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INFLUENCE OF GLUCOSE ON *IN VITRO* DEVELOPMENT RATE OF *Mus domesticus domesticus* 1-CELL STAGE EMBRYOS IN HEPES SUPPLEMENTED KSOM OR HTF MEDIA

INFLUÊNCIA DA GLICOSE SOBRE O DESENVOLVIMENTO *IN VITRO* DE EMBRIÕES *Mus domesticus domesticus* A PARTIR DO ESTÁDIO DE 1-CÉLULA EM MEIO KSOM OU HTF SUPLEMENTADO COM HEPES

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ABSTRACT

In spite of the considerable progress obtained in the past decades with the *in vitro* techniques for the culture of mammalian embryos, the development of one-cell embryos to the stage of hatching blastocyst was accomplished only in a few species. The mechanism throughout glucose would exert this inhibitory effect on the *in vitro* development of mammalian embryos is not clear yet.The objective of these experiments was to determine influence of glucose on the *in vitro* development rate of *Mus domesticus domesticus* (CF1 vs. Swiss strain) one cell embryos to hatched blastocysts cultured in KSOM or HTF media supplemented with HEPES. In the first experiment, the embryos were exposed to glucose (0,20 mM in KSOM and 2,78 mM in HTF) since the start of the culture. In the second experiment, the embryos were transferred to droplets of medium with glucose 24 h after the beginning of the culture, and in the third experiment, the transfer of embryos was carried out after 48h of culture. In experiment 1, 61.7% (261/423) and 53.5% (176/332) of the embryos cultured in KSOM and HTF media, respectively, reached the hatched blastocyst stage. In experiment 2, the addition of glucose after 24h led to a higher embryo development rate in KSOM (59%, 134/227) than in HTF (40.0%, 79/197). In experiment 3, 50.8% (233/458) of the embryos cultured in KSOM and 50.8% (248/488) of those cultured in HTF reached the stage of hatched blastocyst. The absence of glucose 24 and 48 hours after the beginning of the *in vitro* culture did not increase the capacity of cultured embryos to reach the hatched blastocyst stage.

Key words: mice, embryos, blastocyst, culture, HEPES.

RESUMO

O desenvolvimento de embriões mamíferos, do estádio de uma célula ao de blastocisto expandido, apesar do considerável progresso obtido nas últimas décadas com as técnicas de cultivo in vitro, foi reportado em um número reduzido de espécies. O objetivo dos experimentos foi determinar a taxa de desenvolvimento *in vitro* de embriões *Mus domesticus domesticus*, (CF1 x SWISS) do estádio de 1-célula ao de blastocisto eclodido, cultivados em KSOM ou HTF, suplementados com HEPES, realizando-se a adição da glicose em diferentes momentos do cultivo. No primeiro experimento, a glicose esteve presente durante todo o período de cultivo *in vitro*. No segundo experimento, os embriões foram transferidos para as gotas de meio com glicose 24h após o início do cultivo e, no terceiro experimento, realizou-se a transferência dos embriões após 48h de cultivo. No experimento 1 eclodiram 61,7% (261/423) e 53,5% (176/332) dos embriões cultivados nos meios KSOM e HTF, respectivamente. Por outro lado, no experimento 2, o cultivo em meio com glicose adicionada após 24h de cultivo, proporcionou desenvolvimento superior aos embriões cultivados em KSOM (59%, 134/227) em relação aos mantidos em HTF (40,1%, 79/197). No experimento 3 eclodiram 50,8% (233/458) dos embriões cultivados em KSOM e também 50,8% (248/488) dos cultivados em HTF. A ausência de glicose por períodos de 24 e 48 horas a partir do início do cultivo *in vitro*, não proporcionou um aumento na capacidade dos embriões cultivados em alcançarem o estádio de blastocisto eclodido.

Descritores: camundongo, embrião, blastocisto, cultivo, HEPES.

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INTRODUCTION

In spite of the considerable progress obtained in the past decades with the *in vitro* techniques for the culture of mammalian embryos, the development of one-cell embryos to the stage of hatching blastocyst was accomplished only in a few species [15]. As early as 1968, Whitten and Biggers [30] described a developmental block at the two-cell stage in *Mus domesticus domesticus* embryos on *in-vitro* cultures. Several years later, it was suggested that the block phenomenon might be caused by inappropriate concentrations of culture medium constituents [20]. These inappropriate culture conditions would result in changes in the expression of the maternal genome, as well as in inadequate activation of the embryonic genome.

In a previous study [1], it was observed a developmental block at the two-cell stage in all cleaved embryos cultured in either KSOM (n=187) or HTF (n=202) and supplemented with 20% of bovine fetal serum (BFS). With the objective of providing conditions for the embryos to overcome the two-cell block, KSOM and HTF were modified by adding HEPES and by replacing BFS with bovine serum albumin (BSA).

The presence of glucose in the culture medium could affect the expression of certain genes, which in turn would lead to the blocking of embryonic development [7]. The maternal genome (probably cytoplasmic factors) has been shown to have a role in the two-cell block [16]. The injection of one-cell embryo cytoplasm of "non-blocking" strains into one-cell embryos of "blocking" strains revealed that small percentages of enzymes or mRNA were sufficient for the embryos to overcome the 2-cell developmental arrest [23]. It was stated that the block occurs when the embryonic genome takes charge of development [20].

The mechanism throughout glucose would exert this inhibitory effect on the *in vitro* development of mammalian embryos is not clear yet. Several authors [26,27] proposed that glucose inhibits the activity of the Krebs Cycle by means of a phenomenon known as 'Crabtree effect'[12],i.e., by inducing an increase in glycolytic activity, thus expending the ATP reserve required for oxidative phosphorylation. The inhibitory effect of glucose on embryonic development has already been described in hamsters [26], in bovines [29], and in humans [11]. However, in the *in-vitro* culture of swine embryos, this effect was not observed [24].

The interaction of glucose with some components of the culture medium, such as NaCl, glutamine, amino acids, phosphate, lactate, and pyruvate, determines the presence or absence of an inhibitory effect [8, 17, 21, 27, 28] . These observations show that the response of the embryo to the presence or absence of glucose is not homogeneous; it is dependent on strain and breed, as well as on the constituents of the medium used [21, 22, 26].

More recently, through the use of a factorial design to examine the effects of 16 combinations of 4 glucose concentrations and 4 potassium di-hydrogen phosphate concentration, it was not observed any significant effects of glucose on blastocyst formation and initiation of hatching. The results also showed, no significant interaction between the effects of glucose and phosphate [4].

The objective of this work was to assess the development of *Mus domesticus domesticus* embryos from the one-cell stage to the stage of hatching blastocyst in KSOM or HTF media supplemented with HEPES and exposed to glucose concentrations at different periods of time during the *in vitro* culture.

MATERIALS AND METHODS

Animals

The embryos were produced by crossing 6 to 8 week old females of the CF1 strain with males from the Swiss strain. The animals were provided by the State Health Research Foundation (*Fundação Estadual de Pesquisa em Saúde da Secretaria de Saúde e Meio Ambiente do Estado do Rio Grande do Sul*, Porto Alegre, Brazil), and were maintained in facilities with controlled lighting

(14 h light : 10 h dark), constant ventilation and temperature between 18 and 22º C. Water and feed were administered *ad libitum*.

Media

Modified phosphate buffered saline (PBSm) was employed for collection of the one-cell embryos [13, 31]. The embryos were cultured in KSOM [20, 25] or HTF [25] supplemented with 4mg/ml of BSA and 25 or 20mM of HEPES, respectively (Anexx 1). In experiment 1, glucose (0,20 mM in KSOM and 2,78 mM in HTF) was present in the media in all phases of the embryonic culture. In experiment 2, the embryos were transferred to the glucose-containing media after 24 hours of culture, and in experiment 3, the embryos were transferred after 48 hours.

The salts were weighed on an analytical balance[1], and later diluted in ultra-purified water[2]. Osmolarity values were between 270 and 290 mOsm/kg[3]. The pH[4] was adjusted to values between 7.10 and 7.20 [6]. The culture media were filtered through 0.22 mm pore membranes[5], and maintained at 4º C for a maximum of two weeks.

Superovulation

CF1 females were superovulated by intraperitoneal injection of 10 IU of equine chorionic gonadotropin (eCG[6]), followed by 10 IU of human chorionic gonadotropin (hCG[7]) after 46 h. Immediately after the second injection, the females were placed with Swiss strain males of proven fertility. The presence of a vaginal plug 18 hours post hCG was the signal of mating occurrence.

Collection of Embryos

For embryo collection, the females were sacrificed by cervical dislocation 30 hours post hCG. The oviducts were removed and maintained in a PBSm solution supplemented with 20% of BFS. After the oviducts were teased using a 30G needle, the one cell embryos were identified under stereomicroscope (40X). The *cumulus oophorus* cells were removed by exposure to 300 IU/mL of hyaluronidase[8] for 20 seconds. After that, the embryos were washed 5 times in PBSm and 3 times in either KSOM or HTF medium before they were placed into culture droplets.

Culture Conditions

Groups of 20 embryos were cultured in 50 ml droplets of medium under mineral oil[8] in a 35 mm-diameter Petri dish[9] for 120 h. The dishes containing the embryos were placed in an incubator[10] at 37 °C, in an humidified environment of 5% CO_2 in air humidity, and the development of the cultured embryos was assessed every 24 hours.

Statistical Analysis

The viable embryos of each donor were randomly distributed among the treatments. Each experiment was replicated at least twice, by the same investigator. The data were analyzed using chi-square test (χ^2) and values were considered significant at $P< 0.05$.

RESULTS

Table 1 shows the percentage of embryos that developed in the presence of glucose since the beginning of the *in-vitro* culture. Embryonic development, assessed at the four-cell stage, blastocyst stage, expanding blastocyst stage, and hatching blastocyst stage was similar in both KSOM and HTFculture media.

Table 1. Development of one-cell *Mus domesticus domesticus* embryos in either KSOM or HTF media supplemented with 25 or 20 mM of HEPES, respectively.

	1-cell	4-cell		Blastocyst		Expanded blastocyst		Hatched blastocyst	
Media	n	n	%	n	%	n	%	n	%
KSOM	423	375[a]	88.6	304[a]	71.8	294[a]	69.5	261[a]	61.7
HTF	332	90[a]	87.3	247[a]	74.4	242[a]	72.9	176[a]	53.0

[a, b] Values with different superscripts in the same column indicate statistical difference (P>0.05).

Until the 4-cell stage, the development rates of embryos cultured in either KSOM or HTF media and exposed to glucose-containing media (0,20 mM in KSOM and 2,78 mM in HTF) after 24 h of culture did not show any significant difference at the 4-cell stage. However, starting at the blastocyst stage, the efficiency of the HTF medium to promote embryonic development was significantly reduced in relation to KSOM (Table 2).

Table 2. Development of one-cell *Mus domesticus domesticus* embryos in either KSOM or HTF media supplemented with HEPES and exposed to glucose after 24 h of culture.

	1-cell	4-cell		Blastocyst		Expanded blastocyst		Hatched blastocyst	
Media	n	n	%	n	%	n	%	n	%
KSOM	227	187[a]	82.4	156[a]	68.7	147[a]	64.7	134[a]	59.0
HTF	197	159[a]	80.7	101[a]	51.2	90[a]	45.7	79[a]	40.1

[a, b] Values with different superscripts in the same column indicate statistical difference (P>0.05).

The transfer of 4-cell embryos to the glucose-containing media 48 h after the beginning of culture resulted in similar embryonic development in both culture media (Table 3).

Table 3. Development of one-cell *Mus domesticus domesticus* embryos in either KSOM or HTF media supplemented with HEPES and exposed to glucose after 48 h of culture.

	1-cell	4-cell		Blastocyst		Expanded blastocyst		Hatched blastocyst	
Media	n	n	%	n	%	n	%	n	%
KSOM	458	400[a]	87.3	332	72.5	314[a]	68.5	233	50.8
HTF	488	428[a]	87.7	328[a]	67.7	319[a]	65.3	248	50.8

[a, b] Values with different superscripts in the same column indicate statistical difference (P>0.05).

DISCUSSION

Several experiments [10, 18] have shown that one and two-cell embryos accumulate glycogen, with is progressively released, and that glucose is used as a primary energetic source after compaction. Other authors [21] suggested that during the embryonic preimplantation stages, glucose is metabolized under different metabolic routes. Glucose may also be stored as glycogen and destined to the Krebs cycle, and it may be expended in the glycolytic route, depending on enzymatic activity.

An inhibitory effect of glucose on the *in vitro* development of human embryos during initial development stages has also been observed with Earle's medium [11]; contradictory to that, the presence of glucose was found to be necessary after the eight-cell stage. The consumption of glucose was minimal (8 pmol/embryo/hour) in the first 48 h, increasing significantly ($p<0.001$) after the fourth (20 pmol/embryo/hour) and fifth culture days (34 pmol/embryo/hour). Better results were obtained on the development of murine embryos when glucose was added to the medium after the embryonic genome had been activated at the 4-cell stage [9].

Other studies [3] suggested that the development block is caused by interruption of most transcription processes after the first cleavage, and is characterized by degradation of maternal mRNA and by activation of the embryonic genome at the end of the two-cell stage, leading to drastic changes in protein synthesis. Studies on the *in vitro* development block in hamster embryos [26, 27] proposed that glucose probably inhibits the activity of the Krebs cycle by means of the 'Crabtree effect' [12]. Seshagiri and Bavister [27] stated that the presence of both glucose and intracellular phosphate (Pi) may stimulate glucolysis at three different levels: while glucose functions as the substrate for glycolysis, Pi stimulates the production of glycolytic enzymes (hexokinase, phosphofructokinase, and glyceraldehyde 3-phosphate dehydrogenase) and is also used in phosphorylation reactions. This network effect directs Pi to glucolysis, causing a destabilization of mitochondrial metabolism, and may result in decreased ATP production, leading to a decrease or block in embryo development. In the absence of glucose, Pi may stimulate the breakdown of the stored glycogen, forming 6-phosphate glucose. Once again, stimulating glucose and deviating Pi would result in insufficient production of ATP and in inhibition of embryonic development. However, in the absence of exogenous phosphate, the glycolytic route is probably not stimulated.

On the other hand, these statements have been disputed by researchers [2] who claim that 1) the activation of the embryonic genome occurs progressively during the entire preimplantation period. Therefore, it is unlikely that the block would occur during a specific developmental stage; 2) in several species, the block coincides with the transition of the development stages from the oviduct to the uterus; and 3) the inadequate production of energy due to inappropriate concentrations of culture medium constituents or energetic substrates or, still, the production of toxic components and free radicals that can cause damage to the cellular membranes, elevate the intracellular pH and/or alter mitochondrial function.

In experiment 1 (Table 1), the embryonic development in glucose-containing KSOM or HTF media supplemented with HEPES was uniform, providing conditions for hatched blastocyst rates above 50%. As observed by others [14, 21] who compared KSOM with other culture media, in the present study the presence of glucose in a medium supplemented with HEPES did not inhibit the development of *Mus domesticus domesticus* embryos from the one-cell stage to the hatched blastocyst stage.

The culture of one-cell embryos for either 24 or 48 hours in glucose-free media (Tables 2 and 3) did not influence the capacity of the embryos to develop to the stage of blastocyst. The analysis of the hatched blastocysts rates in the three experiments reveals that the embryos cultured in KSOM had a tendency to have their hatching capacity reduced when exposed to glucose after 48 hours of culture (50.8%) when compared to the embryos exposed to glucose from the start of culture (61.7%). In turn, the embryos cultured in HTF reached hatching unevenly; after 24 hours, viability was reduced in the group of embryos exposed to glucose (40.1%). This was not observed in the group

of embryos exposed to glucose 48 hours after the beginning of culture, since 50.8% reached hatching.

CONCLUSIONS

The strategy of exposing embryos to glucose at different moments of *in-vitro* culture did not prove to be efficient to increase the rates of development of hatching blastocysts. HEPES-supplemented KSOM or HTF in the presence of glucose allowed the *in vitro* embryonic development of one-cell *Mus domesticus domesticus* embryos to the hatched blastocyst stage.

SOURCES AND MANUFACTURES

[1]Kern 410: http://www.Kern.de
[2]Milli-Q: Millisul Comércio de Equipamentos para Laboratório LTDA.Rua Dr. Eduardo Chartier, 647, C.E.P. 90520-000, Porto Alegre - RS.
[3]Precision Systems: http://www.precisionsystems.com
[4]Millipore: Millisul Comércio de Equipamentos para Laboratório LTDA.
[5]Folligon-Intervet: http://www.intervet.com
[6]Pregnyl-Organon: http://www.organon.com
[7]Sigma: http://www.sigma-aldrich.com.br.
[8]Costar: http://www.corning.com
[9]Forma Científica: http://www.thermo.com

REFERENCES

1 **Basile L.F. 1999.** Viabilidade *in vitro* após vitrificação de blastocistos de *Mus domesticus domesticus* cultivados a partir de embriões de 1-célula. 62 f. Curitiba, PR. Dissertação (Mestrado em Ciências Veterinárias) - Curso de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Paraná.

2 **Bavister B.D. 1995.** Culture of preimplantation embryos: facts and artifacts. *Human Reproduction Update*. 91-148.

3 **Bensaude O., Babinet C., Morange M. & Jacob F. 1983.** Heat shock proteins: first major products activity in mouse embryos. *Nature*. (305): 331-333.

4 **Biggers J.D. & McGinnis L.K. 2001.** Evidence that glucose is not always an inhibitor of mouse preimplantation development *in vitro*. *Human Reproduction Update*. 16: 153-163.

5 **Bolton V.N. & Braude P.R. 1987.** Development of the human preimplantation embryo *in vitro*. *Current Topics in Development Biology*. 23: 93-114.

6 **Brinster RL. 1965.** Studies on the development of mouse embryos *in vitro.* I - Effect of osmolarity and hydrogen ion concentration. *The Journal of Experimental Zoology*. 158: 49-58.

7 **Brown J.J.G. & Whittingham DG. 1992.** The dynamic provision of different energy substrates improves development of one-cell random-bred mouse embryos *in vitro*. *Journal of Reproduction Fertility*. 95: 503-511.

8 **Chatot C.L., Ziomek C.A., Bavister B.D. Lewis J.L & Torres I. 1989.** An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *Journal of Reproduction Fertility*. 86: 679-688.

9 **Chatot C.L., Tasca R.J. & Ziomek CA. 1990.** Glutamine uptake and utilization by preimplantation mouse embryos in CZB medium. *Journal of Reproduction Fertility*. 89: 335-346.

10 **Chi M.M.Y., Manchester J.K., Yang V.C., Curato A.D., Strickler R.C & Lowry O.H. 1988.** Contrast in levels of metabolic enzymes in human and mouse ova. *Biology of Reproduction*. 39:295-307.

11 **Conagahn J., Handyside A.H., Winston R.M.L. & Leese H.J. 1993.** Effects of pyruvate and glucose on the development of human preimplantation embryos *in vitro*. *Journal of Reproduction Fertility*. 99: 87-95.

12 **Crabtree H.G. 1929.** Observations on the carbohydrate metabolism of tumors. *Biochemistry*. 23: 536-545.

13 **Dulbecco R. & Vogt M. 1954.** Plaque formation and isolation of pure lines with poliomyelitis viruses. *Journal of Experimental Medicine*. 99: 167-182.

14 **Erbach G.T., Lawitts J.A., Papaioannou V.E. & Biggers J.D. 1994.** Differential growth of the mouse preimplantation embryo in chemically defined media. *Biology of Reproduction*. 50: 1027-1033.

15 **Gardner D.K. & Leese H.J. 1990.** Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism *in vitro*. *Journal of Reproduction Fertility*. 88: 361-368.

16 **Goddard M.J. & Pratt H.P.M. 1983.** Control of events during early cleavage of the mouse embryo: An analysis of the '2-cell block'. *Journal of Embryology Experimental and Morphology.* 73: 111-133.

17 **Ho Y., Wigglesworth K., Eppig JJ. & Schultz R.M. 1995.** Preimplantation development of mouse embryos in KSOM: Augmentation by amino acids and analysis of gene expression. *Molecular Reproduction and Development*. 41: 232-238.

18 **Hsieh B., Chi M.M.R. & Knor O.H. 1979.** Enzymes of glycogen metabolism and related metabolites in preimplantation mouse embryos. *Development of Biology*.72: 342-349.

19 **Lawitts J.A. & Biggers J.D. 1991.** Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. *Biology of Reproduction*. 45: 245-251

20 **Lawitts J.A. & Biggers J.D. 1993.** Culture of preimplantations embryos. *Methods in Enzymology.* 225: 153-164.

21 **Leppens-Luisier G. & Sakkas D. 1997.** Development, glycolytic activity and viability of preimplantation mouse embryos subjected to different periods of glucose starvation. *Biology of Reproduction*. 56: 589-596.

22 **Loutradis D., John D. & Kiessling A.A. 1987.** Hypoxanthine causes a 2-cell block in random-bred mouse embryos. *Biology of Reproduction*. 37: 311-316.

23 **Muggleton-Harris A., Whittingham D.G. & Wilson L. 1982.** Cytoplasmic control of preimplantation development *in vitro* in the mouse. *Nature*. (299): 460-462.

24 **Petters R.M., Johnson B.H., Reed M.L & Archibong A.E. 1990.** Glucose, glutamine, and inorganic phosphate in early development of the pig embryo *in vitro*. *Journal of Reproduction Fertility*. 89: 269-275.

25 **Quinn P., Kerin J.F. & Warnes G.M. 1985.** Improved pregnancy rate in human in vitro fertilization with use of a medium based on the composition of human tubal fluid. *Journal of Reproduction Fertility.* 44: 493-498.

26 **Schini S.A. & Bavister B.D. 1988.** Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. *Biology of Reproduction*. 39: 1183-1192.

27 **Seshagiri P.B. & Bavister B.D. 1991.** Glucose and phosphate inhibit respiration and oxidative metabolism in cultured hamster eight-cell embryos: evidence for the "Crabtree Effect". *Molecular Reproduction and Development*. 30: 105-111.

28 **Summers M.C., Bhatnagar P.R., Lawitts J.A. & Biggers J.D. 1995.** Fertilization *in vitro* of mouse ova from inbred and outbred strains: complete preimplantation embryo development in glucose-supplemented KSOM. *Biology of Reproduction*. 53: 431-437.

29 **Takahashi Y. & First N.L. 1992.** *In vitro* development of bovine one-cell embryos : influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology*. 37: 963-978.

30 **Whitten W.K. & Biggers J.D. 1968.** Complete development *in vitro* of the preimplantation stages of the mouse in a simple, chemically defined medium. *Journal of Reproduction Fertility*. 17: 399-401.

31 **Whittingham D.G. 1971.** Culture of mouse ova. *Journal of Reproduction Fertility.* 14 (Suppl): 7-21.