Effect of Exogenous Fatty Acids on *in vitro* Development of Rat Embryos

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ABSTRACT : Studies were made to evaluate the specific and combined effects of different fatty acids on the *in vitro* development of 8-cell rat embryo in culture media with and without carbohydrate substrate. Palmitic, oleic, linoleic and arachidonic acids were added singly and in combination to media which contained fatty acid-free BSA. Cell numbers in blastocysts cultured in the media were counted and compared with cell numbers in blastocysts at the corresponding stage collected from the uterus. Oleic, linoleic and arachidonic acids promoted the rat embryo development from 8-cell to the blastocysts, especially in the absence of carbohydrate substrates. Among these three, oleic acid was the most effective but embryo development was not accelerated by the addition of palmitic acid in either the presence or the absence of carbohydrate substrates. Addition of the mixture of four fatty acids was more effective for rat embryo development than single treatment with any of fatty acids tested. Cell numbers per blastocyst in the presence and absence of carbohydrate substrate were similar, and did not differ from those for blastocysts obtained from the uterus. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 2 : 169-173*)

Key Words : Rat Embryo, Fatty Acid, Embryo Development, *In Vitro* Culture

INTRODUCTION

The oviductal and uterine fluids of mammals are physiologically important for the embryo *in vivo*. For studies on embryo development *in vitro*, the formulation of the culture medium is usually based on the composition of these fluids (Tervit et al., 1972; Quinn et al., 1985; Gardner and Leese, 1990).

We have reported the composition characteristics of several kinds of saturated and unsaturated fatty acids in rat embryos, oviductal and uterine fluids contained several kinds of saturated and unsaturated fatty acids (Khandoker et al., 1997). We have also reported that rat embryo development was severely reduced when fatty acids bound to BSA in the culture medium were removed together with the carbohydrate substrate (Khandoker et al., 1995). Further, our unpublished data show that preimplantation stage rat embryos utilize the exogenous fatty acids as the materials for the synthesis of different lipids needed for the embryo development as well as a source of energy through their oxidation.

In view of the above, the object of this experiment was to evaluate the contribution of fatty acids in the culture medium to the *in vitro* development of 8-cell rat embryos. By the reference to the fatty acid composition of lipids in oviductal and uterine fluids (Khandoker et al., 1997), palmitic, oleic, linoleic and arachidonic acids were selected and supplemented individually or in combination to the culture medium in which fatty acids bound to BSA were removed.

MATERIALS AND METHODS

Animals and embryo collection

Two to three month old Wistar-Imamichi strain rats, weighing 220 to 260 g, were used. They were kept in controlled environmental conditions with room temperature at 21±1℃ and a cycle of 12 h light (lights on at 06:00 h) and 12 h dark. The animals were allowed free access to a commercial pellet diet (Labo MR Breeders; Nihon Nosan Kogyo, Co. Ltd., Yokohama) and tap water at all times. The reproductive state of females was checked by daily vaginal smearing, and on the day of vaginal proestrus females were joined with males overnight. On the following morning, females were checked for the presence of vaginal plugs and/or vaginal spermatozoa, and the day of finding the plug or spermatozoa was designated day 1 of pregnancy. Mated rats were killed by cervical dislocation between 06:00 and 07:00 h on day 4 of pregnancy (about 76 h after ovulation) and 8-cell embryos were collected from the oviducts by flushing. Phosphate buffered saline (PBS) with polyvinyl alcohol (Sigma Chemical Co. Ltd. St. Louis, USA) was used to flush the oviduct. Embryos collected were washed 3-4 times by PBS and appropriate culture media and used in the experiments.

Culture medium

Brinster ovum culture-III (BMOC-III; Brinster, R. L., 1972) was used as the basic culture medium, although BSA essentially free of fatty acid (BSA FAF; Sigma Chemical Co. Ltd. St. Louis, USA) was substituted for fraction V BSA (BSA F-V; Nacalai Chemical Co., Kyoto, Japan). The culture medium was prepared for the experiment with or without carbohydrate substrates. Fatty acids were added to culture media according to the protocol of Lui and Meizel (1977). Extra pure grade preparations of palmitic, oleic, linoleic and arachidonic acids (Sigma Chemical Co. Ltd. St. Louis, USA) were dissolved in hexane separately and added to celite (Sigma Chemical Co. Ltd. St. Louis, USA). Hexane was evaporated, and 100 mg of the celite particles coated with 0.01 mmol fatty acid were added to 2 ml of medium containing 20 mg/ml BSA FAF (<0.005%, Sigma Chemical Co. Ltd. St. Louis, USA). The media containing fatty acid-coated celite particles were vortexed

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for several minutes, incubated at 37℃ for 30 minutes, and left to stand overnight at 4℃ to allow the celite to settle. Supernatant was decanted, sterilized by passage through a cellulose acetate 0.45 μm Millipore filter (Toyo Roshi Co. Ltd. Tokyo, Japan), and the clear solution obtained was kept in cold as stock. Two ml of the stock solution was mixed with 6 ml of culture medium without BSA FAF immediately before use. Final fatty acid concentration was 1.25 mM and the molar ratio of fatty acid to albumin was about 15.7. Control medium was prepared similarly, except that the coating of celite particles with fatty acid was omitted. The medium containing all 4 fatty acids was prepared by incorporating palmitic, oleic, linoleic and arachidonic acids in the ratio by volume of 1.0:1.5:1.0:1.0.

Embryo culture and evaluation of embryo development

Culture of 10 embryos was repeated 4-5 times for each medium. The development of embryos was monitored at 24 and 48 h of culture by observation under a phase contrast microscope, and proportions of embryo developed to morula and blastocyst stages were recorded. Data were presented as mean±SEM for each treatment group. Cell numbers were counted on blastocysts cultured in the media containing fatty acid mixture with and without carbohydrate substrates. Cell numbers of blastocysts that had developed in utero were also counted for comparison.

Statistical analysis

For the statistical analyses, all rates of embryo development were transformed to angles, and comparisons between group means were made by Fisher's protected least significant difference (FPLSD) method following one-way ANOVA by using the STATVIEW computer program (Abacus Concepts, Inc., Berkeley, CA, 1992).

RESULTS

The effects of the addition of palmitic, oleic, linoleic and arachidonic acids singly and as a mixture on the rates of development of 8-cell rat embryos to the morula and blastocyst stages are summarized in tables 1 and 2.

In the presence of carbohydrate the rates of development to both morula and blastocyst were greater in embryos cultured in media with oleic, linoleic and arachidonic acids than in those in the BSA FAF-fatty acid-free medium, although the difference reached significance only with oleic acid (table 1). On the other hand, rates of embryo development were slightly lower in the medium with palmitic acid than in the fatty acid-free medium, but the difference was not significant. Embryo development when the four fatty acids were all added to the culture medium was significantly more rapid than with other groups, except for the group cultured in the medium with added oleic acid.

In a further set of experiments in which carbohydrate substrate was deleted from the culture media similar results were obtained, though the rates of embryo development were generally slower than in cultures with carbohydrate (table 2). The rates of development to the morula and blastocyst were significantly higher in embryos cultured with oleic, linoleic and arachidonic acids when compared with those in the fatty-acid free medium but development was not accelerated by the addition of palmitic acid.

Table 1. Effects of exogenous fatty acids on rat embryo development from 8-cell stage onwards during 48h *in vitro* culture in the presence of carbohydrate substrates

Medium*	Development % (mean±SEM)	
	Morula	Blastocyst
BSA FAF	50.0 ± 4.5^{ac}	30.0 ± 4.5^{ad}
BSA FAF; Palmitic	40.0 ± 4.5^{a}	24.0 ± 5.1^{a}
BSA FAF; Oleic	64.0 ± 5.1^{d}	46.0 ± 5.1^{bc}
BSA FAF; Linoleic	56.0 ± 4.0^{bc}	38.0 ± 3.7^{bd}
BSA FAF; Arachidonic	54.0 ± 5.1^{bc}	36.0 ± 5.1^{bd}
BSA FAF; Mixture	72.0 ± 3.7^{d}	52.0 ± 3.7^{c}

* BSA FAF (5 mg/ml) was substituted for BSA F-V (5 mg/ml) in BMOC-Ⅲ basic medium.
Each medium contained 1.25 mM of palmitic, oleic, linoleic or arachidonic acid or their mixture for which 4 fatty acids were mixed in the ratio of 1.0:1.5:1.0:1.0.
Culture of 10 embryos was repeated 4-5 times for each medium.
Significant difference was found between the means having different alphabet in their superscripts in the same column ($p<0.05$ in FPLSD-test).

Table 2. Effects of exogenous fatty acids on rat embryo development from 8-cell stage onwards during 48h *in vitro* culture in the absence of carbohydrate substrates

Medium*	Development % (mean±SEM)	
	Morula	Blastocyst
BSA FAF	22.0 ± 3.7^{a}	14.0 ± 2.5^{a}
BSA FAF; Palmitic	22.5 ± 2.5^{a}	12.5 ± 2.5^{a}
BSA FAF; Oleic	48.0 ± 3.7^{b}	30.0 ± 6.3^{b}
BSA FAF; Linoleic	44.0 ± 2.5^{b}	24.0 ± 2.5^{b}
BSA FAF; Arachidonic	42.0 ± 3.7^{b}	22.0 ± 3.7^{ab}
BSA FAF; Mixture	64.0 ± 5.1^{c}	48.0 ± 3.7^{c}

* BSA FAF (5 mg/ml) was substituted for BSA F-V (5 mg/ml) in carbohydrate free BMOC-Ⅲ medium.
Each medium contained 1.25 mM of palmitic, oleic, linoleic or arachidonic acid or their mixture for which 4 fatty acids were mixed in the ratio of 1.0:1.5:1.0:1.0.
Culture of 10 embryos was repeated 4-5 times for each medium.
Significant difference was found between the means having different alphabet in their superscripts in the same column ($p<0.05$ in FPLSD-test).

Again, among individual fatty acids, oleic acid was the most effective in promoting embryo development. The order of the effectiveness of individual fatty acids

was the same in the cultures with and without carbohydrate substrates. When all 4 fatty acids were added, development to both morula and blastocyst were promoted more than by any of the individual fatty acids.

The effect of the addition of fatty acid mixture on the cell number of rat blastocysts in the presence and absence of carbohydrate substrates is shown in table 3. Cell numbers per blastocyst in the two groups were similar, and did not differ significantly from those of blastocysts obtained from the uterus at the corresponding stage of embryo development.

Table 3. Cell number of rat blastocysts cultured for 48 h in the medium containing fatty acids with or without carbohydrate substrates

Medium	Carbo-hydrate	Lipid	Cell Number/ Blastocyst (mean ± SEM)
BSA FAF	+	Fatty acid mixture	42.6 ± 2.2
BSA FAF	-	Fatty acid mixture	41.4 ± 1.7
In vivo			46.7 ± 1.9

Blastocysts of the corresponding groups in tables 1 and 2 were used for counting cell number.

DISCUSSION

The development to morula and blastocyst was promoted by the addition of oleic, linoleic and arachidonic acids to culture medium in both the presence and the absence of carbohydrate substrates. The results of the present experiments demonstrated that fatty acids found predominantly in rat oviductal and uterine fluids have a considerably beneficial effect on rat embryo development *in vitro*. This finding is consistent with the results of our previous studies which had demonstrated that fatty acids bound to BSA do have a beneficial effect on *in vitro* rat embryo development (Khandoker et al., 1995). Considering these results together with those of our unpublished data on fatty acid metabolism, the beneficial effect of fatty acids might be explained by their utilization as an energy source, especially in the absence of carbohydrate substrates, and for the synthesis of phospholipids and glycolipids required for the formation of cellular membranes.

When both carbohydrate substrates and fatty acids bound to BSA were removed from the culture medium, embryo development was severely impaired, perhaps owing to a shortage of energy. Brinster (1973) characterized the basic requirements of carbohydrate substrates in the culture medium for embryo development. In the absence of carbohydrate, most of the fatty acids provided either singly or in combination had much greater beneficial effects on rat embryo development than when carbohydrate substrates were present. This result clearly indicates that the primary function of the fatty acids with carbohydrate absent was to act as a source of energy. Kane (1979) also reported that exogenously supplied myristic, palmitic, stearic, oleic and linoleic acids considerable stimulated the growth and development of rabbit embryo by their use as energy source.

How much of the fatty acids were oxidized and used for the generation of energy in developing embryos in the presence of carbohydrate substrates? Glucose and pyruvate are usually used as the major energy substrate for the *in vitro* development of most of mammalian embryo. However, active utilization of glucose through glycolysis and the TCA cycle is reported to start before blastocoel formation in the rat embryo (Sugawara and Takeuchi, 1973). Brison and Leese (1991), using glucose and pyruvate as substrates in the experiments on energy metabolism in rat blastocysts cultured *in vitro*, also found that the utilization of glucose through glycolysis and TCA cycle did not develop to an extent sufficient to explain the amount of ATP generation until the stage of early blastocyst. Consequently, it was supposed that acetyl CoA formed by the β-oxidation of fatty acids and entering the TCA cycle was the cause of this excess amount of ATP generation. If it is correct, the role of fatty acids as energy source is important even in the presence of carbohydrate substrates.

Aside from the role of fatty acids as metabolic energy, their use as materials for the membrane formation and for various bioactive compounds may well play an important part in the embryo development. Our unpublished data show that radionuclide-labelled fatty acid was incorporated both into neutral and phospholipid fractions of embryo lipids. The proliferation and integrity of cellular membranes may be an important controlling factor in the differentiation of embryonic cells. Growth of the embryo requires the assembly of new intracellular and plasma membranes (Calarco and Brown, 1969 and Thompson, G. A., 1989). These changes may explain the increased requirement of fatty acids for phospholipid synthesis in embryo. In the present experiment, an obvious increase in the rate of morula and blastocyst formation was caused by the addition of fatty acids even in the presence of carbohydrate.

Among the three fatty acids which were found to have beneficial effects on the embryo development, the effect of oleic acid was the most prominent. This finding is consistent with the results of Quinn and Whittingham (1982) which showed that oleic acid was a very effective fatty acid for mouse embryo development *in vitro*. It was reported that oleic acid had an important role in elongation and desaturation of fatty acids as a metabolic precursor in human lymphocyte (Anel et al., 1990) and mammalian liver (Jeffcoat, R., 1979). The fatty acid may have a specific role in controlling embryo development because the poly-unsaturated linoleic and arachidonic acids are nutritionally essential (Bailey, J. M., 1980; Ganong, W. F., 1993). Essential fatty acids cannot be synthesized in animal body, but are necessary precursors for a range of hormones that include prostaglandins, leukotriens, thromboxanes and lipoxins. It was reported that linoleic acid functioned as growth factor for Chinese hamster cells in culture (Ham, R. G.,

1963). Moreover, linoleic and arachidonic acids have a specific effect on mitochondrial function in cultured HeLa cells (Gerschenson et al., 1967). When incubated in lipid-free medium, the cells showed consistently an uncoupling of oxidative phosphorylation and a loss of respiratory control. The addition of either linoleic or arachidonic acid partially prevented this impairment.

In the present experiment, no acceleration and indeed a slight suppression of development was observed in embryos cultured with palmitic acid. Palmitic acid, a 16 carbon saturated fatty acid, can be synthesized in mammalian tissues, and its addition in high concentration may disturb the balance of saturated fatty acid in tissue or cells. In this regard, it has been reported that in the culture of skin fibroblasts, triacylglycerol buildup promoted by only one specific saturated fatty acid resulted in damage to the cells (Rosenthal and Geyer, 1978). In addition, reduced growth of mouse LM cells was found from an imbalance in saturated fatty acid contents of membrane phospholipids (Doi et al., 1978). It is possible to speculate, therefore, that accumulation of triacylglycerols or phospholipids of an imbalanced fatty acid composition is responsible for the reduction in embryo growth. Embryos cultured for 48 h with the high concentration of palmitic acid in the present experiment appear to provide evidence of the imbalance effect of the compound.

The mixture of four fatty acids gave more definite beneficial effects on rat embryo development. The combined effect of oleic and palmitic acids on mouse embryo development was reported by Quinn and Whittingham (1982). A balance between saturated and unsaturated fatty acids may be important.

The present study shows definitely that rat embryos are capable of utilizing exogenous fatty acids for their growth and development *in vitro*. The results obtained are useful information for the improvement of culture media for rat embryos in order to enhance the rate and quality of their development *in vitro*.

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REFERENCES

Anel, A., Naval, J., Gonzalez, B., Uriel, J. and Pineiro, A. 1990. Fatty acid metabolism in human lymphocytes. II. Activation of fatty acid desaturase-elongase systems during blastic transformation. Biochim. Biophys. Acta. 1044:332-339.

Bailey, J. M. 1980. Essential fatty acid requirements and metabolism of cells in tissue culture. APTRDI. 6:549-565.

Brinster, R. L. 1972. Cultivation of mammalian egg. In: Rothblat, G. and Christofalo, V. [eds.] Growth, Nutrition and Metabolism of cells in culture, vol. 2, pp. 251-286. Academic Press, New York.

Brinster, R. L. 1973. Nutrition and metabolism of the ovum, zygote and blastocyst. In: Green, R. O. and Astwood, E. B. [eds.] Handbook of Physiology, Section 7, vol.11, pp. 165-185, American Physiological Society, Washington.

Brison, D. R. and Leese, H. J. 1991. Energy metabolism in late preimplantationi rat embryos. J. Reprod. Fertil. 93:245-251.

Calarco, P. G. and Brown, E. A. 1969. An ultrastructural and cytological study of preimplantation development of the mouse. J. Exp. Zool. 171:253-284.

Doi, O., Doi, F., Schroeder, F., Alberts, A. W. and Vagelos, P. R. 1978. Manipulation of fatty acid composition of membrane phospholipid and its effect on cell growth in mouse LM cells. Biochim. Biophys. Acta. 509:239-250.

Ganong, W. F. 1993. Essential fatty acids. In: Dolan, J. and Langan, C. [eds.] Review of Medical Physiology, Energy balance, metabolism and nutrition, pp. 253-286. Appleton and Lange, Norwalk, Connecticut.

Gardner, D. K. and Leese, H. J. 1990. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism *in vitro*. J. Reprod. Fertil. 88:361-368.

Gerschenson, L. E., Harary, I. and Mead, J. F. 1967. Studies in vitro on single beating rat-heart cells. X. The effect of linoleic and palmitic acids on beating and mitochondrial phosphorylation. Biochim. Biophys. Acta. 131:50-58.

Ham, R. G. 1963. Albumin replacement by fatty acids. Science. 140:802-803.

Jeffcoat, R. 1979. The biosynthesis of unsaturated fatty acids and its control in mammalian liver. Essays Biochem. 15:1-36.

Kane, M. T. 1979. Fatty acids as energy sources for culture of one-cell rabbit ova to viable morulae. Biol. Reprod. 20: 323-333.

Khandoker, M. A. M. Y., Nishioka M. and Tsujii, H. 1995. Effect of BSA binding fatty acids on mouse and rat embryo development. J. Mamm. Ova Res. 12:113-118.

Khandoker, M. A. M. Y., Tsujii, H. and Karasawa, D. 1997. Fatty acid composition of blood serum, oocytes, embryos and reproductive tract fluids of rat and comparison with BSA. Anim. Sci. Techol. 68:1070-1074.

Lui, C. W. and Meizel, S. 1977. Biochemical studies of the *in vitro* acrosome reaction inducing activity of bovine serum albumin. Differentia. 9:59-66.

Purdon, A. D. and Rao, A. K. 1989. Interaction of albumin, arachidonic acid and prostaglandins in platelets. Prostaglandins-Leukotrienes-Essential fatty Acids. 35:213-218.

Quinn, P. and Whittingham, D. G. 1982. Effect of fatty acids on fertilization and development of mouse embryos *in vitro*. J. Androl. 3:660-666.

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

Reichman, H., Maltese, W. A. and DeVivo, D. C. 1988. Enzymes of fatty beta-oxidation in developing brain. J. Neurochem. 51:339-344.

Rosenthal, M. D. and Geyer, R. P. 1978. Incorporation of moderate and excessive levels of fatty acids by human skin fibroblasts. Fed. Proc. 37:1832 (Abstr.).

Stanke, D. F., Sikes. J. D., DeYoung, D. W. and Tumbleson, M. E. 1974. Proteins and amino acids in bovine oviductal

fluid. J. Reprod. Fertil. 38:493-496.

Sugawara, S. and Takeuchi, S. 1973. On glycolysis in rat eggs during preimplantation stages. Tohoku J. Agric. Res. 12:17-28.

Telford, N. A., Watson, A. J. and Schultz, G. A. 1990. Transition from maternal to embryonic control in early mammalian development : a comparison of several species. Mol. Reprod. Dev. 26:90-100.

Tervit, H. R., Whittingham, D. G, and Rowson, L. E. A. 1972. Successful culture *in vitro* of sheep and cattle ova. J Reprod. Fertil. 30:493-497.

Thompson, G. A. 1989. Lipid molecular species retailoring and membrane fluidity. Trans. Biochem. Soc. 17:286-289.