Reprod Dom Anim **36**, 189–194 (2001)

ISSN 0936-6768

Effects of Acetoacetate on *in vitro* Development of Bovine Embryos in Medium Containing Citrate and Myo-inositol

E Gómez, P Duque, E Díaz and C Díez

Servicio Regional de Investigación y Desarrollo Agroalimentario-Centro de Selección y Reproducción Animal (SERIDA-CENSYRA), Somió, Gijón, Asturias, Spain

Contents

This study investigated bovine embryo development *in vitro* in the presence of acetoacetate in serum-free medium. *In vitro*-matured and fertilized oocytes from ovaries of slaughtered cows were cultured in synthetic oviduct fluid (SOF) containing citrate, myo-inositol, lactate and pyruvate. In the medium with acetoacetate this compound replaced both lactate and pyruvate as energy sources. Three experiments were carried out: (1) to test development in medium with acetoacetate and bovine serum albumin; (2) to analyse the effects of acetoacetate that were dependent upon citrate and myo-inositol; and (3) to determine the effects of acetoacetate in the presence of serum. Blastocyst development was recorded at day 8 and the number of cells of expanded blastocysts obtained were counted. Blastocysts development was reduced in medium with 1.8, 3.6 or 7.2 mM acetoacetate in comparison with the control with or without lactate and pyruvate. The detrimental effect of acetoacetate was independent of the presence of citrate and myo-inositol, but serum added to culture medium protected against this effect. Citrate and myo-inositol did not improve blastocyst formation. Morphological quality and cell number of blastocysts were similar between groups.

Introduction

Research in the past decade has largely focused on the availability of energy substrates and their role in promoting embryo development in an effort to improve the culture conditions of bovine embryos. The embryonic requirements for specific energy substrates such as glucose, pyruvate, lactate and glutamine (Takahashi and First 1992; Kim et al. 1993; Matsuyama et al. 1993; Rosenkrans et al. 1993; Pinyopummintr and Bavister 1996) were examined through the use of simple, defined culture media. In adult tissues, the ketone bodies acetoacetate and D-β-hydroxybutyrate may be utilized in aerobic metabolism and are quantitatively important as energy sources; in fact, the heart and kidney cortex utilize acetoacetate in preference to glucose.

Recently, it has been shown that bovine embryos can be cultured up to the hatched blastocyst stage by using either acetoacetate or D-β-hydroxybutyrate (Gómez 1997) at any time during *in vitro* development (Gómez and Díez 1998). In these two studies embryos reached post-compaction stages in synthetic oviduct fluid medium as modified by Takahashi and First (mSOF: Takahashi and First 1992) with fetal calf serum (FCS), which is known to give nonrepeatable results and increase replicate variation in embryo development (Thompson et al. 1998). Although this inconvenience could be overcome by using simple media under defined conditions, actually protein supplementation has been shown to be beneficial for embryo development *in vitro* (reviewed by Gómez and Díez 2000; Thompson 2000). In the last year, a modification of SOF medium containing citrate and myo-inositol (SOFaaci; Holm et al. 1997, 1999) has been shown to yield consistent proportions of morphologically good blastocysts in the authors' laboratory. Therefore, this medium with albumin was chosen to test the effects of acetoacetate in serum-free culture conditions and, subsequently, to test the effects of serum itself. Apart of the presence of trisodium-citrate and myo-inositol themselves, SOFaaci concentrations of lactate and pyruvate are two-fold greater than those in mSOF. A concentration of acetoacetate 3.6 mM has been demonstrated to be beneficial for bovine embryo development *in vitro* (Gómez 1997). However, as SOFaaci contains substances that could be able to act as substrates which are not included in mSOF (i.e. citrate and myo-inositol), a redosage of acetoacetate is suggested. As a consequence, the objectives of this work were to investigate the requirements of acetoacetate as incorporated in the SOFaaci culture system, and its dependence upon citrate plus myoinositol and serum.

Materials and Methods

Bovine ovaries recovered from slaughtered Asturiana de los Valles cows were placed in 0.9% (wt/vol) NaCl containing antibiotics (Penicillin, 100 U/ml and streptomycin sulphate, 100 mg/ml) and maintained at 30–35°C until recovery of cumulus-oocyte complexes (COCs). Ovaries were washed twice in distilled water and once in saline and antibiotics. The COCs were aspirated from follicles (2–8 mm) through an 18-gauge needle and placed in a 50 ml plastic tube (Nunc, Roskilde, Denmark). The follicular fluid and COCs were placed in an embryo filter and rinsed with holding medium, consisting of 4.2 mM $NaHCO_3$ containing TCM-199 (No. 31100027, Gibco, Life Technologies, Barcelona, Spain), 20 mM HEPES (Sigma, H-3375, Madrid, Spain) and 2 IU/ml heparin.

In vitro maturation

Only oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were selected for maturation. The COCs were washed three times in the holding medium and twice in the maturation medium, which consisted of Medium 199 (Sigma), 10% v/v fetal calf serum (FCS, F-4135, Sigma), FSHp (1 μg/ml, Sigma), LH (5 μg/ml, Sigma), 17β-estradiol (1 μg/ml, Sigma) and cysteamine 100 μM (Sigma; M-9768, De Matos et al. 1995).

Maturation was performed by culturing approximately 50 COCs in 500 μl of maturation medium in four-well dishes (Nunc) at 39°C in 5% CO_2 under air and high humidity for 23–24 h.

In vitro fertilization

In vitro fertilization was carried out using sperm separated by a swim-up procedure similar to that previously reported by Parrish et al. (1986). Briefly, semen from one frozen straw of a single bull was thawed in a water bath and added to a polystyrene tube containing 1 ml of pre-equilibrated Sperm-tyrode albumin lactate pyruvate (TALP). After 1 h of incubation, 700 μl of the upper layer of supernatant containing the motile sperm was removed. The sperm were centrifuged for 7 min at $700 \times \boldsymbol{g}$ and the supernatant aspirated to leave a pellet approximately 100 μl in volume. Sperm concentration was determined with a haemocytometer. After maturation, the COCs were washed three times in holding medium, once in fertilization medium and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 μg/ml, Calbiochem, 375095, La Jolla, CA, USA). Spermatozoa were then added to a concentration of 2×10^6 cells/ml in 500 μl of medium per well containing 100 COCs. *In vitro* fertilization was accomplished by incubating oocytes and sperm cells together for 20 h at 39°C in 5% CO_2 in air and high humidity.

Preparation of acetoacetate

Sodium acetoacetate was obtained by hydrolysis of ethyl acetoacetate (Sigma) with equimolar NaOH solution with further lyophilization to eliminate water and the ethanol formed; the crystallized sodium acetoacetate prepared in this manner was stored frozen at −20°C. Thawed aliquot samples of the synthetic product were analysed by MS-GC, by using a capillary polyethylene glycol phase modified n-phtalic acid (FFAP) column (50 m × 0.21 mm inside diameter; 0.3 μm thickness phase) from Teknokroma, Barcelona (Spain). Quantification was performed in SCAN mode, taking into account the main ion (mw 43). Samples were dissolved in 6% ethanol, filtered trough 0.45 μm-cellulosa acetate and injected onto the column.

In vitro embryo culture

Embryo cultures were performed in SOFaa containing citrate and myo-inositol as a basic medium, as reported by Holm et al. (1997, 1999) with minor modifications. This culture medium contained bovine serum albumin (BSA) (8 mg/ml, Sigma) and was further supplemented with FCS (5% v/v), where indicated, 48 h post-insemination. In culture media containing acetoacetate this compound replaced lactate and pyruvate. Culture media were renewed on days 3 and 6 of culture (fertilization: day 0). Media were freshly prepared and had a pH of 7.2–7.3 and osmolality of 280–290 mOsm. Fifty microlitre droplets (2 μl/embryo, approximately) of the corresponding culture medium were prepared in four-well culture dishes under mineral oil and allowed to equilibrate in the incubator for at least 2 h before the addition of embryos. Fertilized oocytes were vortexed for 3 min in holding medium to separate cumulus cells, rapidly washed three times in holding medium and twice in the corresponding culture medium prior to the 8-day culture period. Culture conditions were 39°C and high humidity in 5% CO_2 in air. Cleavage and 5 to 8 cell stage, morulae, day 7 blastocysts and day 8 blastocysts were evaluated at 72, 144, 168 and 192 h post-insemination (PI), respectively.

Experiment I: effect of increasing concentration of acetoacetate in SOFaaci + BSA

Three concentrations of acetoacetate were tested in medium containing 8 mg/ml BSA in the absence of serum. Development of embryos cultured in SOFaaci containing three concentrations of acetoacetate (1.8, 3.6 and 7.2 mM) was compared with that of embryos in medium containing lactate 7.02 mM + pyruvate 0.72 mM and no substrate as the controls.

Experiment II: effect of acetoacetate in the presence or absence of myo-inositol and citrate

This experiment evaluated the effect of acetoacetate on embryo development, dependent upon the citrate plus myo-inositol contained in serum-free SOFaaci. Thereby, culture groups lactate 7.02 mM + pyruvate 0.72 mM, acetoacetate 3.6 mM and no substrate were tested in the presence or the absence of citrate + myo-inositol. Cell numbers of expanded blastocysts obtained at day 8 PI were counted as reported previously (Gómez 1997). Briefly, blastocysts were maintained for 20 min in hypotonic solution (0.9% w/v of sodium citrate in water) fixed with methanol, acetic acid and water (3 : 2 : 1), and stained with Giemsa (1/20, v/v) for 15 min. Cell counting was carried out at × 200 under a light microscope.

Experiment III: effect of adding serum on the toxicity of acetoacetate in SOFaaci

The effect of serum on embryos developing in SOFaaci containing acetoacetate was tested in a 3 × 2 factorial design. Embryos were cultured in the following three conditions: (1) lactate 7.02 mM + pyruvate 0.72 mM; (2) acetoacetate 3.6 mM; and (3) no substrate. Each condition was tested in the presence or absence of 5% FCS.

Statistical analysis

Data were arc-sine transformed and analysed by one-way analysis of variance (ANOVA). The numbers of replicates and treatments were considered as fixed effects. Appropriate tests for means used to estimate significance are cited under tables. Data were expressed as mean percentages ± SEM of the cultured oocytes.

Results

A total of 1799 presumptive zygotes were put in culture within all experiments. Synthesized sodium acetoacetate

Table 1. Effect of acetoacetate concentration on *in vitro*-development of bovine embryos cultured in mSOF + aa containing citrate and myo-inositol with bovine serum albumin (8g/l) in the absence of serum

Group	n	R	Percentage 5–8 cell	Percentage morulae	Percentage blastocysts	
					Day 7	Day 8
Lactate/pyruvate	88	4	79.2 ± 4.6^{a}	39.1 ± 3.9^{a}	23.4 ± 8.2^{a}	31.4 ± 4.5^{a}
No substrate	89	4	57.1 ± 4.5^{ab}	19.0 ± 3.6^{b}	9.9 ± 3.0	12.3 ± 3.3^{b}
Acetoacetate 1.8 mM	64	3	45.9 ± 11.8^{bc}	12.3 ± 2.5^{bc}	3.1 ± 1.5^{b}	3.1 ± 1.5^{c}
Acetoacetate 3.6 mM	93	4	36.9 ± 8.8^{bc}	8.6 ± 5.8^{bc}	0.0^{bd}	2.5 ± 1.4^{cd}
Acetoacetate 7.2 mM	65	3	29.6 ± 5.2^{c}	3.4 ± 3.3^{c}	0.0^{bd}	1.7 ± 1.6^{cd}

n, number of oocytes; R, number of replicates. ANOVA and Duncan's test: $^{abc}p < 0.05$; $^{ad}p < 0.01$.

Table 2. Effects of citrate + myo-inositol and acetoacetate on *in vitro*-development of bovine embryos cultured in mSOF + aa containing BSA (8g/l) in the absence of serum

Energy substrates	Citrate + Myo-inositol	n	R	Percentage morulae	Percentage blastocysts	
					Day 7	Day 8
Lactate/pyruvate	+	92	4	31.9 ± 8.3^{a}	23.6 ± 5.7^{a}	25.5 ± 6.8^{a}
Lactate/pyruvate	−	67	3	33.0 ± 1.2^{a}	22.6 ± 1.7^{a}	22.6 ± 1.7^{a}
Acetoacetate	+	52	3	7.4 ± 3.6^{b}	0.0^{bd}	0.0^{bd}
Acetoacetate	−	47	3	9.2 ± 4.1^{b}	0.0^{bd}	0.0^{bd}
No substrate	+	87	4	9.7 ± 3.9^{b}	5.1 ± 3.6^{b}	6.3 ± 3.2^{b}
No substrate	−	93	4	9.5 ± 3.2^{b}	1.0 ± 0.9^{b}	1.0 ± 0.9^{b}

n, number of oocytes; R, number of replicates. ANOVA and test Ryan-Einot-Gabriel-Welsch Multiple F-Test (REGWF) $^{abc}p < 0.05$; $^{ad}p < 0.01$.

Table 3. Development of *in vitro*-produced bovine embryos in SOFaaci + BSA (8g/l) with lactate/pyruvate, acetoacetate or no substrate either in presence or in absence of fetal calf serum (FCS)

Group	FCS	n	R	Percentage 5–8 cell	Percentage morulae	Percentage blastocysts	
						Day 7	Day 8
Lactate/pyruvate	+	174	6	60.0 ± 4.3^{x}	36.5 ± 3.7^{x}	24.2 ± 3.4^{x}	25.2 ± 3.4^{x}
Lactate/pyruvate	−	131	5	75.5 ± 3.1	35.2 ± 5.7^{x}	21.0 ± 5.7^{x}	30.2 ± 6.8^{x}
Acetoacetate	+	260	9	55.4 ± 3.2	36.2 ± 2.8^{x}	20.7 ± 3.1^{xy}	22.7 ± 2.6^{xy}
Acetoacetate	−	93	4	36.9 ± 8.8^{y}	8.7 ± 5.9^{y}	0.0^{z}	2.5 ± 1.4^{z}
No substrate	+	177	6	55.1 ± 7.3	28.8 ± 3.3^{x}	16.1 ± 1.9^{xy}	17.2 ± 2.0^{y}
No substrate	−	127	5	47.2 ± 7.1	13.5 ± 4.2^{y}	6.6 ± 2.8^{xy}	9.0 ± 2.9^{y}

n, number of oocytes; R, number of replicates. ANOVA and test Ryan-Einot-Gabriel-Welsch Multiple F-Test (REGWF). Superscripts indicate significant differences: $^{xy, yz}p < 0.01$; $^{xz}p < 0.001$.

samples contained less than 0.1% ethyl acetoacetate ($\leq 2.9 \times 10^{-6}$ mM ethyl acetoacetate in culture medium). No other compounds were found.

Experiment I

Results are shown in Table 1. A detrimental effect of acetoacetate was observed at all tested concentrations from the 5 to 8 cell stage. At the cleavage stage, differences were only detected between lactate/pyruvate and acetoacetate 7.2 mM (91.7 and 78.1, respectively), whereas no differences were seen within the remaining experiments (cleavage rates ranged from 80.5 to 90.5).

Experiment II

As shown in Table 2, the detrimental effect of acetoacetate was independent of the presence of citrate and myo-inositol in the culture medium. These two compounds did not improve blastocyst development in the conditions studied.

Experiment III

The results of this experiment are shown in Table 3. The presence of serum in culture allowed blastocysts to form in medium containing acetoacetate. Some blastocysts appeared on day 6 in all groups containing serum (11.4 ± 3.2, 10.3 ± 2.3 and 5.0 ± 2.5 for lactate/pyruvate, acetoacetate and no substrate, respectively) and in lactate/pyruvate alone (8.7 ± 5.3), but no difference was observed between groups.

Table 4. Cell counts of embryos produced in SOFaaci + BSA (8g/l)

Supplementation	Embryos counted	Nuclei
Lactate/pyruvate	9	96.5 ± 8.6
Lactate/pyruvate + 5% serum	9	97.6 ± 13.0
Acetoacetate + 5% serum	11	93.4 ± 7.34

ANOVA and Duncan's test. No significant differences: $p > 0.05$.

No significant differences were found in cell number of expanded day 8 blastocysts between groups (Table 4).

Discussion

Increased levels of ketone bodies in the plasma of lactating cows are physiologic and related to evidence of lipid breakdown. Free nonesterified fatty acids are at the origin of the production of ketones from fat (Rukkwamsuk et al. 1999; Sato et al. 1999). In order to be oxidized, fatty acids must first enter the mitochondria,

where ketone bodies are generated, which can be either readily metabolized or diffuse to be used in other organs. Concentrations of ketone bodies in plasma of 1 mM or higher are indicative of mobilization of fat reserves in dairy cows (Lean 1987), and are characteristic of an increased postpartum anoestrus interval (Verkerk and Guiney 1999).

The role of ketone bodies during the embryonic development is widely unexplored. In the present work, mainly conceived to test the effect of acetoacetate in serum-free conditions during early bovine embryo development *in vitro*, the basic culture medium was modified by comparison with the authors' previous studies (Gómez 1997; Gómez and Díez 1998). Therefore a new dose–response experiment was designed in this modified medium. In those conditions, acetoacetate was revealed as an embryo-toxic compound. Interaction between citrate, myo-inositol and acetoacetate were then tested as those compounds were not present in the previous study. Experiments run in parallel into which the embryos developed in medium mSOF, exhibited a similar detrimental effect of the acetoacetate preparation in the absence of serum (Díez et al. 2000). This toxicity could not be overcome by varying the acetoacetate concentrations and was independent of the presence of citrate and myo-inositol. The remaining ethyl acetoacetate present in the acetoacetate preparation could be responsible for toxicity, as reported for 3.6 mM ethyl acetoacetate during bovine embryo culture *in vitro* (Gómez 1997). However, together with the low concentration reached once in culture in the present work (less than 2.9×10^{-6} mM), ethyl acetoacetate was first shown to be toxic in the presence of serum, which strongly contrasts with the present results and suggests some other origin for the toxicity. In any case, testing the effects of both low amounts of ethyl acetoacetate and other acetoacetate derivatives (e.g. lithium acetoacetate) on embryo development in serum-free conditions will be accomplished in further experiments.

Citrate has been previously shown to exert an embryotrophic effect when added to completely defined medium (Keskintepe et al. 1995; Holm et al. 1999). However, citrate has been found to be a common contaminant of albumin preparations (Gray et al. 1992), which makes it difficult to analyse the effects of defined concentrations of citrate in culture medium containing protein. As the internal citrate increases in the presence of acetoacetate and D-β-hydroxybutyrate in somatic cells *in vitro* (Yudkoff et al. 1997), an optimal concentration of acetoacetate that would be supportive of embryonic development in SOFaaci had to be established. This balancing would also be suggested as citrate promotes fatty acid synthesis (Goodridge 1973), although the presence of free acetoacetate is commonly associated with previous lipid breakdown. If acetoacetate and D-β-hydroxybutyrate were derived from endogenous lipids the survival after freezing and thawing of embryos is likely to increase, as is the case following the mechanical delipidation of IVP bovine embryos (Leibo et al. 1995; Díez et al. 1996; Ushijima et al. 1996, 1999). Attempts to improve freezability by triggering lipid breakdown and subsequent fatty acid β-oxidation in embryonic cells using epinephrine have been carried out, so far without positive results (Gómez and Díez 1997).

Myo-inositol and its derivatives exert a major role as cell signals, and some metabolites have a mitogenic effect (Downes and McPhee 1990). As an osmolite, myo-inositol may affect the pre-implantation period, being active in rabbit embryos (Li and Foote 1995). However, myo-inositol did not protect mouse embryos against raised osmolarity (Dawson and Baltz 1997), even though some myo-inositol transport can be demonstrated in mouse embryos (Van Winkle et al. 1985). Unlike in mice, the concentration of myo-inositol correlated with high embryotrophic activity in batches of human serum (Chiu and Tam 1992).

In the present study, neither myo-inositol nor citrate affected blastocyst development in a medium with BSA and no serum. This finding partially agrees with previous data from Holm et al. (1999), who reported no effect of myo-inositol on blastocyst development in SOF-containing citrate and BSA. Nevertheless, these authors did find a stimulating effect of myo-inositol in medium containing polyvinyl alcohol (PVA) and reported that minute concentrations of myo-inositol could be present in commercial BSA preparations. This contamination is likely to have a biological action that would mask the effects of myo-inositol added on purpose. The present findings fit well with the possibility of citrate and myo-inositol contaminations in the BSA preparation used, as any significant effect could be attributed to the simultaneous presence of these compounds in culture. As a way to accomplish a more defined study about the effects of citrate and myo-inositol in medium with protein, albumin might be dialysed to remove citrate and other putative contaminants.

In the present study the presence of serum in culture when acetoacetate was tested as an energy source did not represent any faster blastocyst development and/or higher blastocyst rates. In contrast to other reports where the presence of serum promoted the earlier appearance of blastocysts (reviewed by Gómez and Díez 2000) the use of SOFaaci seemed to preserve blastulation from the stimulative effects of the serum. The present results partially agree with those from Holm et al. (1999), who found that serum did not affect blastocyst rates although it did increase the cell numbers on day 7.

In the present study, fetal calf serum overcame the embryotoxic effect of the acetoacetate preparation in culture. The need for serum further complicates the analysis of the role of acetoacetate during the early bovine embryo development *in vitro*. To the authors' knowledge, the protective effect of serum upon acetoacetate is unexplained and controversial, as serum has been shown to contain a wide variety of compounds. Both genomic imprinting (Young and Fairburn 2000) and mitochondria (Bavister 2000) are affected by the presence of serum in the culture medium. A possible protective mechanism for serum upon acetoacetate toxicity could be promoting incorporation of acetoacetate into the embryonic lipid. This would circumvent in part the direct metabolization of the ketone that is expected to occur in the absence of serum, since it is well documented that culture media supplemented with

serum leads to excessive accumulation or synthesis of lipids in pre-implantatory bovine embryos (Ferguson and Leese 1999; Abe et al. 1999). In this respect, oleic acid incorporated from culture media by bovine embryos produced *in vitro* in serum-containing medium accounted for 58.4% of embryonic triglycerides (Abd El Razek et al. 2000). Another possible explanation for serum protection would lie in providing substances to maintain redox potential. In fact, balanced concentrations of lactate and pyruvate in culture medium exert this role and such compounds act not only as energy sources. Acetoacetate alone would not be able to preserve the appropriate redox conditions, which would be detrimental to the embryos in the absence of putative redox couples from serum. In human endothelial cells elevated levels of acetoacetate generated oxygen radicals and caused lipid peroxidation (Jain et al. 1998). The oxygen concentration used in the present experiments (5% CO_2 in air) could have also led to the formation of free oxygen radicals in the embryonic cells.

In accordance with findings from Holm et al. (1999), the blastocysts produced in SOFaaci in the present experiments, both in the presence of acetoacetate plus serum and in media containing no acetoacetate, appeared like *in vivo* embryos. Together with a light colour, tight compaction and integrity of the inner cell mass, expanded blastocysts cell counting showed no differences between groups analysed.

It was observed that citrate and myo-inositol did not improve blastocyst development under the conditions of the present work. Acetoacetate showed severe embryo toxicity, which could not be prevented by reducing acetoacetate concentration and was independent of the presence of citrate and myo-inositol. Fetal calf serum prevented the toxic effect of acetoacetate during early bovine embryo development *in vitro*.

Acknowledgements

The authors are grateful to Dr I. Donnay for critical review of the manuscript and valuable suggestions, Dr. Piccinelli for chromatographic analysis and N. Facal for excellent laboratory assistance. This work was supported by the research project 1FED97–0023 (CICYT-FEDER).

References

Abd El Razek IM, Charpigny G, Kodja S, Marquant-Leguienne B, Mermillod P, Guyader-Joly Humblot P, 2000: Differences in lipid composition between *in vivo*- and *in vitro*-produced embryos. Theriogenology **53,** 346 (Abstract).

Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H, 1999: Ultrastructure of bovine embryos developed from *in vitro* matured and fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. Mol Reprod Dev **53,** 325–335.

Bavister BD, 2000: Interactions between embryos and the culture milieu. Theriogenology **53,** 619–626.

Chiu TT, Tam PP, 1992: A correlation of the outcome of clinical *in vitro* fertilization with the insitol content and embryotrophic properties of human serum. J Assist Reprod Genet **9,** 524–530.

Dawson KM, Baltz JM, 1997: Organic osmolytes and embryos: substrates of the gly and β transport systems protect mouse zygotes against the effects of raised osmolarity. Biol Reprod **56,** 1550–1558.

De Matos DG, Furnus CC, Moses DF, Baldassarre H, 1995: Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*. Mol Reprod Dev **42,** 432–436.

Díez C, Le Bourhis D, Heyman Y, Renard JP, 1996: Effect of partial lipid removal from *in vitro* produced bovine zygotes on further development *in vitro* and on the freezing tolerance of blastocysts. Theriogenology **45,** 166 (Abstract).

Díez C, Hidalgo C, Gómez E, 2000: *In vitro*-produced bovine zygotes cultured in simple medium containing acetoacetate need serum to develop. Theriogenology **53,** 293.

Downes CP, McPhee CH, 1990: Myo-inositol metabolites as cellular signals. Eur J Biochem **193,** 1–18.

Ferguson EM, Leese HJ, 1999: Triglyceride content of bovine oocytes and early embryos. J Reprod Fertil **116,** 373–378.

Gray CW, Morgan PM, Kane MT, 1992: Purification of embryotrophic factor from commercial bovine serum albumin and its identification as citrate. J Reprod Fert **94,** 471–480.

Gómez E, 1997: Acetoacetate and β-D-hydroxybutyrate as energy substrates during early bovine embryo development *in vitro*. Theriogenology **48,** 63–74.

Gómez E, Díez C, 1997: Effects of epinephrine and lactate/pyruvate during the first 48 hours of culture on the freezability of IVF bovine blastocysts. Theriogenology **47,** 344 (Abstract).

Gómez E, Díez C, 1998: Bovine embryos can use ketone bodies as energy substrates at different developmental stages *in vitro*. Theriogenology **49,** 232.

Gómez E, Díez C, 2000: Effects of glucose and protein sources on bovine embryo development *in vitro*. Anim Reprod Sci **58,** 23–37.

Goodridge AG, 1973: Regulation of fatty acid synthesis in isolated hepatocytes. Evidence for a physiological role for long chain fatty acyl coenzyme A and citrate. J Biol Chem **248,** 4318–4326.

Holm P, Booth PJ, Vajta G, Callesen H, 1997: A protein-free SOF system supplemented with amino acids, sodium citrate and myo-inositol for bovine embryo culture. In: Foundation Marcel Merieux, Lyon, France (eds), Proc. 13th AETE Meeting, Lyon, 1997. p. 158.

Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H, 1999: High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology **52,** 683–700.

Jain SK, Kannan K, Lim G, 1998: Ketosis (acetoacetate) can generate oxygen radicals and cause increased lipid peroxidation and growth inhibition in human endothelial cells. Free Radic Biol Med **25,** 1083–1088.

Keskintepe L, Brunley CA, Brackett BG, 1995: Production of viable bovine blastocysts in defined *in vitro* culture conditions. Biol Reprod **52,** 1410–1417.

Kim JH, Niwa K, Lim JM, Okuda K, 1993: Effects of phosphate, energy substrates and amino acidos on development of *in vitro*-matured, *in vitro*-fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol Reprod **48,** 1320–1325.

Lean I, 1987: Nutrition of Dairy Cattle. University of Sydney Post-Graduate Foundation in Veterinary Science, Sydney, Australia, p. 485.

Leibo SP, Pollard JW, Martino A, 1995: Chilling and freezing sensitivity of 'reassembled' *in vitro*-derived bovine embryos. Theriogenology **43,** 265 (Abstract).

Li J, Foote RH, 1995: Effect of inositol and glycine with increasing sodium chloride and constant osmolality on development of rabbit embryos. J Assist Reprod Genet **12,** 141–146.

Matsuyama K, Miyakoshi H, Fukui Y, 1993: Effect of glucose levels during the *in vitro* culture in synthetic oviduct fluid medium on *in vitro* development of bovine oocytes matured and fertilized *in vitro*. Theriogenology **40,** 595–605.

Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL, 1986: Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology **25,** 591–600.

Pinyopummintr T, Bavister BD, 1996: Energy substrate requirements for *in vitro* development of early cleavage-stage bovine embryos. Mol Reprod Dev **44,** 193–199.

Rosenkrans CFJ, Zeng GO, McNamara GT, Schoff PK, First NL, 1993: Development of bovine embryos *in vitro* as affected by energy substrates. Biol Reprod **49,** 459–462.

Rukkwamsuk T, Wensing T, Kruip TAM, 1999: Relationship between triacilglycerol concentration in the liver and first ovulation in postpartum dairy cows. Theriogenology **51,** 1133–1142.

Sato H, Matsumoto M, Hanasaka S, 1999: Relations between plasma acetate, 3-hydroxybutyrate, FFA, glucose levels and energy nutrition in lactating dairy cows. J Vet Med Sci **61,** 447–451.

Takahashi Y, First NL, 1992: *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate and vitamins. Theriogenology **37,** 963–978.

Thompson JG, 2000: *In vitro* culture and embryo metabolism of cattle and sheep embryos – a decade of achievement. Anim Reprod Sci **60–61,** 263–275.

Thompson JG, Allen NW, McGowan LT, Bell ACS, Lambert MG, Tervit HR, 1998: Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development *in vitro* and following transfer. Theriogenology **41,** 1239–1249.

Ushijima H, Yamakawa H, Nagashima H, 1996: Cryopreservation of bovine IVM/IVF embryos at early cleavage stage following removal of cytoplasmic lipid droplets. Theriogenology **45,** 159 (Abstract).

Ushijima H, Yamakawa H, Nagashima H, 1999: Cryopreservation of bovine pre-morula-stage *in vitro*-matured/*in vitro*-fertilized embryos after delipidation and before use in nucleus transfer. Biol Reprod **60,** 534–539.

Van Winkle LJ, Christensen HN, Campione AL, 1985: Na^+-dependent transport of bassic, zwitterionic, and bicyclic amino acids by a broad-scope system in mouse blastocysts. J Biol Chem **260,** 12118–12123.

Verkerk GA, Guiney JC, 1999: Can β-D-hydroxybutyrate levels predict the post-partum anoestrous interval of dairy cows? Proc NZ Soc Anim Prod **59,** 205–207.

Young LE, Fairburn HR, 2000: Improving the safety of embryo technologies: possible role of genomic imprinting. Theriogenology **53,** 627–648.

Yudkoff M, Daikhin Y, Nissim I, Grunstein R, Nissim I, 1997: Effects of ketone bodies on astrocyte amino acid metabolism. J Neurochem **69,** 682–692.

Submitted: 28.06.2000

Author's address: Enrique Gómez, Centro de Selección y Reproducción Animal, Camino de los Claveles, Somió s/n, 33203 Gijón, Asturias, Spain