韓國家畜繁殖學會誌 19(3) : 227~234(1995)
Korean J. Animal Reprod.

Effect of Glucose Exposure on the Development of the Mouse Preimplantation Embryo *In Vitro*

Kim, S. E., S. J. Uhm, E. Y. Kim, S. H. Yoon*, S. P. Park,
K. S. Chung and J. H. Lim***
Maria Infertility Medical Institute, Seoul, Korea

착상전 생쥐배아의 Glucose에 대한 노출이 체외 배발생에 미치는 영향

김선의 · 엄상준 · 김은영 · 윤산현* · 박세필 · 정길생 · 임진호***
마리아 불임클리닉 기초의학연구소

요 약

본 연구는 1-세포기배의 glucose에 대한 노출이 상실배기 이후의 배발생에 미치는 영향을 검토하고자 실시되었다. hCG 주사 후 24~25시간 째에 F_1 hybrid (C57BL/6, ♀ × CBA/N, ♂) 계통 생쥐를 도살하여 1-세포기배를 회수한 후 0.1% hyaluronidase로 처리하여 난구세포를 제거하였다. 1-세포기배는 hCG 주사 후 72시간째 다양한 농도의 glucose (5.5, 16.5, 27.5 및 38.5mM)에 1분 노출 후 glucose가 첨가되어 있거나 혹은 첨가되지 않은 CR_{1aa} 배양액에서 계속 배양함으로써 배발생을 유도하였다.

이 실험의 결과를 요약하면 다음과 같다.

1. M2 배양액에서 회수한 후 3mg/ml의 Fatty-acid free BSA가 첨가된 배양액에서 배양한 경우 27.5%의 확장배반포까지의 배발생율과 16.6%의 탈출 배반포까지의 배발생율을 나타낸 반면, TL HEPES 배양액에서 회수한 경우는 전혀 상실배기 이후의 배발생이 나타나지 않았다.
2. hCG 주사 후 72시간째에 단 1분간의 27.5mM glucose에 대한 노출만으로도 68.8% (CR_{1aa}+BSA)와 77.1% (CR_{1aa}+FBS)의 확장배반포까지의 발생을 유도할 수 있었다. 그러나 1분 노출과 이후 계속되는 노출간에는 배발생에 있어서 유의차는 인정되지 않았다.
3. hCG 주사 후 72시간째에 5.5, 16.5, 27.5 및 38.5mM의 glucose 첨가에 따른 확장 배반포까지의 배발생율은 45.7~61.5%로 각 처리간의 유의차는 없었으며, 따라서 고농도의 glucose 첨가에 따른 저해효과는 확인할 수 없었다.

I. INTRODUCTION

It is known from studies of mammalian embryo culture that mouse zygotes require pyruvate to support the first cleavage division (Biggers et al., 1967) and glucose as sole energy source is unable to support development until the late four-cell to early eight-cell stage (Brinster & Thomson, 1966). Cross and Brinster (1973) showed that zygotes of a random-bred strain can develop through the 2-cell block in a medium

* 마리아 산부인과 (Maria Obs. & Gyn.)
** 건국대학교 축산대학 (College of Animal Husbandry, Kon-Kuk University)

containing lactate and pyruvate, but no glucose. Leese and Barton (1984) reported the uptake of pyruvate and glucose by small numbers of mouse ova and preimplantation embryos using an ultramicrofluometric technique ; pyruvate uptake exceeded that of glucose in unfertilized and fertilized ova to the morula stage but glucose utilized of the predominent substrate during blastocyst development. Glucose removal has been shown to facilitate the development of hamster (Schini & Bavister, 1988; Seshagiri & Bavister, 1989), cattle (Takahashi & First, 1992), and human (Conaghan et al., 1993) embryos.

At the viewpoint, the role of glucose in mouse embryo development has been extensively studied. But, it was not recognized until recently that exposure of early mouse embryos to glucose is detrimental (Chatot et al., 1989). Nasr-Esfahani et al., (1992) showed that embryos must be exposed to the medium containing glucose at some point within 70 h after hCG if good rates of blastocyst formation are to be achieved ultimately, even if the exposure to glucose is restricted to the period of recovery from the oviduct. Chatot et al. (1994) found that exposure to 27mM glucose for only 1 min. at the four-cell stage was sufficient to overcome the two-cell block and to promote development from morula to blastocyst stage in embryos from a blocking strain. A more recent study by Martin and Leese (1995) has shown that mouse embryos from an non-blocking strain have an obligatory requirement for glucose at the early developmental stages in order to develop to the blastocyst stage.

On the other hand, Lawitts and Biggers (1991) reported that glucose is indeed inhibitory to development through the 2-cell stage in the random-bred mice cultured in medium M16, but the inhibitory effect of glucose is also reduced when the concentration of sodium chloride in the medium is reduced. A possible explanation for the result is that 95mM NaCl present in M16 may be particularly detrimental to glucose-6-phosphate isomerase function (Lawitts and Biggers, 1991). CZB medium, lacks glucose, promotes development of one-cell embryos to morula stage in strains of mice that exhibit a "two-cell block" to development, while in the absence of glucose, morulae failed to cavitate normally and degenerated (Chatot et al., 1994). However, the development of embryos from morula to blastocyst promoted when exposed to glucose at the four- to eight-cell stage (48 h of culture) (Chatot et al., 1989, 1990).

The basis for this glucose requirement is not still clear. However, it may be important for the onset of active glucose transport and utilization, which is around the 8-cell stage (Brinster & Thomson, 1966; Wales & Brinster, 1968; Gardner & Leese, 1986, 1988).

The present study was designed to examine the effects of exposure time in CR_{1aa}, chemically defined medium supplemented with amino acids (Rosenkrans & First, 1993), containing various concentration of glucose on the development of one-cell mouse embryo beyond morula to hatching blastocyst *in vitro*.

Ⅱ. MATERIALS AND METHODS

1. Animals

All mice of this study were maintained on 14 h light and 10 h dark (lights on at 0600 h). Four to six weeks old, F_1 female mice from C57BL/6, ♀ × CBA/N, ♂ (B6CBA F_1) were superovulated by intraperitoneal (i.p.) injection of 5 i.u. pregnant mare serum gonadotrophin (PMSG ; Sigma, St. Louis, MO) at 1430 h, followed by 5 i.u. human chorionic gonadotrophin (hCG ; Sigma) 48 h later. Females, hCG injected, were immediately caged with the same strain (≥ 8 weeks old) and checked the next morning (day 1)

for the presenceof a vaginal plug.

2. Embryo collection

Mice were sacrificed at 24~25 h after hCG to obtain fertilized oocytes at the pronuclear stage. The embryos were recovered by tearing the oviduct with medium M2 or TL-HEPES containing 4 mg/ml BSA (fraction V, Sigma), respectively (Table 1). Cumulus cells were removed with 0.1% hyaluronidase and then washed 3 times.

3. Preparation of culture media

Media were pipetted in 50μl drops of CR_{1aa} (Table 1) containing 3mg/ml fatty-acid free BSA (FAF-BSA, Sigma) or 10% FBS into Falcon 60-mm petri dishes and overlaid with mineral oil. Culture drops that required an exposure to glucose were injected directly with 0.5, 1.5, 2.5 and 3.5μl of a 100mg/ml glucose solution in water (5.5, 16.5, 27.5 and 38.5mM final medium concentration). Controls received an identical injection of water. Media drops in culture dishes were equilibrated with 5% CO_2 at 37℃ for overnight before use.

Table 1. Composition of media used in this experiment

Component	Concentration (mM)		
	M2	TL HEPES	CR_{1aa}
NaCl	94.66	114	114.7
KCl	4.78	3.2	3.1
$CaCl_2 \cdot 2H_2O$	1.71	2.0	–
$NaHCO_3$	4.15	0.4	26.2
NaH_2PO_4	–	0.34	–
KH_2PO_4	1.19	–	–
$MgCl_2 \cdot 6H_2O$	–	0.5	–
$MgSO_4 \cdot 7H_2O$	1.19	–	–
HEPES	20.85	10	–
Na lactate	23.28	10	5.0
Na pyruvate	0.33	0.22	0.4
L-Glutamine	–	–	1.0
Glucose	5.56	–	–

* CR_{1aa} is added BME (Basal Medium Eagle's) and MEM (Minimum Essential Medium)

4. Embryo culture

The embryos from all mice were pooled and subsequently divided into groups of 20~30 and cultured at 37℃ in a humidified atmosphere of 5% CO_2 in air. The embryos were either, transferred at 72 h post hCG to CR_{1aa} containing various concentration of glucose, or were placed in the same media containing glucose for 1 min. and then returned to the fresh culture drop of CR_{1aa} (without glucose). Embryos were scored for developmental stages at 96 (day 4) and 144 h (day 6) of culture.

5. Statistics

The results were analysed by chi-squre test using SAS Institute software package (SAS Institute Inc., 1985).

Ⅲ. RESULTS AND DISCUSSION

1. Effect of exposure of glucose during recovery of one-cell zygote

This experiment was undertaken to determine whether transient glucose exposure during recovery of one-cell zygote from oviduct affects the embryo development to expanded blastocyst and hatching blastocyst stage (Fig. 1).

The developmental rates of B6CBA F_1 embryos that flushed in M2 and cultured in CR_{1aa} with 3mg/ml FAF-BSA were 25.7% of expanded blastocyst (day 4) and 17.6% of hatching blastocyst stage (day 6), but those of embryos that flushed in TL HEPES were 0% and 0%, respectively. And embryos cultured in the absence of glucose underwent a distinct morphological degeneration at the morula stage. However, no differences were observed between development to the hatching blastocyst stage in

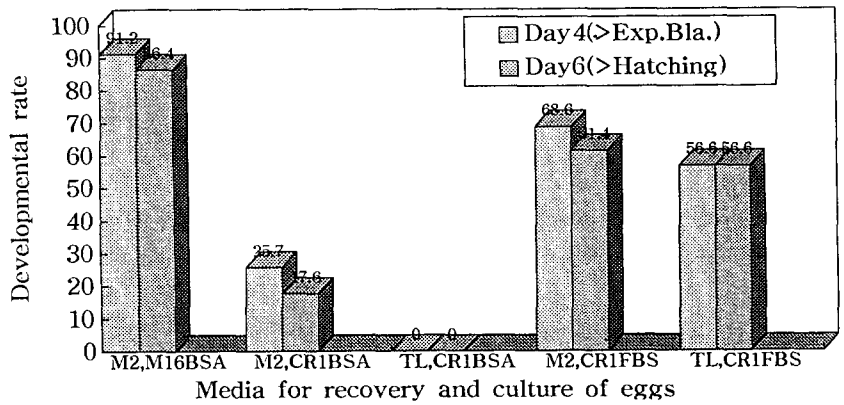

Fig. 1. Effect for egg which is recovered in M2 or TL HEPES from oviduct and cultured in CR_{1aa} supplemented with FAF-BSA or FBS on the development *in vitro*.

M2 (61.4%) orTL HEPES (56.6%), when the embryos were cultured in CR_{1aa} supplemented containing 10% FBS. The effectiveness of culture in CR_{1aa} with 10% FBS may reflect glucose or unknown factors in FBS.

This result reconfirmed that one-cell embryos from B6CBA F_1 can be developed to the blastocyst stage, even if the exposure to glucose is restricted in the medium used for flushing from the oviduct (Nasr-Esfahani et al., 1992). Also, two-cell embryos from a nonblocking strain were able to develop to the blastocyst stage in glucose-free medium with only pyruvate and lactate as exogenous energy source, but these may have been exposed to exogenous glucose during first cleavage *in vivo* (Biggers et al., 1965; Brinster, 1965; Brown & Whittingham, 1991; Martin & Leese, 1995).

The requirement for glucose to support the morula to blastocyst transition as early as day 1 may be explained in terms of glycogen synthesis and storage. The glycogen begins to accumulate rapidly at the time of the first cleavage and reaches a maximum by the 8-cell stage. The time of greatest synthesis of glycogen, is temporally related to the "block" found in the development *in vitro* of the early 2-cell embryo (Stern & Biggers, 1968). Also, the measurement of activities of glycogen phosphorylase and synthase suggest that glycogen synthesis is favored during early development (Hsieh et al., 1979).

However, glucose as the sole energy source is unable to support development until the late 4- to 8-cell stage due to a block to glycolysis (Barbehenn et al., 1974). Barbehenn et al. (1978) reported that the nature of the block in glycolysis which prevents glucose from acting as a sufficient energy source for early embryos may be due to inhibition by the very high citrate levels present. Glycolysis is turned on late in preimplantation development by the rise in fructose-6-p, a deinhibitor of p-fructokinase (Barbehenn et al., 1978). So, the breakdown of glycogen stores could provide a potential supply of glucose for use by the embryo at later developmental stage (Martin & Leese, 1995).

2. Effect of exposure time to glucose

To determine the effect of exposure to glucose, embryos were either, placed in CR_{1aa} containing 27.5mM glucose for 1min, and subsequen-

tly returned to the fresh culture medium (without glucose), or were transferred to the same media containing glucose at 72 h post hCG (Table 2).

When the embryos were cultured in CR_{1aa} with FAF-BSA, a 1 min. exposure of embryos to 27.5mM glucose at 72 h post hCG supported the significantly higher development rates to expanded blastocyst (day 4, 81.3%) and to hatching blastocyst stage (day 6, 68.8%) than those of embryos cultured in CR_{1aa} without glucose (day 4, 13.6% and day 6, 6.8%), but no significantly differences were observed between embryos exposed for 1 min. or transferred at 72 h (day 4, 78.8% and day 6, 66.7%). Also, in the embryos cultured in CR_{1aa} supplemented with 10% FBS, there were no significantly differences between embryos exposed for 1 min. (day 4, 77.1% and day 6, 83.6%) or transferred at 72 h (day 4, 77.1% and day 6, 78.7%).

The results of this experimant showed that the preimplantation mouse embryos must be exposed to glucose for only 1 min. at 72 h post hCG in order to develop optimally from the one-cell to the blastocysts *in vitro*. At the view point, the results are similar to that of Chatot et al. (1994) who found that exposure to 27.5mM glucose for only 1 min, at between 58 and 80 hpost hCG, was sufficient to promote 67~73% of development to the blastocyst stage in embryos from a blocking strain. Also, Martin and Leese (1995), using cultured embryos from B6CBA F_1 mice, reported a smiliar result. However, this is in contrast to the embryos of other species (hamster, Schini & Bavister, 1988; cattle, Takahashi & First, 1992; and human, Conaghan et al., 1993), where glucose has been showed to be inhibitory to development *in vitro*.

The importance of glucose before the morula stage may be related to synthesize glycogen for the maintenance of compaction in that one-cell embryos cultured in the absence of glucose but then decompact before degenerating (Brown & Whittingham, 1991; Martin and Leese, 1995). Due to the specific timing of the requirement of glucose (between 76 and 82 h post hCG), it seems unlikely that this is related to a sudden switch from a pyruvate/lactate-based metabolism to one solely dependent on glucose (Brown & Whittingham, 1991). Glucose, and/or its metabolites, may therefore be required for the synthesis of some stage-specific, developmentally essential embryonic component(s) involved in the morula to blastocyst transition (Johnson et

Table 2. Effect of one-min. exposure or transfer to 27.5mM glucose at 72 h post hCG on the development of one-cell embryos

Sample	Exposure to glucose	No. of embryos	No. of embryos developed to	
			Day 4 (>EB)*	Day 6 (>Hing)*
CR_1 + FAF-BSA	−	59	8 (13.6)[a]	4 (6.8)[a]
	One-min.	48	39 (81.3)[b,c]	33 (68.8)[b,c,d]
	Transfer	66	52 (78.8)[c]	44 (66.7)[b,d]
CR_1 + 10% FBS	−	55	33 (60.0)[d]	29 (52.7)[d]
	One-min.	48	37 (77.1)[b,d]	37 (77.1)[b,c]
	Transfer	61	51 (83.6)[b,c]	48 (78.7)[b,c]

[a,b,c & d] ; Different superscripts in the same column were significantly different ($p<0.05$)

* EB : Early Blastocysts

* Hing : Hatching Blastocysts

Table 3. Effect of one-min. exposure or transfer to CR_{1aa} + FAF-BSA with various concentration of glucose at 72 h post hCG on the development of one-cell embryos

Exposure to glucose	Sample	No. of embryos examined	No. of embryos developed to Day 4 (>EB)*	No. of embryos developed to Day 6 (>Hing)*
One-min.	5.5 mM	53	23 (52.8)	27 (50.9)
	16.5 mM	52	32 (61.5)	22 (42.3)
	27.5 mM	59	32 (54.2)	29 (49.2)
	38.5 mM	49	26 (53.1)	21 (42.9)
Transfer	5.5 mM	35	16 (45.7)	15 (42.9)
	16.5 mM	33	17 (51.5)	14 (42.4)
	27.5 mM	36	20 (55.6)	13 (36.1)
	38.5 mM	35	17 (48.6)	13 (37.1)

* NS : Not significantly different from each other

al., 1984; Brown & Whittingham, 1991, 1992).

3. Effect of exposure to various concentration of glucose

To determine if concentration in the injection protocol was affected to embryos in culture, the one-cell embryos were either, transferred at 72 h post hCG to CR_{1aa}+FAF-BSA containing various concentration of glucose, or were placed in the same media containing glucose for 1 min. and then returned to the fresh culture drop of CR_{1aa} (without glucose) (Table 3).

Regardless of glucose concentration, 45.7~61.5% of embryos developed beyond the blastocyst stage and 36.1~50.9% of hatching blastocyst. There were no significant differences between any of the treatments on the development to expanded blastocyst and hatching blastocyst stage.

Similar finding reported by Chatot et al., (1990) also showed that all glucose levels (5.5 to 49.5mM), with the exception of the highest concentration tested, supported 65~85% of development to the morula and blastocyst stages. At the highest glucose concentration (49.5mM glucose) used, embryo development was generally slowed at the 3-cell to morula stage, but there were no significant differences between any of the treatments on day 4 (morula and blastocyst) or day 5 (blastocyst).

Therefore, this result showed that the detrimental effect of highly concentration was not appeared on the development of one-cell embryos, exposed from 72 h post hCG, beyond blastocyst stage.

Ⅳ. SUMMARY

This study was carried out to investigate the effects according to the time course of glucose exposure on the development of one-cell embryos beyond morula in CR_{1aa} medium. One-cell zygotes from B6CBA F_1 mice were recovered at 24~25h after hCG and cumulus cells were removed with 0.1% hyaluronidase. The embryos were pooled and subsequently divided into each groups and cultured in CR_{1aa} at 37℃ in 5% CO_2 in air.

The embryos were either, placed in CR_{1aa} containing various concentration (5.5, 16.5, 27.5 and 38.5 mM) of glucose for 1 min. and subsequently returned to the fresh culture medium (without glucose), or were transferred to the same media containing glucose at 72 h post hCG.

The results obtained in these experiments were summarized as follows:

1. The development rates of zygotes, recovered from the oviducts in M2 and cultured in CR_{1aa} with 3mg/ml FAF-BSA, to expanded blastocysts (25.7%) and hatching blastocysts (17.6%) were significantly higher than those of zygotes recovered in TL Hepes (0% and 0%, respectively).
2. The development rates of one-cell embryos exposed to 27.5 mM glucose at 72 h post hCG for 1 min. were 68.8% (CR_1+BSA) and 77.1% (CR_1+FBS) of expanded blastocyst stage, but there were no significant differences between the embryos exposed for 1 min. or transferred at 72 h.
3. Regardless of glucose concentration (5.5, 16.5, 27.5 & 38.5mM), 45.7~61.5% of embryos developed to the blastocyst stage. There were no significant differences between any of the treatments on the development of one-cell embryos. Therefore, the detrimental effect of highly concentration was not appeared.

V. REFERENCES

1. Barbehenn, E.K., R.G. Wales and O.H. Lowry. 1974. The explanation for the blockade of glycolysis in early mouse embryos. Proc. Natl. Acad. Sci. USA, 71, 1056-1060.
2. Barbehenn, E.K., R.G. Wales and O.H. Lowry. 1978. Mesurement of metabolites in single preimplantation embryos ; a new means to study metabolic control in early embryos. J. Embryol. Exp. Morph. 43, 29-46.
3. Biggers, J.D., B.D. Moore and D.G. Whittingham. 1965. Development of mouse embryos *in vivo* after cultivation from two-cell ova to blstocysts *in vitro*. Nature. 206, 734-735.
4. Biggers, J.D., D.G. Whittingham and R.P. Donahue. 1967. The pattern of energy metabolism in the mouse oocyte and zygote. Proc. Natl. Acad. Sci. USA. 58, 560-567.
5. Brinster, R.L. 1965. Studies on the development of mouse embryos *in vitro*. IV. Interaction of energy sources. J. Reprod. Fertil. 10, 227-240.
6. Brinster, R.L. and J.L. Thomson. 1966. Development of eight-cell mouse embryos *in vitro*. Exp. Cell Res. 42, 308-315.
7. Brown, J.J.G. and D.G. Whittingham. 1991. The roles of pyruvate, lactate and glucose during preimplantation development of embryos from F_1 hybrid mice *in vitro*. Development 112, 99-105.
8. Brown, J.J.G. and D.G. Whittingham. 1992. The dynamic provision of different energy substrates improves development of one-cell random-bred mouse embryos *in vitro*. J. Reprod. Fertil. 95, 503-511.
9. Chatot, C.L., J.L. Lewis, I. Torres and C.A. Ziomek. 1990. Development of 1-cell embryos from different strains of mice in CZB medium. Biol. Reprod. 42, 432-440.
10. Chatot, C.L., J. Lewis-Williams, I. Torres and C.A. Ziomek. 1994. One-minute exposure of 4-cell mouse embryos to glucose overcomes morula block in CZB medium. Mol. Reprod. Dev. 37, 407-412.
11. Chatot, C.L., C.A. Ziomek, B.D. Bavister, J. L. Lewis and I. Torres. 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. J. Reprod. Fertil. 86, 679-688.
12. Conaghan, J., A.H. Handyside, R.M.L Winston and H.J. Leese. 1993. Effects of pyruvate and glucose on the development of human preimplantation embryos *in vitro*. J. Reprod. Fertil. 99, 87-95.
13. Cross, P.C. and R.L. Brinster. 1973. The sensitivity of one-cell mouse embryos to pyru-

vate and lactate. Exp. Cell. Res. 77, 57-62.
14. Gardner, D.K. and H.J. Leese. 1986. Noninvasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. Human Reprod. 1, 25-27.
15. Gardner, D.K. and H.J. Leese. 1988. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation embryos. Development 104, 423-429.
16. Hsieh, B., N.M.Y. Chi, J. Knor and O.M. Lowry. 1979. Enzymes of glycogen metabolism and related metabolites in preimplantation mouse embryos. Dev. Biol. 72, 342-349.
17. Johnson, M.H., J. McConnel and J. Van Blerkon. 1984. Programmed development in the mouse embryo. J. Embryol. Exp. Morph. 83, Supplement, 197-231.
18. Lawitts, J.A. and J.D. Biggers. 1991. Overcoming the 2-cell block by modifying standard components in amouse embryo culture medium. Biol. Reprod. 45, 245-251.
19. Leese, H.J. and A.M. Barton. 1984. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. J. Reprod. Fertil. 72, 9-13.
20. Martin, K.L. and H.J. Leese. 1995. Role of glucose in mouse preimplantation embryo development. Mol. Reprod. Dev. 436-443.
21. Nasr-Esfahani, M.H., N.J. Winston and M. H. Johnson. 1992. Effects of glucose, glutamine, ethylenediaminetetraacetic acid and oxygen tension on the concentration of reactive species and on development of the mouse preimplantation embryo *in vitro*. J. Reprod. Fertil. 96, 219-231.
22. Rosenkrans, C.F. and N.L. First. 1993. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. J. Ani. Sci. 72, 434-437.
23. Schini, S.A. and B.D. Bavister. 1988. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol Reprod 39, 1183 1192.
24. Seshagiri, P.B. and B.D. Bavister. 1989. Glucose inhibits development of hamster 8-cell embyos *in vitro*. Biol. Reprod. 40, 599-606.
25. Stern, S. and J.D. Biggers. 1968. Enzymatic estimation of glycogen in the cleaving mouse embryo. J. Exp. Zool. 168, 61-66.
26. Takahashi, Y. and N.L. First. 1992. *In vitro* development of bovine one-cell embryos : Influence of glucose, lactate, pyruvate, amino acids & vitamins. Theriogenology 37, 963-978.
27. Wales, R.G. and R.L. Brinster. 1968. The uptake of hexoses by preimplantation mouse embryos *in vitro*. J. Reprod. Fertil. 15, 415-422.