A Kinetic Study of Fatty Acid Composition of Embryos, Oviductal and Uterine Fluids in the Rabbit

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ABSTRACT : The different developmental stage embryos and oviductal and uterine fluids of rabbit were analyzed by gas chromatography. Myristic (C 14 : 0), palmitic (C 16 : 0), palmitoleic (C 16 : 1), stearic (C 18 : 0), oleic (C 18 : 1), linoleic (C 18 : 2), linolenic (C 18 : 3), arachidic (C 20 : 0), arachidonic (C 20 : 4), docosahexaenoic (C : 22 : 6) and lignoceric (C 24 : 0) acids were the common fatty acid constituents with little exception. In most of the samples palmitic, oleic, linoleic and arachidonic acids were observed in high concentration. Moreover, linoleic, linolenic and arachidonic acids were the three poly-unsaturated fatty acids in both type sample except day-1 oviductal fluids. Similarly, in both day-1 and day-2 oviductal and uterine fluids myristic, palmitoleic, stearic, linolenic, arachidic and docosahexaenoic acids were in less composition or undetected.
(Key Words: Rabbit, Fatty Acid, Embryo, Oviductal Fluid, Uterine Fluid)

INTRODUCTION

Lipids not only provide a source of nutrient to the cell, but also play a vital role in modifying the physical properties and functions of biological membranes, and have potential effects on cell-cell interactions, cell proliferation and transport (Stubbs and Smith, 1984). Moreover, the activities of membrane proteins depends on their immediate lipid environment, and the ability of hormone receptor complexes to bind to the effector molecules may be modulated by the headgroup and fatty acid composition of membrane lipids (Hirata et al., 1979 and Galo et al., 1981). The principal component, associated with most lipids are fatty acids and usually they are esterified to glycerol and glycerophospholipids. The importance of fatty acids in maintenance of normal cell function is therefore unequivocal.

It was reported that exogenously supplied fatty acids are beneficial for growth and continued development of rabbit ova (Kane M. T., 1979 and Kane and Headon 1980). Recent studies revealed that BSA binding fatty acids had considerable effect on mouse and rat (Khandoker et al., 1995) and cow (Ohboshi et al., 1996) embryo development. Moreover, mouse ova can synthesize sterols and lipids (Pratt H. P. M., 1978) and metabolize fatty acids (Flynn and Hillman, 1980). Bovine serum albumin (BSA) is commonly used as the macromolecular component for culturing mammalian eggs. The information in the literature on the binding of low molecular weight compounds to albumin indicates that commercial BSA is a heterogeneous and ill-defined product and normal BSA contains several saturated and unsaturated fatty acids (Kane and Headon, 1980; Menezo et al., 1982 and Khandoker et al., 1995) and it is reasonable to assume that albumin in culture media and different reproductive fluids *in vivo* supplies different fatty acids. The oviductal and uterine fluids of mammals are of physiological interest, because they provide environment to the embryo *in vivo*. The culture media is important for *in vitro* embryo development (Bavister, 1995) and usually devised based on the composition of reproductive tract fluids (Stubbs and Smith, 1984 and Quinn et al., 1985).

Data concerning amino acids, proteins, RNA, DNA, sugars and trace elements are available for numerous species. Although a number of reports have documented in lipid analysis, roles and metabolism in rabbit (James R. B., 1973; Kane M. T., 1979; Kane and Headon, 1980 and Khandoker et al. 1996) but few have presented an extensive biochemical lipid profile. Therefore, the aim of the present study was to use Gas chromatography to identify the methylated products from embryos (one-cell, morula, day 7 and 9 late blastocyst) and the oviductal and uterine fluid lipid extracts of rabbit.

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MATERIALS AND METHODS

Sample collection

Reproductive ducts were collected from superovulated Japanese White does which were maintained singly in metal cages. Superovulation was performed according to the method of Hafez (1970), 50 iu pregnant mare serum gonadotrophin (PMSG) was injected subcutaneously for 3 days followed by 1 day later simultaneous intravenous injection of 50 iu human chronic gonadotrophin (hCG). Immediately after the injection of hCG, the animals were allowed to mate with two fertile bucks. One-cell, early morula and late blastocysts (day 7 and 9) were collected for this experiment. At the specific stage the donors were sacrificed by bleeding, then the reproductive ducts were collected and dissected free of other tissues and rinsed thoroughly in 0.9% (w/v) NaCl, dried on a paper towel and the ovary, oviducts and uterus were separated. Phosphate buffered saline (PBS; supplemented with 1 mg/ml polyvinyl alcohol; Sigma Chemical Co., St. louis, USA) was used to flush the embryos from the reproductive tract. One cell and early morulae were collected, 20-21 h and 44-48 h after hCG injection, respectively. They were washed to remove oviductal debris and the first washing of 1-cells and morula was collected as day-1 and day-2 oviductal fluid, respectively.

The uterus was also flushed with PBS and the uterine fluids of both 1-cell and morula stages were collected. The oviductal and uterine fluids were then filtered, using 75 μm filter paper (Toyo Roshi Co Ltd. Tokyo, Japan). In order to collect the day 7 and 9 late blastocysts, the uterus was cut at appropriate day of pregnancy and a strip of the uterine wall was peeled off with a fine forceps. Then the embryos were taken in a test tube without any fluid. Three hundred 1-cell, 200 morulae, 10-15 each of day 7 and 9 late blastocysts were collected and pooled for each sample. The samples were stored at -20℃ until further preparation for analysis.

Sample Preparation

Lipid extraction was carried out with chloroform and methanol according to the method of Bligh & Dyer (1959). Each sample is homogenized with a mixture of chloroform and methanol (1:2 ratio). To the mixture is then added more 1 unit chloroform and after mixing, 1 unit distilled water is added and homogenized again. The homogenate is filtered through filter paper (Toyo Roshi Co Ltd. Tokyo, Japan). The filtrate is transferred to a graduated cylinder, and, after allowing a few minutes for complete separation and clarification, the alcoholic layer removed by aspiration. A small volume of the chloroform layer is also removed to ensure complete removal of the top layer. The chloroform layer contains the purified lipid. Lipid extracts was then evaporated to dryness by vacuum aspiration and weighed. The fatty acid methylation was performed according to the method described by Luddy et al. (1968). Briefly, 1 ml benzene and 1 ml sodium methoxide (GL Science, Tokyo, Japan) were added for each 10-30 mg total lipid extract in a stoppered centrifuge tube and the tube was heated to 80℃ and maintained for 15-20 min in a water bath for complete methylation. It was then cooled at room temperature, and 3 ml each of diethyl ether and distilled water were added and mixed well. The lower aqueous layer was removed and the top solvent layer was washed more than two times with 2-3 ml distilled water and dried overnight by adding anhydrous Na_2SO_4 at 4℃. After drying by vacuum aspiration, the residue containing the fatty acid methyl esters were dissolved in ethyl acetate (5 mg/ml) and used for gas chromatographic analysis.

Gas chromatography

The fatty acid analysis was carried out as described else where (Khandoker et al., 1998). Briefly, the composition of fatty acid was analyzed by a GC-14 A gas chromatograph (Shimadzu Co., Ltd. Kyoto, Japan) equipped with a capillary column (liquid phase, film thickness 0.25 μm, 30 m long and 0.25 mm i. d; split ratio 1/100; GL. Science, Tokyo, Japan). The chromatograms were recorded on a data processing recorder (Chromatopac, C-R 1 B, Shimadzu, Kyoto, Japan). The initial column temperature was, 150℃ and final 210℃, with helium as the carrier gas at a constant flow rate of 25 ml/min. Individual fatty acids were identified on the basis of their retention times with that of authentic standards (Sigma Chemical Co., St. louis, USA).

Statistical Analysis

Fatty acid peaks determined by gas chromatography were expressed as a percentage of the total lipid components (Slover and Lanza, 1979). Percentage area composition data were arcsine transformed prior to statistical assessment. Comparisons between samples were made using Student's t test. All values presented in the tables are mean ± SEM of the nontransformed data.

RESULTS

The fatty acid profiles of rabbit embryos of different developmental stages are shown in table 1. Myristic (C 14:0), palmitic (C 16:0), palmitoleic (C 16:1), stearic (C 18:0), oleic (C 18:1), linoleic (C 18:2), linolenic (C 18:3),

Table 1. Fatty acid constituents and relative compositions of different developmental stage rabbit embryos

Fatty acid	Relative percentage of fatty acid in methylated sample			
	One-cell	Morula	Blastocyst (d-7)	Blastocyst (d-9)
Myristic	1.31 ± 0.12^{a}	8.26 ± 0.16^{b}	1.65 ± 0.13^{ac}	1.91 ± 0.05^{a}
Palmitic	2.89 ± 0.13^{a}	12.25 ± 0.15^{b}	16.62 ± 2.85^{c}	18.41 ± 0.30^{c}
Palmitoleic	1.32 ± 0.22^{a}	4.25 ± 0.13^{b}	3.82 ± 0.16^{b}	3.16 ± 0.15^{c}
Stearic	5.38 ± 0.23^{a}	1.16 ± 0.16^{b}	3.00 ± 0.29^{c}	3.40 ± 0.23^{c}
Oleic	5.96 ± 0.03^{a}	18.34 ± 0.16^{b}	20.51 ± 0.29^{c}	30.49 ± 0.32^{d}
Linoleic	8.02 ± 0.21^{a}	9.51 ± 0.36^{b}	12.15 ± 0.13^{c}	16.50 ± 0.29^{d}
Linolenic	1.66 ± 0.15^{a}	32.99 ± 0.49^{b}	3.55 ± 0.29^{c}	2.11 ± 0.25^{a}
Arachidic	8.66 ± 0.15^{a}	1.66 ± 0.09^{b}	15.49 ± 0.28^{c}	9.49 ± 0.30^{a}
Arachidonic	28.23 ± 0.15^{a}	6.63 ± 0.15^{b}	9.43 ± 0.32^{c}	8.60 ± 0.15^{c}
Docosahexaenoic	9.64 ± 0.14^{a}	2.80 ± 0.15^{b}	3.66 ± 0.15^{c}	1.60 ± 0.19^{d}
Lignoceric	26.50 ± 0.29^{a}	1.03 ± 0.15^{b}	8.80 ± 0.44^{c}	3.23 ± 0.15^{d}

Values are the means ± SEM of three analyses and are expressed as a % of total fatty acid.
^{a,b,c,d} : Means with different superscripts within the same row differ significantly ($p < 0.05$).

Table 2. Fatty acid constituents and relative compositions of rabbit oviductal and uterine fluids of day-1 and day-2 pregnancy

Fatty acid	Relative percentage of fatty acid in methylated sample			
	Oviductal fluid[1]	Uterine fluid[1]	Oviductal fluid[2]	Uterine fluid[2]
Myristic	1.61 ± 0.06^{a}	1.64 ± 0.10^{a}	1.43 ± 0.30^{a}	4.93 ± 0.13^{b}
Palmitic	23.49 ± 0.30^{a}	21.24 ± 0.50^{b}	18.53 ± 0.40^{c}	13.40 ± 0.20^{d}
Palmitoleic	6.29 ± 0.01^{a}	3.53 ± 0.15^{b}	3.15 ± 0.15^{c}	2.80 ± 0.20^{c}
Stearic	3.60 ± 0.13^{a}	9.14 ± 0.20^{b}	4.32 ± 0.20^{c}	5.61 ± 0.20^{d}
Oleic	32.30 ± 0.51^{a}	33.62 ± 0.20^{a}	13.23 ± 0.20^{b}	14.66 ± 0.20^{c}
Linoleic	24.90 ± 0.13^{a}	22.96 ± 0.02^{b}	8.42 ± 0.26^{c}	6.11 ± 0.06^{d}
Linolenic	6.90 ± 0.46^{a}	5.34 ± 0.24^{b}	6.61 ± 0.21^{ac}	2.14 ± 0.14^{d}
Arachidic	UD	UD	5.34 ± 0.22^{a}	3.33 ± 0.16^{b}
Arachidonic	UD	1.54 ± 0.24^{a}	18.64 ± 0.13^{b}	25.44 ± 0.31^{c}
Docosahexaenoic	UD	UD	8.42 ± 0.23	8.60 ± 0.23
Lignoceric	UD	UD	11.44 ± 0.64	11.98 ± 1.02

The superscripts 1 and 2 indicated day-1 and day-2 of pregnancy, respectively.
Values are the means ± SEM of three analyses and are expressed as a % of the total fatty acid. UD = Undetectable.
^{a,b,c,d} : Means with different superscripts within the same row differ significantly ($p < 0.05$).

arachidic (C 20 : 0), arachidonic (C 20 : 4), docosahexaenoic (C 22 : 6) and lignoceric (C 24 : 0) acids were the common fatty acids detected in all samples. In 1-cell stage embryo, arachidonic acid composition was the highest, followed by lignoceric acid (both are significantly different from others; $p < 0.05$). The composition of docosahexaenoic, arachidic, linoleic, oleic and stearic acids were in medium level (5.38 to 9.64%). On the other hand, myristic, palmitoleic and linolenic acid composition were comparatively lower (1.31 to 1.66%). In morula, linolenic acid composition was the highest, followed by oleic and palmitic acid (all differ significantly from others; $p < 0.05$). The myristic, linoleic and arachidonic acid composition were moderate (7.63 to 9.51%). But, palmitoleic, stearic, arachidic, docosahexaenoic and lignoceric acid composition was less (1.03 to 4.25%). In both day 7 and day 9 late blastocysts, the oleic acid composition was the highest (significantly different from others; $p < 0.05$), followed by palmitic and either linoleic or arachidic acid (all differ significantly from each others except palmitic acid; $p < 0.05$). The other fatty acids were less or moderate (1.60 to 9.43 %) in both samples.

In reproductive tract fluids the fatty acid content was almost similar to that of embryos, but there was

difference in the composition (table 2). In day-1 oviductal and uterine fluids oleic acid composition was the highest (not differ significantly from each others; $p < 0.05$), followed by linoleic and palmitic acid (both are differ significantly; $p < 0.05$). The composition of palmitoleic and linolenic acids were in moderate level (3.53 to 6.90%). Where as, the compositions of other fatty acids were very low or not detected. In day-2 stage, the long chain fatty acids of carbons 20, 22 and 24 were detected and interestingly the level of arachidonic acid was the highest in both oviductal and uterine fluids (differ significantly; $p < 0.05$). These was followed either palmitic or oleic acids (both are differ significantly; $p < 0.05$). The level of other fatty acids were in moderate or less compositions.

Linoleic, linolenic and arachidonic acids were the three poly-unsaturated fatty acid detected in all samples analyzed, except day-1 oviductal fluid. The palmitic acid composition increased gradually from 1-cell to day 9 blastocysts and showed a decreasing trend from oviductal to uterine fluids of both stages although no direct comparison has been made between samples.

DISCUSSION

The results of this study provide a detailed fatty acid compositions of different developmental stage embryos and reproductive fluids of rabbit. The common constituents of fatty acid in 1-cell to day 9 blastocysts and oviductal and uterine fluids indicated that the fatty acids may be originated from the same source, the blood (Oliphant et al., 1984) and these results further confirm this statement. There was a difference in the composition of fatty acids content (%) but the number of fatty acids detected remain constant in all these samples analyzed except one cell stage reproductive tract fluids, which can be attributed to the similar fatty acid requirement for embryo development. While the difference in the percentage of fatty acids indicated that the secretion and transudation process may be different (Leese, 1988).

Oleic, linoleic and palmitic acids were observed in descending order of abundance in day 1 of reproductive tract fluids. Similarly, arachidonic, palmitic and oleic acids were observed in descending order of abundance in day 2 of the same. These findings shows a close similarity with our previous report (Khandoker et al., 1998). Moreover, it was reported that oleic, palmitic and linoleic acids are capable of supporting growth of 1-cell rabbit ova to viable morulae (Kane M. T., 1979). So, also, the combination of palmitic and oleic acid had synergetic effect in mouse embryo development (Quinn and Whittingham, 1982). The higher composition of palmitic and oleic acids in the reproductive fluids and embryos may serve as a storage pool of metabolic precursors, as both these fatty acids are reported to be essential for fatty acid elongation and desaturation in embryo development (Jeffcoat R., 1979). The three poly-unsaturated fatty acids (linoleic, linolenic and arachidonic acids) were detected in all except day-1 oviductal fluid samples, which corroborate well with the results of Yao et al. (1980) and Khandoker et al. (1998). Moreover, these three fatty acids are nutritionally essential (McDonald et al., 1973) and the role of arachidonic acid as the precursor for the biosynthesis of prostaglandin have been implicated in ovarian function (Kennedy et al., 1993).

The arachidonic acid composition was high in one cell and less in morulae to day 9 blastocysts and correspondingly undetected or very less in day-1 but higher in day-2, reproductive tract fluids (table 1 & 2). So, it is possible to speculate that the higher endogenous embryonic arachidonic acid shows lesser requirement exogenously and vise versa. Kane (1979) observed that arachidonic acid was unable to support the growth of 1-cell rabbit ova to viable morulae. The absence or low percentage of myristic, palmitoleic, stearic, linolenic, arachidic and docosahexaenoic acids in both day-1 and day-2 reproductive tract fluids were in concurrence with the previous studies (Yao et al., 1980; Khandoker et al., 1998). Thus it may be due to the less importance of these fatty acids for the congenial environment of reproductive organ. The palmitic acid composition increased gradually from 1-cell to day 9 embryo and followed decreasing trend from oviductal to uterine fluids (table 1 & 2). On the basis of this results, we hypothesize that changes in fatty acid requirement occur during the early preimplantation development *in vitro* but need more systematic investigation.

Determination of the biochemical needs of the embryo and an examination of its environment *in vivo* have equal merit in designing defined embryo culture system (Thompsom, 1996). So, the results of this report could assist in the development of a defined culture medium for rabbit which would enhance the quality and speed of embryo development *in vitro*.

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