USE OF KETONE BODIES AS ENERGY SUBSTRATES AT DIFFERENT DEVELOPMENTAL STAGES AND UNDER DIFFERENT OXYGEN TENSIONS BY *IN VITRO*-PRODUCED BOVINE EMBRYOS*

UTILIZACIÓN DE CUERPOS CETÓNICOS COMO SUSTRATO ENERGÉTICO EN DIFERENTES ESTADIOS Y BAJO DIFERENTES CONDICIONES ATMOSFÉRICAS DURANTE EL DESARROLLO EMBRIONARIO BOVINO *IN VITRO**

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ADDITIONAL KEYWORDS

Cattle-embryology. Acetoacetate. β-hydroxybutyrate. Lipid.

PALABRAS CLAVE ADICIONALES

Embriología del ganado vacuno. Acetoacetato. b-D-hidroxibutirato. Lípido.

SUMMARY

In vitro-produced bovine embryos can develop into hatched blastocysts in the presence of acetoacetate and β-hydroxybutyrate, which may be derived from lipid breakdown. The effects of these ketone bodies, added during different periods of early bovine embryo development and under different atmospheric conditions, were examined. *In vitro* produced bovine zygotes were cultured in modified-Synthetic Oviduct Fluid medium. Suplementation with 3.6 mM acetoacetate or β-hydroxybutyrate for 3 periods of culture gave blastocyst and expanded blastocyst rates comparable to controls with lactate/pyruvate. However, lactate/pyruvate produced higher blastocyst formation and expansion rates than ketone bodies when different proportions (10:1 and 1:10) of 3.6 mM acetoacetate/β-hydroxybutyrate and 3.6 mM lactate/pyruvate were used with either 20 percent O_2 or 5 percent O_2. However, no differences in development were attributable to redox status. When ketone bodies were added together in culture, lower blastocyst formation rates were obtained than using the same molecules as single substrates. It is concluded that bovine embryos can utilize ketone bodies at any period of early development and can develop withstanding different redox status.

*This work was supported by a grant from FICYT, project PA-AGR96-01. Part of this work was presented to the European Association of Embryo Transfer meeting in 1997 **(table IV)** and to the International Embryo Transfer Association meeting in 1998 **(table I)**.

RESUMEN

Los embriones bovinos producidos *in vitro*

pueden desarrollarse hasta la eclosión del blastocisto en presencia de acetoacetato y b-D-hidroxibutirato, los cuales pueden proceder de la degradación de lípidos.

Se estudiaron los efectos de estos cuerpos cetónicos, añadidos durante diferentes períodos del desarrollo embrionario preimplantatorio bajo diferentes condiciones atmosféricas. Zigotos bovinos producidos *in vitro* se cultivaron en medio modified-Synthetic Oviduct Fluid. La suplementación con acetoacetato o β-D-hidroxibutirato 3,6 mM en cada uno de los 3 períodos de cultivo considerados resultó en proporciones de blastocistos y de blastocistos expandidos comparables a los controles con lactato/piruvato. Sin embargo, la mezcla lactato/piruvato 3,6 mM dió lugar a índices de formación de blastocistos y de expansión más altos que los de los acetoacetato/β-D-hidroxibutirato 3,6 mM cuando los elementos de cada par se utilizarón en proporciones 10:1 ó 1:10, tanto con una concentración de O_2 del 20% como del 5%. El estado redox no dió lugar a diferencias en el desarrollo embrionario. Cuando los cuerpos cetónicos se añadieron juntos en cultivo, los porcentajes de blastocistos fueron inferiores a los obtenidos utilizando cada molécula por separado. En conclusión, los embriones bovinos pueden utilizar cuerpos cetónicos durante cualquier período del desarrollo embrionario preimplantatorio y pueden desarrollarse bajo diferentes estados redox.

INTRODUCTION

In vitro produced (IVP) bovine embryos can utilize different exogenous energy substrates during their development (Peura, 1990; Rieger *et al.*, 1992; Takahashi *et al.*, 1992; Rosenkrans *et al.*, 1993; Pinnyopummintr and Bavister, 1995; Thompson *et al.*, 1996a). In the absence of exogenous energy sources, few embryos are able to complete the first cleavage stage (Pinnyopummintr and Bavister, 1995) and reach the blastocyst stage (Gómez, 1997). However, it has been suggested that bovine embryos might metabolize endogenous substrates (Gordon, 1995; Gómez, 1997; Gómez and Díez, 1997; Rieger *et al.*, 1997), although their potential contribution as energy sources has tended to be ignored (Leese *et al.*, 1993). Moreover, endogenous lipids could be metabolically useful at different developmental stages, as evidenced by the lipid content in preblastocyst stage pig embryos being higher than that in the more advanced stages (Dobrinsky, 1996). Bovine embryos cultured *in vitro* can develop up to the hatched blastocyst stage by using either acetoacetate or β-hydroxybutyrate as single energy substrates at concentrations of 3.6 mM (Gómez, 1997). The blastocyst formation rates and cell number were comparable to those obtained in modified Synthetic Oviduct Fluid (mSOF). Concentrations of 3.3 mM lactate and 0.3 mM pyruvate, normally contained in mSOF, have also been reported to be optimal for ovine embryos (Thompson *et al.*, 1993) and allow for the reliable culture of bovine embryos (Takahashi and First, 1992).

If acetoacetate and 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). 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).

Intracellular β-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios are related to the $NADH/NAD^+$ ratios in the mitochondria and cytoplasm, respectively. The relative concentrations of these substrates can alter via the respective redox couples and may affect embryo development. At the same time, oxygen concentration influences the redox status and, as a consequence, preferential utilization of substrates might occur.

The aim of this work was to characterize bovine embryo development *(in vitro* 1) in the presence of acetoacetate and β-hydroxybutyrate during three periods of culture (0 to 48 h, 48 to 120 h and 120 to 216 h); and 2) in the presence of different proportions (10:1 and 1:10) of acetoacetate/β-hydroxybutyrate (3.6 mM) and lactate/pyruvate (3.6 mM) under 5 percent CO_2 in air and 5 percent CO_2, 5 percent O_2 and 90 percent N_2.

MATERIALS AND METHODS

OOCYTE RECOVERY

Bovine ovaries recovered from a slaughterhouse were placed in NaCl solution (9 mg ml^{-1}) containing antibiotics (Penicillin, 100 u.i. ml^{-1} and streptomycin sulfate, 100 µg ml^{-1}) and maintained at 30-35ºC until recovery of cumulus-oocyte complexes (COCs). Ovaries were washed twice in distilled water and once in freshly prepared saline and antibiotics. The COCs were aspirated from follicles (2-7 mm) through an 18-gauge needle connected to a vacuum system and recovered into a 50-ml plastic tube (Nunc, Roskilde, Denmark). Follicular fluid and COCs were placed in an embryo filter (Em-Con, Eurofomento Pecuario, Madrid, Spain) and rinsed 3 times with holding medium, consisting of PBS, pyruvate 0.16 mM, BSA 3 mg ml^{-1} and gentamycin solution 5 µl ml^{-1} (Gibco, Barcelona, Spain).

IN VITRO MATURATION

Only oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were selected for maturation. The COCs were washed 3 times in maturation medium, which consisted of Medium 199 (Sigma, Madrid, Spain), fetal bovine serum (FBS, 10 percent v/v), FSHp (1 µg ml^{-1}, Sigma), LH (5 µg ml^{-1}, Sigma) and 17β-estradiol (1µg ml^{-1}, Sigma). Maturation was performed by culturing approximately 50 COCs in 500 µl of maturation medium in four-well dishes at 39ºC in 5 percent CO_2 under air and high humidity for 23 to 24 h.

IN VITRO FERTILIZATION

In vitro fertilization was carried out using a swim-up procedure similar to that previously reported by Parrish *et al.* (1986). Briefly, semen from 1 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-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 600 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 3 times in holding medium and placed in four-well culture

Table I. In vitro *development up to the hatched blastocyst stage of bovine embryos cultured in modified Synthetic Oviduct Fluid with acetoacetate or β-hydroxybutyrate replacing lactate and pyruvate for three different periods of culture.* (Desarrollo *in vitro* hasta el estadio de blastocisto eclosionado de embriones bovinos cultivados durante tres periodos diferentes en mSOF con acetoacetato o β-hidroxibutirato como sustitutos del lactato/piruvato).

Culture period/ Experimental Groups	Embryos	percent Blastocysts Day 8 (168 h)	percent Expanded blastocysts	percent Hatched Blastocysts
0-48 h/	Presumptive zygotes			
Acetoacetate	106	36.8±9.0	34.9±7.9	24.5±9.8
β-hydroxybutyrate	111	27.1±2.9	19.3±3.1	13.8±2.9
Lactate/Pyruvate	108	37.6±0.8	34.5±2.4	27.3±4.5
48-120 h/	8-16 cell			
Acetoacetate	89	60.0±11.7	48.5±7.7	19.7±1.2
β-hydroxybutyrate	90	52.8±4.7	33.0±6.8	15.8±6.2
Lactate/Pyruvate	90	46.5±7.1	34.2±5.5	18.4±5.3
120-216 h /	Morulae + Early Blastocysts			
Acetoacetate	58	74.7±6.5	56.5±16.4	21.9±9.1
β-hydroxybutyrate	56	51.1±12.7	49.3±13.8	15.2±10.1[a]
Lactate/Pyruvate	56	64.3±8.6	53.6±5.8	27.4±6.9[b]

Period 0-48 h: 3 replicates; period 48-120 h: 4 replicates; period 120-216 h: 3 replicates.
Values with different superscripts within each column and period of culture differ statistically ($p<0.05$)

dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 µg ml^{-1}, Calbiochem, La Jolla, CA). Spermatozoa were then added to a concentration of 2 x 10^6 cells ml^{-1} in 500 µl of medium per well containing 100 COCs. *In vitro* fertilization was accomplished by incubating oocytes and sperm cells together for 18 h at 39ºC in 5 percent CO_2 in air and high humidity.

In vitro EMBRYO CULTURE

Embryo cultures were performed in mSOF (Takahashi and First, 1992), with cysteamine (50 µM, Sigma) and BSA (3 mg ml^{-1}, Sigma), to which FBS (10 percent v/v) was added 48 h post-insemination. Lactate and pyruvate were substituted with sodium acetoacetate and/or β-hydroxybutyrate where indicated. β-hydroxybutyrate was purchased as a powder (Sigma). Sodium acetoacetate was obtained by hydrolysis of ethyl acetoacetate (Sigma) with equimolar NaOH solution with further lyophylization to eliminate water and the ethanol formed; the crystallized sodium acetoacetate prepared in this manner was stored frozen at -20ºC. Culture

Table II. In vitro *development up to the hatched blastocyst stage of bovine embryos cultured in lactate/pyruvate and acetoacetate/β-hydroxybutyrate in 5 percent CO_2 in air.* (Desarrollo hasta el estadio de blastocisto eclosionado de embriones bovinos cultivados en lactato/piruvato y acetoacetato/β-hidroxibutirato en 5 percent CO_2 en aire).

Experimental Groups	Oocytes	Cleaved percent	percent Blastocysts Days 7 and 8	percent Expanded blastocysts	percent Hatched Blastocysts
Lactate/Pyruvate 3.3mM/0.3mM	139	84.4±4.2	34.7±7.5[a]	27.2±6.1[a]	13.0±5.9
Lactate/Pyruvate 0.3mM/3.3 mM	136	89.2±2.0	33.4±3.3	22.6±5.2	10.4±5.4
Acetoacetate/ β-hydroxybutyrate 3.3mM/0.3mM	137	87.0±2.0	24.5±6.7	13.4±5.5[b]	3.7±2.1
Acetoacetate/ β-hydroxybutyrate 0.3mM/3.3mM	139	85.1±3.2	19.2±6.6[b]	14.0±4.7	6.3±3.8

Values with different superscripts within each column differ statistically ($p<0.05$); Data from 4 replicates.

media were renewed on Day 3 and 6 of culture (Fertilization: Day 0). Media were freshly prepared and had a pH of 7.2-7.3 and osmolality of 270-280 mOsm. Fifty to 70 µl droplets (2 µl embryo^{-1}) of the correspondent culture medium were prepared in 4-well culture dishes under mineral oil and allowed to equilibrate in the incubator for at least 2h 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 a 9-d period of culture. Embryo development was assessed at 48, 144, 168, 192, and 216 h of culture. Culture conditions were 39º C and high humidity throughout, either in 5 percent CO_2 in air (Experiments I and III) or both in 5 percent CO_2 in air and in 5 percent CO_2, 5 percent O_2 and 90 percent N_2 (see Experiment II).

EXPERIMENT I

The effects of 3.6 mM acetoacetate and 3.6 mM β-hydroxybutyrate on blastocyst development were evaluated for 3 different periods of *in vitro* culture 1) up to 48 h, 2) from 48 h to 120 h, and 3) from 120 h up to 216 h of culture. A lactate/pyruvate (3.3 mM/0.3 mM) solution in mSOF acted as a control (LP) throughout.

Presumptive zygotes, 8-16 cell embryos and morulae + blastocysts were used for periods 1, 2 and 3 respectively.

EXPERIMENT II

Both pairs of substrates, aceto-

Table III. In vitro *development up to the hatched blastocyst stage of bovine embryos cultured in lactate/pyruvate and acetoacetate/β-hydroxybutyrate in 5 percent CO_2 , 5 percent O_2 and 90 percent N_2*. (Desarrollo hasta el estadio de blastocisto eclosionado de embriones bovinos cultivados en lactato/piruvato y acetoacetato/β-hidroxibutirato en 5 percent CO_2, 5 percent O_2 y 90 percent N_2).

Experimental Groups	Oocytes	Cleaved percent	percent Blastocysts Days 7 and 8	percent Expanded blastocysts	percent Hatched Blastocysts
Lactate/Pyruvate 3.3mM/0.3mM	237	78.5±3.5	29.3±4.1[a]	16.1±2.5[a,b]	6.5±1.8
Lactate/Pyruvate 0.3mM/3.3 mM	238	76.3±4.7	28.0±4.8	17.5±2.4[a]	11.2±3.4
Acetoacetate/ β-hydroxybutyrate 3.3mM/0.3mM	232	73.6±4.8	19.5±3.9	9.2±2.4[b,c]	3.6±1.3
Acetoacetate/ β-hydroxybutyrate 0.3mM/3.3mM	230	74.3±3.5	18.4±4.6 [b]	8.1±2.3[c]	5.3±2.3

Values with different superscripts within each column differ statistically ($p<0.05$).
Data from 7 replicates, except of percent Hatched Blastocysts which were from 6 replicates.

acetate/β-hydroxybutyrate and lactate/pyruvate, were added at concentrations of 3.3 mM/0.3 mM and 0.3mM/3.3 mM (four experimental groups) during the whole *in vitro* culture. To test effect of the oxygen tension, which might affect the NADH/NAD+ ratios, two trials were conducted: *trial 1*) culture at 39ºC under 5 percent CO_2 in air, and *trial 2*) culture at 39ºC under 5 percent CO_2 and 5 percent O_2.

Experiment III

Experiment III arose from the results of Experiment II, which showed that lactate/pyruvate supported higher rates of blastocyst development than acetoacetate/β-hydroxybutyrate. A direct comparison between acetoacetate and β-hydroxybutyrate added singly at 3.6 mM and as combined (acetoacetate 3.3 mM + β-hydroxybutyrate 0.3 mM) substrates during the whole *in vitro* culture was carried out.

Statistical analysis

Data were analyzed by one-way ANOVA (SPSS Inc. 1994, Chicago, Ill, Ver 6.1.2) and expressed as mean percentages ± SEM. The number of replicates and treatments were considered as fixed effects. Tukey's Test for the different variables was used to estimate the significance of mean values.

RESULTS

A total of 2,868 oocytes were

***Table IV**. Cleavage and blastocyst formation rates of bovine embryos cultured in modified Synthetic Oviduct Fluid with acetoacetate and β-hydroxybutyrate added as either single or combined substrates.* (Porcentaje de división y tasas de desarrollo (p.100 de blastocistos) de embriones bovinos cultivados en mSOF en presencia simple o combinada de acetoacetato y β-hidroxibutirato).

Experimental groups	oocytes	percent cleaved	percent blastocysts days 7 and 8
Acetoacetate (3.6 mM)	94	77.4±6.2	31.9±2.8[a]
β-hydroxybutyrate (3.6 mM)	91	63.1±11.4	24.0±5.8
Acetoacetate (3.3 mM) + β-hydroxybutyrate(0.3 mM)	97	60.4±6.7	19.3±6.9[b]

Values with different superscripts within each column differ statistically ($p<0.05$); Data from 4 replicates.

distributed amongst the 3 different experiments with 25 independent replicates.

EXPERIMENT I

The results are given in **table I**. There were no significant differences ($p>0.05$) between groups, except for the hatching rate between β-hydroxybutyrate and lactate/pyruvate during the period of culture 120-216 h (15.2 percent ± 10.1 and 27.4 percent ± 6.9, respectively).

EXPERIMENT II

The results are shown in **tables II** and **III**. Blastocyst development was similar in both atmospheric conditions (5 percent CO_2 in air and 5 percent CO_2, 5 percent O_2). For each pair of substrates, neither an excess of the reduced nor the oxidized component significantly affected the blastocyst development. As reported above, combinations of lactate and pyruvate promoted rates of blastocyst development and expansion significantly higher than those achieved when combinations of ketone bodies were used.

EXPERIMENT III

Table IV shows a significant reduction in blastocyst formation rate on days 7 and 8 when acetoacetate and β-hydroxybutyrate were added together in culture as opposed to singly.

DISCUSSION

Under our experimental conditions, culture of bovine embryos *in vitro* with acetoacetate and/or β-hydroxybutyrate gave consistent blastocyst and expanded blastocysts rates, irrespective of the period at which these substrates replaced lactate and pyruvate as energy sources. This indicates that the early bovine embryo can use ketone bodies as a primary energy source at any time during *in vitro* development. The benefits of ketone bodies to embryo development between 120 h and 216 h are not surprising, since lipid turnover has been reported to occur in

porcine embryos within this period (Dobrinsky, 1996). This depletion of intracellular lipids store coincides with a considerable demand for ATP, required for Na^+ transport into the blastocoele (Leese, 1991). Furthermore, an increase in oxygen consumption after the initiation of compaction and a large dependence of bovine embryos on oxidative phosphorylation *in vitro* (Thompson *et al.*, 1996a) and *in vivo* (Thompson *et al.*, 1996b), together with a negligible oxidation of glucose in bovine post-compaction embryos (Rieger and Guay, 1988; Rieger *et al.*, 1992; Waugh and Wales, 1993) also suggest the strong possibility of the lipid breakdown. Moreover, the ability to use acetoacetate/β-hydroxybutyrate is strong evidence that bovine embryos can use intracellular lipids as energy sources. Embryos could use endogenous substrates when the exogenous substrate availability is low (Thompson, 1996). Acetoacetate and β-hydroxybutyrate, being present in culture as single substrates, were able to promote *in vitro* development before and after genome activation, which occurs at the 8-16 cell stage (Telford *et al.*, 1990). In other words, ketone bodies are important to the bovine embryo, since other energy substrates (e.g. glucose), negatively affect development when added during the initial periods of the *in vitro* culture (Kim *et al.*, 1993; Furnus *et al.*, 1997).

Lipids in IVP embryos may be derived either from the oocyte or synthesized by the embryo in culture as occurs in oviduct cells cultured *in vitro* (Walter, 1995). The *in vitro* culture systems continually provide subtrate with which embryos may synthesize glycogen (Ozias, 1973; Houghton *et al.*, 1996) and lipids (Heyman, personal communication). In addition, culture in presence of serum led to embryos with abundant lipid inclusions in sheep (see Gordon, 1995, for review), although relationship among energy substrates, serum and lipids remain still quite unexplored.

There were other three findings in this study. First, the carbohydrates pyruvate and lactate promoted blastocyst and expanded blastocyst formation rates higher than those with ketone bodies. This is not in accordance with a previous report (Gómez, 1997), in which 3.6 mM acetoacetate enabled blastocysts to form at a rate similar to that in 3.3 mM lactate/0.3 mM pyruvate, in spite of lactate or pyruvate accounts for 1 Acetyl-CoA entering Krebs cycle and each ketone body yields twice this quantity. In the same work, a concentration of 1.8 mM acetoacetate, stechiometrically equivalent to 3.3 mM lactate/0.3 mM pyruvate, was insufficient to satisfy the energy requirements of the embryos. Therefore, ketone bodies might be concerned by other factors non strictly energetic in nature. Moreover, subsequent testing of single against combined ketone bodies revealed that an interaction exists. In this respect, notwithstanding much later in development, some workers have reported teratogenic damages in rats when combining ketotic factors (Zusman and Ornoy, 1987; Sadler *et al.*, 1988). Second, embryo development with a ratio of 10:1 for lactate: pyruvate concentrations, as found in oviductal fluid following estrus in the ewe and reported to be succesful in

other species (Wales and Whittingham, 1973; Thompson *et al.*, 1993), is not optimal in bovine. It is well known that these levels change with estrous cycle stage and location within oviduct and uterus (Thompson, 1996). More research is needed to optimise the concentrations of pyruvate and lactate in bovine *in vitro* culture. Third, neither oxygen concentration nor a high ratio of oxidized or reduced substrates gave different rates of embryo development. This indicates that IVP bovine embryos are able to adapt to different redox status. Thus, equivalent embryo development was obtained in the most reductive (i.e., high lactate or β-hydroxybutyrate in 5 percent O_2) and most oxidative situations (i.e., high pyruvate or acetoacetate in air). It would be interesting to discover the limits of the bovine embryos response to extreme redox conditions. The apparently lower proportions of blastocyst development obtained in air with regard to those in 5 percent O_2 could be attributed either to the different batches of oocytes and media used or to the different incubators utilized in each experiment (Eckert *et al.*, 1996) or both, since it is agreed that an oxygen tension below 10 percent is associated with increased rates of development (see Thompson, 1996, for review).

In conclusion, we have provided strong evidence that IVP bovine embryos can utilise the lipid breakdown products, ketone bodies acetoacetate and β-hydroxybutyrate, at any period of the early development and this is independent of the oxygen tension of the medium (5 percent or 20 percent).

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Recibido: 1-10-98. Aceptado: 19-5-99.