ACETOACETATE AND β -D-HYDROXYBUTYRATE AS ENERGY SUBSTRATES DURING EARLY BOVINE EMBRYO DEVELOPMENT IN VITRO

E. Gómez

Centro de Investigación Aplicada y Tecnología Agroalimentaria
Centro de Selección y Reproducción Animal, Gijón, Asturias, Spain

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ABSTRACT

We examined the effects of acetoacetate and other metabolic products of fatty acid oxidation on early bovine embryo development. In vitro produced bovine zygotes were cultured in modified-synthetic oviduct fluid medium supplemented with acetoacetate, acetoacetate derivatives, acetyl CoA precursors and lithium chloride. Acetoacetate and all acetoacetate derivatives, with the exception of the ethyl ester, supported in vitro development up to the hatched blastocyst stage at rates similar to that of controls supplemented with lactate/pyruvate. The optimal concentration of acetoacetate in supporting embryo development was 3.6 mM; addition of 1.8 and 3.6 mM lithium chloride did not significantly affect embryo development, while 7.2 mM was inhibitory. Hatched blastocysts cultured with 3.6 mM acetoacetate contained a similar number of cells as the lactate/pyruvate control group. It can be concluded that in vitro produced bovine embryos can develop using ketone bodies as energy substrates, which could be derived in vivo from endogenous lipids.

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Key words: acetoacetate, hydroxybutyrate, bovine, embryo, lipid

INTRODUCTION

In vitro produced (IVP) embryos display cryobiological features different from their in vivo counterparts (13,21). Bovine IVP morulae show poor survival after freezing (15,20), although survival after freezing/thawing increases as IVP morulae develop to the hatched blastocyst stage, particularly when the blastocysts are frozen by vitrification

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(3,5,15). Mechanical delipidation of IVP bovine embryos leads to an increased survival after freezing and thawing (4,14,30). If embryos could be induced to use endogenous lipids as energy sources, this might represent a way of decreasing their fat content and increasing their ability to survive cryopreservation. It would also improve embryo quality generally. Bovine IVP embryos can utilize different exogenous energy substrates during their development (18,19,22,23,28), but the potential role of fatty acid oxidation and of the metabolic products, acetoacetate and β -D-hydroxybutyrate, during early bovine development has not been examined. The aim of this work was therefore to characterize bovine embryo development in the presence of 1) different acetoacetate derivatives; 2) acetyl CoA precursors (acetate, acetoacetate and β -D-hydroxybutyrate); 3) different concentration of acetoacetate; and 4) different concentrations of lithium, since one derivative of acetoacetate is the lithium salt.

MATERIALS AND METHODS

Oocyte Recovery and Maturation

Ovaries recovered from slaughtered Asturiana de los Valles and Holstein Friesian cows and heifers were placed in 0.9% (wt/vol) NaCl containing antibiotics (penicillin, 100 units/ml and streptomycin sulfate, 100 mg/ml) and maintained at 30 to 33°C 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 2- to 7-mm antral follicles through an 18 G 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 ovum concentrator (Em-Con, Eurofomento Pecuario, Madrid, Spain; 8) and rinsed 3 times with holding medium (consisting of PBS, pyruvate 0.16 mM, BSA 3g/L and gentamycin solution [Gibco, Paisley, Scotland] 5 μ l/ml). Only oocytes enclosed in a compact cumulus with and evenly granulated cytoplasm were selected for maturation. The COCs were washed 3 times in maturation medium comprised of Medium 199 (Sigma, Madrid, Spain), fetal bovine serum (FBS, 10% v/v) and FSHp (20 μ g/ml; Schering-Plough Animal Health, Madrid, Spain; 25). Maturation was performed by culturing approximately 100 COCs in 500 μ l of maturation medium in four-well dishes. Culture conditions were 39°C, 5% CO₂ in air and high humidity throughout.

In Vitro Fertilization

In vitro fertilization was carried out using procedures similar to those previously reported by Parrish et al. (17). Frozen semen from a single bull was thawed into a warmed tube, transferred to the top of a Percoll 45 to 90% gradient contained in a 15-ml polystyrene tube, and centrifuged at 700 x g for 30 min. The sperm pellet was diluted with 5 ml of sperm-TALP (sp-TALP), centrifuged for 10 min at 200 x g and the

supernatant aspirated to leave a pellet approximately 100 μ l in volume. Sperm concentration was determined with a hemocytometer. Fertilization medium (fert-TALP) with heparin (10 μ g/ml; Calbiochem, La Jolla, CA) was used to dilute the spermatozoa. After 23 to 24 h of maturation, the COCs were washed 3 times in wash-TALP and placed in four-well culture dishes containing pre-equilibrated fertilization medium. Spermatozoa were then added to a concentration of approximately 2×10^6 cells/ml in 500 μ l of medium per well containing 100 COCs. In vitro fertilization was accomplished by incubating oocytes and sperm cells together for 18 h.

In Vitro Embryo Culture

Embryo cultures were carried out in modified synthetic oviduct fluid medium (mSOF; 28), with cysteamine (50 μ M; Sigma) and BSA (3 g/L; Sigma), to which FBS (10% v/v) was added 48 h post insemination. Lactate and pyruvate were substituted by other putative energy substrates where indicated. Culture media were renewed on Days 3 and 6 of culture. Media were prepared weekly and used fresh at pH 7.2 to 7.3 and osmolality of 270 to 280 mOsm. Then 50- to 70- μ l droplets (2 μ l/embryo) of the correspondent 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 2 min in holding medium at medium speed to separate cumulus cells, rapidly washed 3 times in holding medium and twice in the corresponding culture medium prior to a 9-d period of culture. Embryo development was assessed on Days 2, 6, 7, 8, 9, and 10 post insemination.

Experiment 1. The following acetoacetate derivatives were tested as putative energy substrates for in vitro embryo culture at a concentration of 3.6 mM in mSOF: 1) lithium acetoacetate (Sigma); 2) ethyl acetoacetate (Sigma); 3) sodium acetoacetate, obtained by the hydrolysis of ethyl acetoacetate 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 and used not more than 7 d later; 4) sodium acetoacetate + ethanol (as in 3) but without lyophilization. A lactate/pyruvate (3.30 mM/0.30 mM) solution in mSOF acted as a positive control (+LP). A negative control (-LP) contained lactate/pyruvate-free mSOF.

Experiment 2. The following acetyl CoA precursors and related compounds were tested for their ability to influence IVP bovine embryo development: 1) sodium acetoacetate (3.6 mM); 2) sodium β -D-hydroxybutyrate (Sigma H-0265, 3.6 mM); and 3) sodium acetate (7.2 mM synthesized from glacial acetic acid treated with equimolar NaOH solution; Panreac, Barcelona, Spain), were tested in comparison to +LP as the control.

Experiment 3. Because acetoacetate at a concentration of 10mM has been reported to retard development of mouse embryos in vitro (16), lower concentrations of sodium acetoacetate were tested in an attempt to overcome this inhibitory effect and to optimize in vitro culture conditions. Acetoacetate concentrations of 1.8, 3.6 and 7.2 mM in mSOF were tested and compared with +LP and -LP as the controls.

Experiment 4. The effects of lithium on in vitro embryo development were tested by replacing sodium chloride with lithium chloride in whole mSOF, to give final concentrations of lithium of 1.8, 3.6 and 7.2 mM; +LP served as the control group.

Cell Counts

The cell numbers in hatched blastocyst obtained at Days 8 and 9 post insemination from Experiments 1, 2 and 3, cultured in whole mSOF (+LP) and 3.6 mM acetoacetate, were determined. Blastocysts were maintained for 20 min in hypotonic solution (0.09% w/v of sodium citrate in double-distilled water); fixed with methanol, acetic acid and double-distilled water (3:2:1); and stained with Giemsa (1/20 v/v) for 15 min. Cell counting was carried out at x 200 with a light microscope.

Statistical Analysis

Data were analyzed by ANOVA. The numbers of replicates and treatments were considered as fixed effects. The Ryan-Einot-Gabriel-Welsch Multiple F test (SAS, 1989) for the different variables was used to estimate the significance of mean values. Data were expressed as mean percentages $\pm$ SEM of the cultured oocytes.

RESULTS

Experiment 1

The results of Experiment 1 are shown in Table 1. Sodium acetoacetate and lithium acetoacetate gave rates of blastocyst formation comparable to the positive control group (+LP, $P < 0.05$), while ethyl acetoacetate was detrimental to embryo development, with no blastocysts being formed. Sodium acetoacetate significantly ($P < 0.05$) improved the rate of hatched blastocysts formation compared with the negative control group (-LP).

Experiment 2

Table 2 shows the results of Experiment 2. No significant differences were obtained between any group for development to the hatched blastocyst stage. β -D-hydroxybutyrate supported embryo developmental rates similar to those observed for

acetoacetate and lactate/pyruvate (+LP control). A nonsignificant, lower blastocyst expansion and hatching rate occurred in the acetate group.

Table 1. Development to the hatched blastocyst stage of IVP bovine oocytes cultured in mSOF with different acetoacetate-based molecules (3.6 mM) as energy sources replacing lactate and pyruvate.

Group	n	% Cleaved	% 5 to 8 Cells	% Blastocysts		
				Total	Expanded	Hatched
Lithium Acetoacetate	114	61.61 ^{ab} ±3.28	43.27 ^a ±4.47	28.27 ^a ±3.06	17.70 ^a ±3.00	13.29 ±2.72
Sodium Acetoacetate (lyophilized)	115	68.51 ^{ab} ±2.01	47.22 ^a ±2.58	27.90 ^a ±3.62	19.12 ^a ±3.17	16.54 ^a ±2.78
Sodium Acetoacetate (+ ethanol)	113	70.64 ^a ±1.61	49.09 ^a ±3.19	28.70 ^a ±3.44	19.61 ^a ±2.42	13.44 ±2.26
Ethyl-ester Acetoacetate	111	29.64 ^b ±1.81	2.61 ^b ±1.60	0.00 ^b	-----	-----
+LP	110	67.25 ^{ab} ±2.33	41.81 ^a ±3.88	29.07 ^a ±3.68	13.57 ±3.06	9.96 ±2.50
-LP	112	54.59 ^c ±2.54	13.47 ^b ±2.21	7.15 ^b ±2.00	2.58 ^b ±1.60	0.00 ^b

n = number of matured oocytes; +LP and -LP represents mSOF with and without lactate/pyruvate added, respectively. Percentages of embryos with 5 to 8 cells, blastocysts, expanded blastocysts and hatched blastocysts were determined on Days 2, 8, 9 and 10 post insemination, respectively.

^{a-c} Mean values with different superscripts are significantly different ($P < 0.05$). Data from 3 replicates.

Experiment 3

Table 3 shows that 3.6 mM was the most favorable concentration of acetoacetate in supporting embryo development. Acetoacetate 7.2 mM was detrimental and acetoacetate 1.8 mM was insufficient to enable blastocysts to form at a rate similar to that in 3.6 mM acetoacetate.

Table 2. Development to hatched blastocyst stage of IVP bovine oocytes cultured in mSOF with different acetyl CoA precursors as energy substrates replacing lactate and pyruvate.

Group	n	% Cleaved	% 5 to 8 Cells	% Blastocysts		
				Total	Expanded	Hatched
Acetoacetate (3.6 mM)	112	59.34 ±3.37	34.67 ±1.97	24.28 ±3.34	15.37 ^a ±2.73	12.59 ±2.53
Hydroxy- butyrate	113	56.94 ±2.75	33.24 ±2.18	20.69 ±3.08	15.29 ±2.57	11.60 ±1.52
Acetate (7.2 mM)	112	54.70 ±3.58	26.94 ±1.86	17.99 ±2.96	8.99 ±2.09	3.60 ±1.30
+LP	110	60.29 ±3.27	37.68 ±1.88	25.75 ±3.54	14.78 ±3.08	9.24 ±2.70

n = number of matured oocytes; +LP represents mSOF with lactate/pyruvate added. Percentages of embryos with 5 to 8 cells, blastocysts, expanded blastocysts and hatched blastocysts were determined on Days 2, 8, 9 and 10 post insemination, respectively. Data from 3 replicates.

Experiment 4

Effects of different concentrations of lithium can be seen in Table 4. Concentrations of 1.8 and 3.6 mM lithium did not affect early embryo development up to the hatched blastocyst stage compared with that of the control (lithium 0 mM), but a concentration of 7.2 mM was inhibitory (6.32% blastocyst formation vs 24.47% for the controls).

Cell Counts

Table 5 shows that there was no significant difference ($P < 0.001$) between the number of cells in blastocysts produced in the presence of 3.6 mM of acetoacetate and lactate/pyruvate.

Table 3. Development to hatched blastocyst stage of IVP bovine oocytes cultured in mSOF with different sodium acetoacetate concentrations as energy substrates replacing lactate and pyruvate.

Group	n	% Cleaved	% 5 to 8 Cells	% Blastocysts		
				Total	Expanded	Hatched
Acetoacetate 1.8 mM	192	62.93 ±4.11	27.95 ±3.09	15.63 ±2.51	10.47 ±2.31	5.42 ±2.11
Acetoacetate 3.6 mM	193	71.54 ±1.73	36.89 ±4.14	21.67 ^a ±3.10	14.24 ^c ±2.64	12.19 ^c ±1.52
Acetoacetate 7.2 mM)	193	61.20 ±3.53	22.01 ±2.23	9.85 ±2.38	5.24 ±2.19	1.52 ^d ±1.50
+LP	193	68.53 ±3.38	34.62 ±4.00	22.38 ^a ±3.40	14.30 ^c ±2.76	8.04 ±2.83
-LP	191	57.21 ±2.42	16.43 ±2.43	7.43 ^b ±1.61	2.18 ^d ±1.53	0.00 ^d

n = number of matured oocytes; +LP and -LP represents mSOF with and without lactate/pyruvate added, respectively. Percentages of embryos with 5 to 8 cells, blastocysts, expanded blastocysts and hatched blastocysts were determined on Days 2, 8, 9 and 10 post insemination, respectively.

Mean values with different superscripts are significantly different (^{a,b} P<0.05; ^{c,d} P<0.01). Data from 5 replicates.

DISCUSSION

It is known that the ketone bodies, acetoacetate and β -D-hydroxybutyrate, may be utilized in aerobic metabolism and are quantitatively important as energy sources. Adult tissues such as heart and kidney cortex utilize acetoacetate in preference to glucose. The present study demonstrates that acetoacetate can support IVP bovine embryo development. Concentrations of acetoacetate (10 mM) and β -D-hydroxybutyrate (>16 mM) higher than those used here adversely affected mouse preimplantation embryo development in vitro (16). Such concentrations are found in human diabetic serum with ketoacidosis (HDS-K; 34). Furthermore, HDS-K yielded teratogenic effects in early somite rat embryos (33). These negative effects on early and late development were mainly attributed to β -D-hydroxybutyrate, while acetoacetate had little or no teratogenic potential in diabetic rats (24), except if added together with β -D-hydroxybutyrate and

Table 4. Development to hatched blastocyst stage of IVP bovine oocytes cultured in whole mSOF (lactate/pyruvate added with different mM concentrations of lithium).

Group	n	% Cleaved	% 5 to 8 Cells	% Blastocysts		
				Total	Expanded	Hatched
Lithium 1.8 mM	111	60.38 ±1.78	38.96 ±2.91	20.86 ^a ±2.05	14.54 ^a ±1.93	6.37 ^a ±2.11
Lithium 3.6 mM	109	60.70 ±3.10	44.22 ±3.85	24.00 ^a ±3.02	14.75 ^a ±2.15	7.36 ^a ±2.08
Lithium 7.2 mM	110	66.56 ±2.14	24.53 ±1.84	6.32 ^b ±1.09	1.75 ^b ±1.23	0.00 ^b
Lithium 0 mM (control)	111	63.04 ±2.33	43.38 ±2.85	24.47 ^a ±2.52	13.49 ^a ±1.54	9.07 ^a ±1.42

n = number of oocytes. ^{a,b} Values with different superscripts are significantly different ($P < 0.05$). Percentages of embryos with 5 to 8 cells, blastocysts, expanded blastocysts and hatched blastocysts were determined on Days 2, 8, 9 and 10 post insemination, respectively.

Data from 3 replicates.

Table 5. Number of cells in hatched blastocyst cultured in acetoacetate 3.6 mM and in whole mSOF (lactate/pyruvate).

Group	Number of blastocysts counted	Number of cells (means±SE)
Acetoacetate 3.6 mM	11	124.09 ± 4.70
Lactate/pyruvate	10	126.00 ± 4.21

Blastocysts were obtained from 7 different replicates ($P < 0.001$).

glucose (32). In the present work, 7.2 mM of acetoacetate significantly reduced blastocyst formation and hatching, and 1.8 mM of acetoacetate was insufficient to satisfy the energy requirements of the embryos; the quality of blastocysts formed with 3.6 mM of acetoacetate, in terms of the number of cells, was comparable to that in the presence of lactate/pyruvate.

Acetoacetate could be derived from either endogenous (embryonic) or exogenous (female tract) sources, or both. Maximal blood levels of ketone bodies in healthy cows are 0.48 mM for acetoacetate and 0.85 mM for β -D-hydroxybutyrate (reviewed by Courcel; 2), or below the concentration required to sustain embryo development. It would be interesting to determine acetoacetate and hydroxybutyrate levels in oviductal fluid, where a variety of lipids have been reported in the bovine (9,11). None of these lipids are required for bovine embryo development (1,26). That IVP bovine embryos are capable of consuming acetoacetate and β -D-hydroxybutyrate could be a means by which their lipid stocks could be degraded. Decreasing lipid content in this way by nonmechanical means might improve the ability of the embryos to be cryopreserved (4,14,30). It has been suggested for ovine embryos that a direct association exists between lipid droplets and mitochondria, which may represent an important source of energy *in vivo* (6). Such lipids may be used not only when substrate availability is low (29), but under normal conditions. In this respect, features of *in vitro*-cultured bovine morulae, such as an absence of compaction, low survival after freezing-thawing, and low buoyant density, could have a common origin related to the continuous presence of a high level of lipid.

The present data showed that acetoacetate-salt derivatives supported *in vitro* embryo development up to the hatched blastocyst stage. Embryo development was significantly decreased by 7.2 mM lithium, but not at the other concentrations tested (1.8 and 3.6 mM). Lithium adversely affects development in post-implantation rat and mouse embryos with 10 ± 2 somite pairs (7) at concentrations of 5 mM and below. Lithium has different growth-stimulating effects on adult cell types *in vitro* (mammary gland explants, cultured fibroblasts and kidney epithelial cells), which may be related to the effect on other intracellular ions (e.g., Ca^{2+} , Mg^{2+} ; 12), which, in turn, are thought to regulate growth. There are no studies on the effect of lithium on preimplantation development. Although the ethyl ester derivative of acetoacetate might have been expected to enter the embryos readily and be hydrolyzed by esterases, it had a negative effect on development, even compared with those embryos cultured in the absence of energy substrates (-LP).

Embryo culture in the presence of other acetyl CoA precursors, β -D-hydroxybutyrate and acetate, also supported development up to the hatched blastocyst stage, though acetate gave statistically non significantly lower embryo development than the other experimental groups. This is consistent with low utilization of acetate reported in ovine and bovine embryos (31), and with low development of IVP bovine (18) and rabbit (10) embryos cultured in medium containing acetate. Because the hydroxybutyrate/acetoacetate ratio depends on the NADH/NAD⁺ ratio in the mitochondria (27), it would be interesting to vary the proportions of both molecules in the culture medium.

In conclusion, IVP bovine embryos can develop to the hatched blastocyst stage in the presence of acetoacetate and β -D-hydroxybutyrate. Embryos developing in 3.6 mM acetoacetate had a cell number similar to those in lactate/pyruvate.

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