South Africa

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The aim of the present investigation was to improve maturation, fertilisation and blastocyst production rates by adding insulin (10 μg/ml), selenium (10 ng/ml) or transferrin (10 μg/ml) individually and in different combinations to *in vitro* culture media. The experiment was divided into three parts with the additives being included to: (A) the maturation medium where effects on maturation rates and the subsequent development of bovine oocytes were measured, (B) the fertilisation medium, assessing fertilisation rates and further development and (C) the culture medium where bovine embryo development was evaluated. Selenium, when added to maturation medium, significantly ($p < 0.01$) improved maturation rates (80.4% vs 61.8%), fertilisation rates (68.0% vs 58.4%) and the percentage of blastocysts produced (24.6% vs 11.5%). None of the treatments had a beneficial effect on fertilisation rate or blastocyst production rate when added to the fertilisation medium. Incorporation of transferrin or transferrin in combination with insulin and selenium in the culture medium, significantly ($p < 0.05$) increased the percentage of blastocysts produced (35.3% and 31.5% vs 18.7%). Transferrin also significantly ($p < 0.05$) increased the percentage of blastocysts that hatched (21.9% vs 14.2%), indicating an improvement in the viability of the blastocysts produced. By adding selenium to the maturation medium and transferrin to the culture medium the *in vitro* production of bovine blastocysts is improved.

Die doelwit van die huidige ondersoek was om die tempo van rypwording, bevrugting en blastosistproduksie te verhoog deur insulien (10 μg/ml), selenium (10 ng/ml) of transferrien (10 μg/ml) afsonderlik, en in verskillende kombinasies by *in vitro* kweek-medium te voeg. Die eksperiment is in drie dele verdeel en die middels is gevoeg by: (A) die rypwordingsmedium, waar die effek op rypwordingstempo en die gevolglike ontwikkeling van bees-oosiete ondersoek is, (B) die bevrugtingsmedium, wat die uitwerking op die bevrugtingstempo en die daaropvolgende ontwikkeling van oosiete ten doel gehad het en (C) die kweekmedium waar die uitwerking op die ontwikkeling van die beesembrio, die mikpunt was. Wanneer selenium by die rypingwordingsmedium gevoeg is, is die persentasie rypwording (80.4% vs 61.8%), die bevrugtingstempo (68.0% vs 58.4%) en die persentasie blastosiste wat geproduseer is (24.6% vs 11.5%) betekenisvol ($p < 0.01$) verhoog. Geeneen van die behandelings het 'n voordelige uitwerking op die tempo van bevrugting of blastosistproduksie gehad, indien dit by die bevrugtingsmedium gevoeg is nie. Wanneer transferrin of transferrin in kombinasie met insulien en selenium by die kweekmedium gevoeg is, is die persentasie blastosiste geproduseer (35.3% en 31.5% vs 18.7%) betekenisvol verbeter ($p < 0.05$). Die byvoeging van transferrin het ook die persentasie blastosiste wat ontwikkel, verhoog (21.9% vs 14.2%; $p < 0.05$), wat dui op 'n verbetering in die lewensvatbaarheid van die blastosiste wat geproduseer is. Om selenium by die rypwordingsmedium by te voeg en transferrin by die kweekmedium, die *in vitro* produksie van beesembrio's verbeter.

Keywords: Bovine blastocyst, *in vitro*, insulin, selenium, transferrin

Introduction

The number of viable embryos produced from the culture of immature oocytes that undergo *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) followed by *in vitro* culture (IVC), plateaus at 20–30%, even with numerous variations on the basic technique. The efficiency (blastocyst yield) of the technique is unsatisfactory because of the limited information available concerning the requirements of bovine oocytes to achieve normal maturation and of embryos for development in culture (Bavister *et al.*, 1992). Failure to achieve high success rates in the culture of oocytes and embryos may reflect the absence in the culture media of factors normally present in the *in vivo* environment of the oocytes or embryos (Rexroad & Powell, 1988). Factors promoting the growth of various types of tissue and cells may also control the growth of early mammalian embryos (Zhang *et al.*, 1994). In somatic cell culture, insulin, selenium and transferrin either individually or in different combinations were found to be essential for many cell lines (Shamsuddin *et al.*, 1994).

Insulin is essential for follicle culture and lack thereof results in follicle degeneration (Roy & Greenwald, 1989). Insulin promotes the uptake of glucose and amino acids and may have a mitogenic effect owing to this property. Selenium functions as a component of glutathione peroxidase which helps remove free radicals of oxygen and thus prevents oxidative damage to cells and tissues. Transferrin binds iron (Fe), making the Fe ions less toxic, but still available to the cell and thus transferrin exerts its supportive effect on *in vitro* development via its chelating effects (Freshney, 1987). Natsoyama *et al.* (1993) showed that transferrin has a prominent growth-promoting activity. It seems rational to study the effects of insulin, selenium and transferrin supplementation in searching for improved culture systems for bovine embryo development (Shamsuddin *et al.*, 1994). It is thus necessary to improve the culture system, not only to increase the rate of blastocyst development, but also to qualify the blastocyst with survivability, comparable to that of *in vivo* produced embryos.

Materials and Methods

Oocyte recovery

Bovine ovaries were removed from culled cows and heifers at a local abattoir. The ovaries were placed in saline with antibiotic (0.5 µg/ml gentamicin; Sigma Chemical Co., USA), maintained at 35°C in a thermoflask and transported to the laboratory. All follicles less than 6 mm in diameter were aspirated using a 10 ml syringe fitted with an 18 gauge needle. The aspirate was placed in a 50 ml centrifuge tube and maintained in a water bath at 35°C until the cellular content settled to the bottom and this was then transferred to a petri dish containing M199 Hepes (with 1 mg/ml bovine serum albumen, fraction V (Sigma); 0.2 mM pyruvate; 25 µg/ml gentamicin and 5 µg/ml heparin). Oocytes which exhibited an evenly granulated cytoplasm and which were completely surrounded by at least three layers of unexpanded cumulus cells were selected.

In vitro maturation procedure

Selected oocytes were washed in maturation medium TCM199, supplemented with 10% FCS (Highveld Biological, RSA), 0.2 mM pyruvate, 25 µg/ml gentamicin, 2.5 µg/ml LH (Sigma), 20 µg/ml FSH (Sigma) and 1 µg/ml oestradiol-17β. Ten oocytes were randomly allocated to each 50 µl drop of TCM199 under mineral oil and incubated at 38.5°C in a humidified incubator, under 5% CO_2 in air, for 24 h. In the first part of the experiment (part A), insulin (10 µg/ml), selenium (10 ng/ml) and transferrin (10 µg/ml) were added individually or in combination to the maturation medium. The eight treatments used in the experiment are set out in Table 1. After 24 h in the maturation medium, oocytes were transferred to the fertilisation medium for 18 h and subsequently to a

Table 1 Treatments used in the experiment

Treatment 1	control
Treatment 2	insulin (I)
Treatment 3	selenium (S)
Treatment 4	transferrin (T)
Treatment 5	I + S
Treatment 6	I + T
Treatment 7	S + T
Treatment 8	I + S + T

culture medium for seven days. In the second (part B) and third (part C) parts of the experiment, treatments were added to the fertilisation medium or the culture medium, respectively. To determine the number of oocytes maturing after 24 h in the maturation medium, a random sample of oocytes was not fertilised, they were transferred to a slide, after being vortexed to remove all cumulus cells. The slide was mounted, transferred to a Coplin jar containing acetic acid and ethanol (1: 3) for fixation, stained with aceto-orcein (2%) and then examined under a microscope. An oocyte was considered to be matured, if it exhibited a metaphase plate and a polar body, indicating that the oocyte is at metaphase II of meiosis.

In vitro fertilisation procedure

In vitro fertilisation was performed using frozen bovine semen from one ejaculate of bull FR1792 (Taurus Co-operative). After 24 h of *in vitro* maturation, oocytes were pooled and washed in fertilisation medium (modified Tyrode's-lactate medium supplemented with 0.2 mM pyruvate, 6 mg/ml fatty acid free BSA (Sigma) and 25 μg/ml gentamicin) and oocytes in groups of 10 were randomly allocated and transferred to 50 μl drops of fertilisation medium under mineral oil. The semen was thawed in water at 35°C and then placed on a percoll gradient (90%,45%) and centrifuged at 2600 rpm for 10 min. At the time of insemination, 5 μl of final concentration heparin (10 μg/ml), 5 μl PHE (20 μM penicillamine, 10 μM hypotaurine and 1 μM epinephrine) and 5 μl of the final sperm suspension were added to each drop, resulting in a final concentration of 1×10^6 spermatozoa/ml of fertilisation medium. Oocytes were coincubated for 18 h with spermatozoa at 38.5°C in a humidified incubator under 5% CO_2 in air.

In the next stage (part B) of the experiment; insulin, selenium and transferrin were added individually or in combinations to the fertilisation medium at a concentration of 10 μg/ml, 10 ng/ml and 10 μg/ml respectively. Treatments were the same as for maturation treatments (Table 1). After 18 h, oocytes were transferred to untreated culture medium. For assessment of the number of oocytes fertilised, a random sample of oocytes was not cultured (control), but transferred to slides, stained and evaluated under a microscope. An oocyte was considered to be normally fertilised if it exhibited two pronuclei and a minimum of one polar body.

In vitro culture procedure

Eighteen hours after addition of the spermatozoa, the oocytes were placed in TCM199 Hepes, vortexed for 2.5 min to remove all cumulus cells and then washed in CR1aa (Charles Rosenkrans medium with amino acids) culture medium. Finally, they were placed in 50 μl drops of CR1aa under mineral oil and incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C. For the final part (part C) of the experiment, the same eight treatments as for IVM and IVF were randomly assigned to each 50 μl culture drop. Initial cleavage (>2 cells) was assessed at 42 h after insemination. On Day 4 of incubation, 5 μl of 10% FCS was added to each drop of culture medium. Criterion for normal development was attainment of the blastocyst stage by Day 7 after insemination.

Statistical analysis

In Parts A and B, there were 5 replicates for Treatment 1 and 8, 20 replicates for Treatment 2,3 and 4, and 10 replicates for Treatment 5, 6 and 7. In Part C, there were 6 replicates for Treatment 1 and

8, 24 replicates for Treatment 2, 3 and 4 and 12 replicates for Treatment 5, 6 and 7. The treatments were randomly assigned to 50 µl drops of each culture medium on a 60 mm Falcon petri dish and each drop contained 10 oocytes. An analysis of variance, using Genstat, was carried out on the number of oocytes matured, number fertilised and the number of blastocysts for each treatment (Steel & Torrie, 1981).

Results

Table 2 demonstrates the results for Part A, where the supplements were added to the maturation medium. The effect on IVM, IVF and blastocyst development was evaluated using 1200 oocytes. The addition of insulin alone, selenium alone and transferrin alone, significantly ($p < 0.05$) increased the maturation rates, compared to the control (Table 2). However, with the insulin and transferrin treatments, effects on fertilisation and blastocyst production were not significantly different. In contrast, selenium alone, when added at 10 µg/ml to the maturation medium significantly ($p < 0.01$) increased the maturation rate (80.4% vs 61.8%), fertilisation rate (68.0% vs 58.4%), cleavage rate (72.6% vs 58.7%) and the blastocyst production rate (24.6% vs 11.5%). Selenium also significantly ($p < 0.05$) decreased the percentage of polyspermy occurring during fertilisation (9.8% vs 11.9%). Insulin plus selenium had no significant effect on maturation or fertilisation rates, but did significantly ($p < 0.01$) increase the percentage of oocytes cleaving and the percentage of blastocysts produced (18.0% vs 11.5%).

In Part B, the insulin, selenium and transferrin treatments were added to the fertilisation medium. The effects on IVF and on blastocyst development were evaluated, with 800 oocytes being used for the evaluations (Table 3). The mean percentage of matured oocytes obtained was 70%. Most treatments (Treatment 2, 4, 5 and 6) significantly ($p < 0.01$) decreased the fertilisation rates and increased the polyspermy rates. However, selenium significantly ($p < 0.01$) increased fertilisation rates, but none of the treatments had an effect on the percentage of blastocysts produced.

The effect of supplementation on blastocyst production was evaluated, with 640 oocytes being analysed for IVC when the treatments were added to the culture medium after IVM and IVF in normal laboratory media (Table 4). The mean percentage of matured and fertilised oocytes was 71% and 68% respectively, and the mean percentage of oocytes exhibiting polyspermy was 7%. The addition of insulin to the culture medium decreased the number of blastocysts produced, while

Table 2 Development of oocytes with supplementation of insulin, selenium or transferrin to the maturation medium in an *in vitro* system

Treatment		Number						
Number	Description	oocytes	Reps	% matured	% polyspermy	% fertilised	% cleaved	% blastocysts
1	control	150	5	61.8±2.9	11.9±1.1	58.4±3.0	58.7±9.1	11.5±1.6
2	insulin (I)	150	20	72.3±1.8*	11.0±1.0	50.6±1.6	67.9±t1.8	11.7±0.8
3	selenium (S)	150	20	80.4±1.8**	9.8±1.0*	68.0±1.6 **	72.6±1.8**	24.6±0.8**
4	transferrin (T)	150	20	72.9±1.8*	11.8±1.0	48.5±1.6**	68.8±1.8	9.7±0.8
5	I + S	150	10	80.8±2.5	8.9±1.5	58.4±2.3	75.0±2.5**	18.0±1.1**
6	I + T	150	10	75.4±2.5	9.1±1.5**	60.5±2.3*	65.6±2.5	10.3±1.1
7	S + T	150	10	86.3±2.5**	13.5±1.5**	49.1±2.3**	75.0±2.5*	10.7±1.1
8	I + S + T	150	5	90.0±4.0	11.8±2.2	65.6±3.0**	71.6±5.9	9.5±1.5

All values are means ± S.E.M. *$p < 0.05$, **$p < 0.01$ compared to the control value.

Table 3 Effect of insulin, selenium or transferrin when included in the fertilisation medium in an *in vitro* system

Treatment		Number					
Number	Description	oocytes	Replicates	% polyspermy	% fertilised	% cleaved	% blastocysts
1	control	100	5	15.9±5.0	58.8±2.5	62.6±6.5	15.0±2.4
2	insulin (I)	100	20	25.4±2.6**	45.3±1.1**	59.2±3.5	10.3±1.0
3	selenium (S)	100	20	20.5±2.6	69.5±1.1**	64.0±3.5	14.1±1.0
4	transferrin (T)	100	20	21.5±2.6	46.2±1.1**	57.5±3.5	11.0±1.0
5	I + S	100	10	23.5±3.6*	450±1.5**	68.1±5.0**	13.0±1.4
6	I + T	100	10	30.8±3.6**	45.6±1.5**	55.7±5.0	12.0±1.4
7	S + T	100	10	20.8±3.6	55.5±1.5	60.4±5.0	12.0±1.4
8	I + S + T	100	5	20.4±5.5	53.3±2.5	60.5±7.1	11.7±2.3

All values are means ± S.E M. *$p < 0$ 05, **$p < 0.01$ compared to the control value.

Table 4 Supplementation of insulin, selenium or transferrin to the culture medium in an *in vitro* system

Treatment		Number				
Number	Description	oocytes	Replicates	% cleaved	% blastocysts	% hatched
1	control	80	6	62.3±1.7	18.7±3.9	14.2±2.3
2	insulin (I)	80	24	70.5±1.9**	17.7±2.0*	15.9±2.2
3	selenium (S)	80	24	70.1±1.9**	23.4±2.0	16.2±2.2
4	transferrin (T)	80	24	71.5±1.9**	35.3±2.0*	21.9±2.2*
5	I + S	80	12	60.0±2.6	18.3±2.9	22.5±3.1*
6	I + T	80	12	68.1±2.6**	20.2±2.9	9.2±3.1**
7	S + T	80	12	68.2±2.6**	27.0±2.9	16.4±3.1
8	I + S + T	80	6	69.5±4.1	31.5±4.0*	18.4±50

All values are means ± S.E.M. *$p < 0.05$, **$p < 0.01$ compared to the control value

transferrin or insulin plus selenium plus transferrin significantly ($p < 0.05$) increased the number of blastocysts produced.

Discussion

Zhang *et al.* (1991) showed that when M199 (maturation medium) is supplemented with insulin, cumulus expansion scores and the level of maturation are improved, but the yield of morulae and development of blastocysts are not affected. This is in agreement with the present results obtained (Table 2), where insulin significantly ($p < 0.05$) increased the maturation rate. However, this hormone did not improve the fertilisation rate or blastocyst production rate.

This study also showed that selenium significantly ($p < 0.01$) increased maturation, fertilisation, cleavage and blastocyst production rates, while polyspermy rates were significantly ($p < 0.05$) decreased. Furthermore, selenium in combination with insulin significantly ($p < 0.01$) improved cleavage rates and blastocyst production rates. This suggests that selenium is beneficial in preparing the oocyte for later development and could be working through its antioxidant actions by sca-

venging super-oxide radicals and sufficiently preventing cell membrane damage (Freshney, 1987; Nasr-Esfahani & Johnson, 1992).

When the fertilisation medium was supplemented with insulin, selenium or transferrin, *in vitro* fertilisation rates were adversely affected (Table 3). The fertilisation rates were significantly ($p < 0.01$) decreased and the polyspermy rates were increased. This is in agreement with Eppig *et al.* (1986) who added insulin, transferrin and selenium to media and found no significant improvement in fertilisation rates. These findings suggest that perhaps spermatozoa are very sensitive to unnatural elements not normally found in the bull ejaculate or in the female reproductive tract. Another explanation is that by addition of these factors, perhaps the oocyte is affected at a very sensitive and crucial stage. The blocks which prevent polyspermy might be affected and once polyspermy has occurred the embryo may not survive many cleavage divisions. However, selenium was seen to significantly ($p < 0.01$) improve fertilisation rate in the present study. This improvement could be accounted for on the basis that selenium deficiencies have been shown to impair sperm motility (Slaweta *et al.*, 1988) and that the addition of selenium to the medium, may be providing an essential element to sperm mitochondria, whereby sperm motility and fertilisation rates could be improved. None of the treatments had a significant effect on blastocyst production rates. The improved fertilisation rates when selenium is added, are most probably due to the action of selenium on the spermatozoa and not the oocytes and so development of the embryo cannot be sustained in the culture medium. From the present results it is thus suggested that none of the above treatments should be added to IVF, as no benefit is observed in terms of the blastocyst production rate.

Treatments added to the culture medium showed that while some (insulin alone, selenium alone, transferrin alone, insulin plus transferrin or selenium plus transferrin) improved the cleavage rates (early embryo development) others favoured blastocyst production rates (late embryo development). This could be due to differences in requirements of the early embryo, compared to the late embryo, especially with regard to glucose utilisation (Harvey & Kaye, 1988). The reason why insulin increased cleavage rates, but decreased blastocyst production, may be because of the post-compacted embryo being highly sensitive to insulin. Harvey and Kaye (1988) also demonstrated a stage-specific binding by insulin which could be related to embryonic metabolism and the switch from lactate to glucose utilisation. Further confirmatory evidence is provided by Kimura and Totsukawa (1995) who observed that the percentage of embryos developing to the 8-cell stage is significantly increased by addition of 10 μg/ml insulin to the culture medium. However, development to the morula stage and blastocyst stage were not significantly increased. Present findings are in agreement with these results (Table 4). Although transferrin was shown to improve blastocyst production rates (Table 4), when combined with insulin, only cleavage rates were improved. Thus, insulin seems to have a strong growth-suppressing effect on the growth of the late stage embryo. Matsui *et al.* (1995) added 5 μg/ml insulin to the culture medium and the percentage of embryos reaching the morula stage was not affected by addition of insulin alone, but was significantly increased when amino acids were included with insulin. A significant increase in the cell numbers of the blastocysts was observed, suggesting that insulin can improve embryonic development by stimulating amino acid transport and/or glucose uptake. This implies that a higher level of amino acids and/or glucose is needed in the culture medium to realise improvements to blastocyst yield by addition of insulin.

The improvement in blastocyst yield where transferrin alone or in combination with insulin and selenium (Treatment 8) were incorporated (Table 4) is supported by the findings of Natsuyama *et al.* (1993) who showed a prominent growth-promoting activity of transferrin when added to IVC media. It was proposed that the growth-promoting effect was due to either an anti-oxidant action or

to the removal of metal ions such as Fe^{3+} (iron). Iron is the fourth most abundant element in the earth's crust (Gutteridge, 1989) and could be present in small amounts in the water used as the base for all media preparations. Owing to normal cell respiration, the reduction of oxygen occurs and forms superoxide radicals which are harmful in large, uncontrolled amounts. In the presence of free radicals, iron is reduced to the ferrous state and together these radicals and iron form a very harmful hydroxy radical via the Fenton reaction. This damages tissues and biological molecules as badly as radiation could (Gutteridge, 1989). An antioxidant like selenium can prevent harmful radicals occurring by scavenging all excess by-products, but when iron is present this cannot be completely controlled by selenium. This is where transferrin is beneficial, as iron can be removed from the media before its breakdown to a ferrous state. In addition, iron will then safely exist within the cell where it can support the synthesis of iron-containing proteins (Gutteridge, 1989).

The selenium plus transferrin treatment (Treatment 7) seems to be operating via this antioxidant and iron chelating effect, as blastocyst production rates were improved from 18.7% to 27.0%. Shamsuddin *et al.* (1994) added ITS (at 5 µg/ml) to cultured IVM/IVF oocytes and noted that the percentage of embryos showing cleavage and blastocyst production did not differ from the control, but the blastocysts exhibited better viability and post-thaw survivability. The present results demonstrate that transferrin also improves the percentage of blastocysts which hatch (21.9% vs 14.2%). Flechon and Renard (1978) believe that the number of blastocysts hatching is a measure of the adequacy of the culture conditions. Viability of the blastocyst is important as an end point when attempting to produce calves and it seems that addition of transferrin to the culture medium would improve blastocyst yields and viability in the IVC system.

To realise improvements in the number of bovine embryos produced in an *in vitro* system, as shown in this investigation, selenium should be added to the maturation medium and transferrin to the culture medium.

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