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Effects of Glucose and Inorganic Phosphate on the Development of Rat 8-Cell Embryos *In Vitro*

Lee, H. M. and D. I. Jin

Department of Food Resources and Manufacturing, Sun Moon University

Glucose와 Inorganic Phosphate가 Rat 8-세포기 난자의 체외배양에 미치는 영향

이 홍 미 · 진 동 일

선문대학교 식량자원식품가공학부

요 약

본 연구는 착상전 Rat 8-세포기 난자의 체외배양시 glucose(5.56 mM와 0 mM)와 phosphate(1.19 mM와 0 mM)의 억제효과를 규명하기 위하여 실시하였다. 48시간 배양후 발육율은 glucose+phosphate군에서는 37%(31/84), glucose군은 70%(64/91), phosphate군은 69% (59/85) 그리고 glucose와 phosphate가 없는 무처리군에서는 77%(67/85)를 나타냈다. Glucose+phosphate군의 발육율은 다른 처리군에 비해 유의적으로 낮았고, 그 외 처리군 사이에는 유의성이 없었다. 발육난자의 핵염색에 의한 세포수의 측정에서는 glucose군에서 가장 많은 세포수를 나타냈고 (29.3±0.97, P<.001), 그 외 처리에서는 유의성이 없었다 (glucose+phosphate, 17.5±1.04; phosphate, 18.6±1.01; no glucosephosphate, 19.8±1.01). 본 실험의 결과로 glucose와 phosphate는 Rat 8-세포기 난자의 발육에 억제효과를 나타냈고, glucose는 난자의 세포분열을 증진시키는 효과가 있는 것으로 나타났다.

I. INTRODUCTION

Recent progress in the examination of physiological relationships between basic culture media components (for example, energy and protein sources) now makes possible the study of embryos that exhibit *in vitro* embryonic developmental blocks(Bavister, 1987, 1988). Glucose and phosphate, in particular, were found to be inhibitory to early cleavege stage hamster embryos (Schini and Bavister, 1988; Seshagiri and Bavister, 1989a,b). Pig embryos appear to be sensitive to lactate and pyruvate with respect to the developmental block at the 4-cell stage, but not to glucose and/or phosphate (Davis, 1985; Petters et al., 1990). Mouse zygotes from several strains, and cattle embryos (during the first 36~48 hr. *in vitro*) are sensitive to the presence of glucose in the medium (Chatot et al., 1989; Ellington et al., 1990). In addition, glutamine has been shown to be an important energy and/or nitrogen source for hamster, mouse and pig embryos(Carney and Bavister, 1987; Chatot et al., 1989, 1990; Petters et al., 1990). Related studies on the *in vitro* development of rat embryos should provide additional information regarding the regulatory roles of various media components on embryonic development.

Development of rat preimplantation embryos

in vitro has been limited by developmental blocks at the 2- and 4-cell stages, with additional difficulties at the 8-cell stage (Bavister, 1987, 1988). Progress has been made towards the development of a consistent *in vitro* culture system for rat embryos; but the reported rates of success between laboratories have been extremely variable (Folstad et al., 1969; Mayer and Fritz, 1974; Horiuchi et al., 1983; Miyoshi et al., 1994, 1995).

The objective of this study was to evaluate the *in vitro* development of rat 8-cell embryos in the presence and absence of glucose and inorganic phosphate. The developmental criteria were embryonic stage (degenerate, 8-cell, morula, and blastocyst) and number of cells (nuclei) at the end of the culture period. Our results suggest that glucose and phosphate together exert an inhibitory effect on 8-cell rat embryo development, while glucose alone resulted in a greater number of cells (nuclei) per embryo.

Ⅱ. MATERIALS AND METHODS

1. Rat embryo culture medium

The medium used in this experiment was similar to that reported by Zhang et al. (1989), and Zhang and Armstrong (1990), with modifications as follows: inclusion of nonessential amino acids (#M-7145, Sigma), substitution of 4mg/ml bovine serum albumin (BSA, fraction V #A-7906, Sigma) in place of polyvinyl alcohol (PVA), and deletion of glucose and potassium phosphate (Table 1). The medium was adjusted to pH 7.4 by addition of 2.5 N NaOH, and sterile filtered. Four treatment media were then made by the addition of glucose(5.56 mM vs. 0 mM) and/or potassium phosphate(1.19 mM vs. 0mM) form 100X stocks for the 2×2 factorial experiment. All media were stored at 4℃ and used within 1 week of preparation. The osmolarities of the treatment media were between

Table 1. Modified Armstong's culture medium for rat 8-cell embryos

Medium Component	Concentration(mM)
NaCl	94.6
KCl	4.77
$CaCl_2$	1.71
$MgSO_4$	1.19
$NaHCO_3$	25.1
Pyruvate(Na)	0.5
Lactate(Na)	20.0
Glucose*	5.56
KH_2PO_4*	1.19
BSA	4 mg/ml
Penicillin/streptomycin	100 U/ml:0.1 mg/ml
Insulin**	5 μg/ml
Transferrin	5 μg/ml
Selenium	5 μg/ml
Ala***	0.1
Arg	0.6
Asn	0.114
As[	0.086
Cys	0.198
Glu	0.079
Gln	2.0
Gly	0.1
His	0.2
ILE	0.397
Leu	0.397
Met	0.101
Phe	0.194
Pro	0.1
Ser	0.1
Thr	0.403
Trp	0.045
Tyr	0.286
Val	0.393

* Treatment media were fotmulated with and without these components.

** Insulin/transferrin/selenium were supplied as ITS (CR-ITS premix, Collabrative Reseach Inc., Bedford, MA).

*** Amino acids (MEM amino aicd solutions, #M-7020 and #M-7145, Sigma, St. Louis, MO).

285 and 295 mOsm. Media components were purchased from either Mallinckrodt Inc. or Sigma.

2. Embryo collection and culture

Mature rats (Sprague-Dawley strain) were housed under a light:dark schedule of 14:10. The animals were fed Purina Laboratory Chow (Diet 5001, Purina Mills) and water *ad libitum*. Donor females were vaginally smeared daily to evaluate stage of the estrous cycle (Baker, 1979). Following breeding, the rats were examined for the presence of copulation plugs of sperm in the vagina (as determined by vaginal smears). Embryos were recovered from the oviducts and the anterior uterine horns of mated rats begining at 0700h on day 4 post coitum. The medium used for embryo recovery and holding prior to allocation into treatments lacked both glucose and phosphate. To control for interdonor variation, all embryos from a single female (only pre- and early compacting 8-cell embryos were used) were allocated randomly by treatment into a single culture dish. The culture dishes were prepared the day prior to embryo recovery as follows: four microdrops (100 μl of each treatment media) were placed into a single dish (60×15mm, Falcon Plastics #1007), covered with 8 ml of washed silicon oil (Aldrich Chemical Company), and placed into the culture incubator under a humid atmosphere of 5% CO_2 and 95% air, at 37℃.

3. Assessment of embryo development and statistical analysis

The embryos were cultured *in vitro* for approximately 48 hours and then evalauted for morphological stage of development (STG) by light microscopy and final cell number (FCN) by staining with trypan blue and Hoeshst 33342 and counting of nuclei under fluorescent microscopy (Pursel et al., 1985).

For statistical analysis, the embryos were assigned a numerical value corresponding to morphological stage of development achieved (STG): degenerate=1; 8-cell=2; morula=3; blastocyst=4. These numerical values and FCN were subjected to analysis of variance using the General Linear Model of the Statistical Analysis System (SAS). The data were analyzed by replication (n=2; 172 embryos from 19 rats, and 173 embryos from 17 rats, respectively), female (n=36), and by treatment (n=4). Comparisons between least square means were made by t-tests.

Ⅲ. RESULTS

The data for embryonic development and number of cells achieved at the end of the *in vitro* culture period are presented in Table 2 and Fig. 1. In two replications, 345 pre- and early compaction stage 8-cell embryos from 36 mature rats were assigned to the four treatments. Statistical evaluation of the data indicated that for STG and FCN, replication was not significant (P>.15) therefore the data were pooled. At the end of the culture period 37%(31/84), 70%(64/91), 69%(59/85), and 77%(67/85) of the cultured 8-cell embryos developed to the blastocyst stage in media with glucose+phosphate, glucose only, phosphate only, and no glucose or phophate, respectively. Embryo development (2.90±0.097 for STG) in the presence of both glucose and phosphate was significantly reduced (P<.001), while no significant differences in development were observed between all other treatment (3.4~3.5±0.093~0.097 for STG). Evaluation of least square mean for FCN between treatments indicated that the greatest mean number of cells (nuclei) resulted in medium with glucose alone (29.3±0.97 cells, P<.001). No significant differences were observed for FCN for the remain-

Table 2. The effects of glucose(±) and phosphate(±) on development of rat 8-cell embryos *in vitro* for 48 h

MEDIA		Number of	Embryo development (cell number)					LS means[1]	
Gluc.	Phos.	embryos	Degenerated	8-cell	Morula	Blastocyst.	% Blast.	STG	FCN
+	+	84	9	20	24(15.0)	31(26.5)	36.9	2.9^{a}	17.5^{b}
+	−	91	5	7	15(18.5)	64(34.4)	70.3	3.5^{b}	29.3^{a}
−	+	85	5	14	7(17.4)	59(21.2)	69.4	3.4^{b}	18.6^{b}
−	−	85	7	6	5(14.4)	67(21.5)	76.5	3.4^{b}	19.8^{b}

[a,b]: Values within columns, with different superscrips and significantly different at P<.001 for STG and P<.001 for FCN.

1: std. error for STG and FCN were ±0.093~0.097 and ±1.01~1.97, respectively.

STG = Developmental stage at end of culture period.

FCN = Cell numbers (nuclei) at end of culture period.

N = 345 embryos from 36 rats.

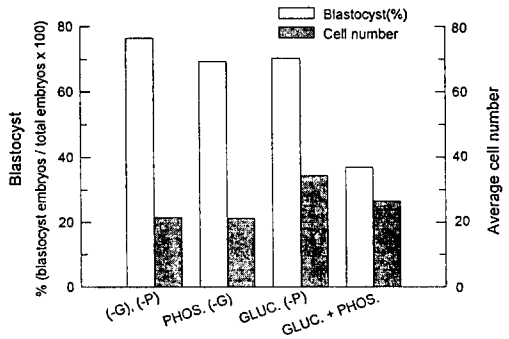

Fig. 1. The effects of glucose and phosphate on development of rat 8-cell embryos (%) and cell numbers of blastocyst stage embryos (cell number). (-G) and (-P) refer to without glucose and without phosphate while GLUC and PHOS refer to with glucose and with phosphate in the medium, respectively.

ing three treatment media (17.5±1.04 cells, 18.6±1.01 cells, and 19.8±1.01 cells for glucose +phosphate, phosphate only, and no glucose or phosphate, respectively).

Approximately 25% of the embryos in the glucose + phosphate medium remained at the 8-cell stage(essentially twice as many as in other media), and blastomeres from many of these embryos appeared to be swollen or distended. During the 48h culture period, embryo hatching from the zona pellucida was not observed, although significant zona thinning did occur with blastocyst expansion.

Significant female effects were observed for STG(P<.001) but not for FCN (P>.14).

Ⅳ. DISCUSSION

The study of rat preimplantation embryonic development *in vitro* has been limited by the difficulties encountered during the culture of 2-, 4-, and 8-cell stages. The successful development of rat 8-cell embryos *in vitro* to the blastocyst stage has been extremely variable. Utilization of the medium described by Zhang and Armstrong (1990) with the inclusion of amino acids and insulin/transferrin/selenium (ITS) has, in our laboratory, significantly improved the *in vitro* developmental success of rat 8-cell embryos (Petters et al, 1988; Petters and Jin, 1989, unpublished observations).

The treatment media used in the present report supported development of 65~87% of the

8-cell embryos to the morula and blastocyst stages combined (37~77% to the blastocyst stage). These data, with the notable exception of the glucose + phosphate treatment, are similar to the morphological development observed for early 8-cell rat embryos by Zhang and Armstrong (1990). The results shown above suggest that the presence of glucose and phosphate together in the treatment medium is inhibitory to the morphological development of 8-cell rat embryos. These results are similar in effect to those observed for development of hamster embryos and rat 1-cell stage embryos *in vitro*, where glucose and phosphate exert a strong inhibitory effect on the cleavage stage embryos (Schini and Bavister, 1988; Seshagiri and Bavister, 1989a,b; Miyoshi et al., 1994, 1995).

The number of cells observed for morulae and blastocysts in this experiment were 1) glucose+ phospahte, ranges = 12~22, and 19~57 cells, respectively, 2) phosphate only, ranges = 14~20, and 14~33 cells, respectively, 3) no glucose or phosphate, ranges = 13~16, and 13~33 cells, respectively, and 4) glucose alone, ranges = 14~25, and 20~58 cells, respectively. These data for cell numbers(nuclei), with respect to stage of development in this experiment, are similar to the number of cells observed in embryos recoverd at the morulae (range =10~18 cells) and blastocyst (range =23~56 cells) stages *in vivo* (Surani, 1975; Schiffner and Spielmen, 1976).

Eight-cell embryos developing to the expanded blastocyst stage under the experimental conditions described, exhibited thinning of the zonae, however no hatching was observed. This observation was not surprising since hatching from or dissolution of the zona pellucida is normally modulated by estrogen interaction(s) with serum and /or uterine fluids (Psychoyos, 1966; Dickmann, 1968; Surani, 1975, 1977). Many of the embryos failing to proceed past the 8-cell stage in medium containing both glucose and phosphate exhibited an abnormal morphology, in that the blastomeres were swollen or distended. This abnormal appearance may be similar to that observed for hamster embryos that block during *in vitro* development (Bavister, 1987). Our results, in reference to the inhibitory effects of glucose + phosphate on 8-cell development are in contrast to those observed by Zhang and Amstrong (1990), where no differnces in morphological development of the 8-cell embryos to the blastocyst stage were observed, either in the presence or absence of glucose and phosphate. This disparity may reflect differences in experimental design or variation between rats (same strain designation, but from differnt supplies). An additional disparity between our protocol and that of Zhang and Armstrong (1990) was the use of embryos from natural ovulating females (our protocol) vs. superovulated immature females.

With regard to experimental design, our protocol was for embryo collection and handling to be carried out in medium free of both glucose and phosphate to eliminate experimental variation due to even a brief exposure to either glucose and /or phosphate. This decision was predicted on the observations that only 0.1mM phosphate was sufficient to mediate the inhibitory effects of glucose on hamster 8-cell embryos (Seshagiri and Bavister, 1989b). Also, our protocol controlled for variation due to female by evaluation of embryos across treatments within individual females. The protocol utilized by Zhang and Armstrong (1990) called for collection of all embryos in Dulbecco's phosphate buffered saline (1.47mM KH_2PO_4 and 8.1mM Na_2HPO_4) supplemented with 21.58mM sodium lactate, 0.5mM sodium pyruvate, and 5.56mM glucose. The embryos were pooled for all females and distributed into treatments within 30

mintutes of collection. The extent to which this protocol(Zhang and Armstrong, 1990) influenced 8-cell embryo development *in vitro*, however, is not clear given the developmental success achieved across all treatments.

In conclusion, the rat 8-cell embryo(pre- and early compaction stages) may be sensitive to the combined presence of glucose and phosphate in the culture medium. Morphological development, as determined by stage of embryonic development achieved after 48h *in vitro*, was significantly inhibited by medium with glucose + phosphate, but not by medium with glucose alone, phosphate alone, or medium without glucose and phosphate. The number of cell divisions achieved *in vitro* was greater in medium with glucose only, while no differences were observed with respect to cell numbers between all other treatments.

V. SUMMARY

This study was designed to evaluate the potential inhibitory effects of glucose (5.56 mM vs. 0 mM) and/or phosphate (potassium phosphate, 1.19 mM vs. 0 mM) on the *in vitro* development of rat 8-cell embryos (n=345 embryos from 36 mature rats). Evaluation of embryos at 48 h for developmental stage (STG) indicated that 37% (31/84), 70% (64/91), 69% (59/85), and 77% (67/85) developed to the blastocyst stage in media with glucose+phosphate, glucose only, phosphate only, and no glucose or phosphate, respectively. Embryo development (2.90±0.097 for STG) in medium with glucose + phosphate was significantly reduced ($P<.001$), while no significant differences were observed between all other media (3.4~3.5±0.093~0.097 for STG). Evaluation of embryos for final cell number (FCN) indicated that the greatest number of cells (nuclei) resulted in medium with glucose alone (29.3±0.97 cells, $P<.001$). No significant differences were observed for FCN for the remaining three media (17.5±1.04 cells, 18.6±1.01 cells, and 19.8±1.01 cells for glucose+phosphate, phosphate only, and no glucose or phosphate, respectively). Our results suggest that glucose and phosphate together exert an inhibitory effect on 8-cell rat embryo development, while glucose alone was beneficial, yielding greater numbers of cells per embryo.

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