Effect of BSA Binding Fatty Acids on Mouse and Rat Embryo Development

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Abstract: *The fatty acid content of bovine serum albumin fraction-V (BSA-V) was analysed and the effect of fatty acids bounded with BSA was evaluated. Gas-liquid chromatography was employed to analyse the fatty acids contents and culture experiments were conducted to evaluate the effect of fatty acids on 4-cell mouse and 8-cell rat embryo development. Linoleic acid was identified to be the major fatty acid (54.55%) bound with BSA, followed by oleic(25.8%), stearic (12.27%) and linolenic(7.36%) acids. BSA binding fatty acids had considerable effect on both mouse and rat embryo development in general but they provided only the rat embryo with energy. The combined effect of different kind of fatty acids (chemically defined lipid concentrate) was more effective in both mouse and rat embryo development.*

Key words: *Mouse and rat embryo, Bovine serum albumin (BSA), Energy free medium, Fatty acid, Chemically defined lipid concentrate.*

The mammalian embryo undergoes qualitative and quantitative changes in energy substrate utilization during the preimplantation period. The mouse embryo, exhibits a characteristic switch in substrate preference; from pyruvate during the early cleavage stage, to glucose after compaction [4, 18]. The same pattern is exhibited though less markedly, by the human preimplantation embryo [12, 13]. The pattern of glucose and pyruvate utilization in rat embryos is quantitatively similar to that observed in mice [9]. The utilization of carbohydrate and protein in oocytes and embryos and also in maternal tissues have been extensively investigated [3, 19]. The studies on the effect of lipid on the same are much more scanty. Crystallized bovine serum albumin (BSA) is commonly used as the macromolecular component in media used for the culture of mammalian eggs. The information in the literature on the binding of low molecular weight compounds to albumin indicate that commercial BSA is an heterogenous and ill-defined product [17]. One-cell rabbit embryos would grow to blastocysts in a complex medium containing 1.5% BSA, amino acids and vitamins, but without carbohydrate type of energy sources [14]. In this situation, either fatty acids bound to the albumin [6, 11, 24], other albumin bound material or the amino acids in the medium might be acting as energy sources. It has been shown that during cleavage, mouse ova synthesize sterols and lipids [25], metabolize fatty acids [8]. Exogenously supplied fatty acids are beneficial for growth and continued development of rabbit ova [16, 17]. Kane [16] indicated that 1-cell rabbit ova grew up to viable morulae in a simple salt solution with normal BSA but in case of defatted BSA this did not happen. This finding indicated that fatty acids might function as energy source for rabbit ova. The involvement of lipid compound in fertilization and early development *in vivo* is probably regulated by endogenous lipids in the fluid of the reproductive tract, but systematic investigation of their role requires *in vitro* studies. Consequently, the study on the fatty acid analysis of BSA-V and the effect of BSA binding fatty acids in mouse and rat embryo development is important.

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Materials and Methods

Fatty acid analysis of BSA: Five gram BSA-V (Nacalai Tesque Inc., Kyoto, Japan) were extracted with diethyl ether. Total lipid extracts were evaporated to dryness under oxygen free nitrogen and added 1 ml benzene and 1 ml of 0.5 N sodium methoxide (GL science Inc., Japan) for each 10–30 mg extract in a vial. Sealed and heated the vial in a bath at 80°C for 15–20 min for methylation. Allowed the vial to cool to room temperature, added 3 ml of water and 3 ml of diethyl ether, and mixed the liquid well. Removed the lower aqueous layer

and washed the top benzene ether layer for 2 times with 2–3 ml water and dried over anhydrous Na_2SO_4. Final evaporation were performed and methylated fatty acids were dissolve in ethyl acetate and Gas-liquid chromatography were performed.

Medium: The medium used for mouse embryo culture was Whittingham's M16 [28]. It contains carbohydrate type of energy sources or none (glucose, pyruvate and lactate) depending on the experimental requirement. The medium also contains 4 mg/ml either fraction V-BSA (BSA-V; No. 012-02, Nacalai Tesque Inc, Kyoto, Japan), essentially fatty acid free BSA (BSA-FAF; <0.02% No. 012-66, Nacalai Tesque Inc, Kyoto, Japan or <0.005% No. A6003, Sigma Chemical Co., St. Louis), or 1 mg/ml polyvinyl alcohol (No. P-8136, Sigma Chemical Co., St. Louis). The available evidence suggests that BSA-FAF is essentially similar to BSA-V, except for its lower fatty acid content [6, 17]. The medium used for rat embryo culture was BMOC-III. BSA or PVA preparations of BMOC-III is similar to that of M16 except the BSA content of the former is 5 mg/ml. In the culture experiment, 1% chemically defined lipid concentrate (No. 11905-015, Gibco BRL, Life Technologies Inc., Grand Island, N.Y., USA) were directly added to the medium. The fatty acid content of lipid concentrate were myristic, stearic, palmitic, palmitoleic, oleic and linoleic acid, 10 mg/ml in all cases. In arachidonic acid, it was 2 mg/ml.

Collection and culture of embryo: Embryos were collected from those animals, maintained under controlled lighting conditions (12 h light: 12 h darkness; lights on at 06•00h). Four-cell mouse embryos were collected from 4–6 week old ICR female mice, which had been superovulated by intraperitoneal injection of 5IU pregnant mare serum gonadotrophin (PMSG) followed 46–50 h later by 5 IU human chorionic gonadotrophin (hCG). After hCG injection the females were placed with fertile males of the same strain, and checked the following morning for copulation plugs (day 1 of pregnancy). Mated mice were killed by cervical dislocation between 04•00 and 05•00h on day 3 of pregnancy (60 h after hCG) and 4-cell embryos were recovered by flushing oviducts with specific treatment medium. Embryos were washed three times, transferred (10–20 embryos) into 100 μl of the same treatment medium and cultured under 5% CO_2 in air at 37°C for 48 h. Each culture medium had previously been covered with mineral oil (E.R. Squibb & Sons, Inc., Princeton, NJ) in 35 mm non-treated polystyrene culture dish and equilibrated with the gas phase and temperature in a CO_2 incubator for 3–5 h. Each medium had a pH of 7.2–7.4 after equilibration. Rat embryos were collected from sexually mature female Wistar rats (2–3 month old) which had been maintained as mouse. At proestrous, which was assessed by examination of vaginal smears, they were naturally mated overnight with male of the same strain. On the following morning, rats were examined for the presence of vaginal plugs or spermatozoa in the vagina (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, washed and cultured as mouse. In this case, 10–15 embryos were transferred into 400 μl of the treatment medium. The culture dish were 60 mm and each medium had a pH of 7.4 after equilibration.

Evaluation of embryo development: Stage prior to 4-cell in mouse and 8-cell in rat were not studied due to avoid *in vitro* block in mouse and rat respectively. Development of embryos were assessed at 24 and 48 h after culture under a phasecontrast microscope. Experiments were replicated for 3–4 times in each and data was analyzed by chi-square analysis to determine the difference between experimental groups.

Results

Fatty acid content of BSA-V is shown in Table 1. It is evident that linoleic acid (54.55%) was the major fatty acid of BSA-V, followed by oleic acid (25.80%). Stearic acid (12.27%) was the only saturated fatty acid content of BSA-V. It also contained less amount of linolenic acid (7.36%).

In the first and second culture experiments, the ability of the various BSA and PVA to support development of embryos to the blastocyst stage, in the absence of extra lipid concentrate were tested. The results are shown in Tables 2 and 3. Development of embryos in complete medium up to blastocyst were higher with BSA-V, followed by BSA-FAF and PVA in both mouse and rat (Table 2). In case of mouse, the difference between the development up to blastocyst with BSA-V and BSA-FAF were not significant ($P<0.05$). On the other hand,

Table 1. The fatty acid composition of BSA-V (chromatographic results)

	Fatty acids			
	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Linolenic (C18:3)
Area (%)	12.27	25.80	54.55	7.36

Table 2. Development of mouse and rat embryos in complete medium containing different BSA or PVA

	Treatment BSA/PVA	No. of Embryos	No. of Morulae (%)	No. of Blastocyst (%)
Mouse	F-V	45	42 (93.3)	38 (84.4)[a]
	FAF (<0.02)	50	45 (90.0)	41 (82.0)[ab]
	FAF (<0.005)	64	61 (95.3)	53 (82.8)[a]
	PVA	40	36 (90.0)	26 (65.0)[b]
Rat	F-V	53	47 (88.7)[a]	36 (67.9)[a]
	FAF (<0.02)	45	26 (57.8)[b]	21 (46.7)[b]
	FAF (<0.005)	34	23 (67.6)[b]	13 (38.2)[b]
	PVA	34	18 (52.9)[b]	14 (41.2)[b]

[a,b]: Superscript values within the same column differ significantly from each other (P<0.05).

Table 3. Development of mouse and rat embryos in energy free medium containing different BSA or PVA

	Treatment BSA/PVA	No. of Embryos	No. of Morulae (%)	No. of Blastocyst (%)
Mouse	F-V	52	35 (67.3)[a]	17 (32.7)[a*]
	FAF (<0.02)	51	24 (47.1)[b]	0 (0.0)[b]
	PVA	46	36 (78.3)[a]	0 (0.0)[b]
Rat	F-V	43	33 (76.7)[a]	27 (62.8)[a]
	FAF (<0.02)	51	19 (37.3)[b]	11 (21.6)[b]
	PVA	32	0 (0.0)[c]	0 (0.0)[c]

[a,b,c]: Superscript values within the same column differ significantly from each other (P<0.05). *: early blastocyst (unable to form blastocyst cavity).

Table 4. Effect of lipid concentrate on rat embryo development in energy free medium

Treatment BSA/PVA	Lipid	No. of Embryos	No. of Morulae (%)	No. of Blastocyst (%)
F-V	−	43	33 (76.7)[a]	27 (62.8)[a]
	+	41	30 (73.2)[a]	28 (68.3)[a]
FAF (<0.02)	−	51	19 (37.3)[b]	11 (21.6)[b]
	+	49	41 (83.7)[a]	34 (69.4)[a]
PVA	−	32	0 (0.0)[c]	0 (0.0)[c]
	+	34	0 (0.0)[c]	0 (0.0)[c]

[a,b,c]: Superscript values within the same column differ significantly from each other (P<0.05).

in rat the difference were significant (P<0.05). In carbohydrate type energy free medium only 32.7% mouse embryos were developed to early blastocyst (unable to form blastocyst cavity) with BSA-V, but with BSA-FAF and PVA the development never reached up to that stage (Table 3). On the other hand, in case of rat both BSA-V and BSA-FAF supported up to blastocyst stage. Number of embryos developed up to blastocyst were 62.8 and 21.6% respectively with the above two treatments and the difference among them are significant (P<0.05). With PVA the embryos although developed up to morulae stage in mouse but, did not develop up to morulae stage at all in case of rat.

The rates of development and the number of blastocyst containing the various BSA and extra lipid concentrate are shown in Tables 4 and 5. The effect of lipid concentrate on rat embryo development without carbohydrate type energy sources medium are shown in Table 4. Blastocysts development were higher with lipid (68.3%) than control (62.8%) in treatment BSA-V but the difference were not significant. In treatment BSA-FAF blastocysts development were significantly higher (P<0.05) with lipid (69.4%) than the control (21.6%). In treatment PVA, the embryos did not develop up to morulae stage at all in both lipid and control groups. In case of mouse, embryos did not develop at all without carbohydrate type of energy supplement (data not shown). In complete medium, the effect of lipid concentrate on mouse and rat embryo development are shown in Table 5. In all treatment groups blastocysts development were higher with lipid concentrate than control in both mouse and rat, except in the treatment with PVA in mouse. However, the difference were not significant except in the treatment BSA-V in mouse (P<0.05).

Discussion

The major fatty acid of BSA-V was linoleic acid, followed by oleic, stearic and linolenic acid. It has been

Table 5. Effect of lipid concentrate on mouse and rat embryo development in complete medium

	Treatment		No. of	No. of	No. of
	BSA/PVA	Lipid	Embryos	Morulae (%)	Blastocyst (%)
Mouse	F-V	−	45	42 (93.3)[a]	38 (84.4)[a]
		+	59	59 (100.0)[b]	59 (100.0)[b]
	FAF(<0.02)	−	50	45 (90.0)[a]	41 (72.0)[ac]
		+	48	45 (93.8)[ab]	41 (85.4)[a]
	PVA	−	40	36 (90.0)[a]	26 (65.0)[cd]
		+	39	37 (94.9)[ab]	20 (51.3)[d]
Rat	F-V	−	53	47 (88.7)[ad]	36 (67.9)[a]
		+	46	43 (93.5)[a]	32 (69.6)[a]
	FAF (<0.02)	−	45	26 (57.8)[b]	21 (46.7)[b]
		+	48	37 (77.1)[cd]	28 (58.3)[ab]
	PVA	−	34	18 (52.9)[b]	14 (41.2)[b]
		+	37	23 (62.2)[bc]	19 (51.4)[ab]

[a,b,c,d]: Superscript values within the same column differ significantly from each other ($P<0.05$).

reported that certain cells in tissue culture have an essential requirement for certain unsaturated fatty acids for growth [7, 10, 20]. It is established that linoleic acid is essentially important for most of the mammals. So, BSA binding linoleic acid might fulfil the essentiality of linoleic acid requirement, because BSA is generally used for embryo culture. Oleic acid composition was also found to be higher in BSA-V. This results, supported by Peters [24], stated that oleic and palmitic acid is among those normally found in highest amounts bound to BSA. But we did not found the presence of palmitic acid. This may be due to the difference of BSA, we analysed in our experiment with that of Peters [24]. The findings of our analysed fatty acid composition is also supported by Arther [1], who stated that lipid are present in serum as albumin-bound free fatty acids (FFA) and as a component of lipoproteins. The organic anion binding site of albumin are composed of two parts, a pocket lined with nonpolar amino acid side chains and a cationic group located at or near the surface of the pocket. FFA binding involves hydrophobic interactions with the hydrocarbon chain and electrostatic interactions with the carboxylate anion.

Our culture experiment demonstrated two points clearly. The first one is that, commercial BSA-V had the effects on the growth of both mouse and rat embryos to blastocysts in culture (Table 2). The second point is BSA binding fatty acids had considerable effect on mouse and rat embryo development in general, as well as had the energy value in rat embryo development in particular. Without carbohydrate type of energy sources BSA binding fatty acids are unable to form blastocysts cavity in mouse (Table 3). This result demonstrated that energy utilization pattern in mouse and rat might be different. Consequently, it is interesting to know the general patterns of energy metabolism operating in embryos of the rat and other mammals. Evidence from the effects of metabolic inhibitors on 1-cell rabbit ova in culture indicated that even at the 1-cell stage there is present an active tricarboxylic cycle and an oxidative phosphorylation system which are essential for ovum growth, but the glucose utilization does not appear to become essential until the onset of blastulation [15]. This picture might be common to other species as well. It is interesting to speculate as to the origin of such a pattern. The mammalian ovum evolved from the amniotic egg with its rich store of yolk [22]. Yolk contains fatty acids as a compact form of energy storage with the change to a viviparous form of reproduction and the reliance on oviductal and uterine secretions for nutriment, it may have been easier to change from the utilization of fatty acid for energy production to the utilization of pyruvate and lactate rather than glucose. Indeed, mammalian ova may still rely on yolk to some degree because yolk is present in mammalian ova generally [2, 27]. In our experiment, although medium with BSA-V lacked energy supplement, mouse embryo were unable to develop blastocyst cavity. This result is supported by Chatot *et al.* [5], they stated that glucose is essential in mouse to blastocoel development. On the other hand, rat embryo developed to blastocyst stage without energy supplement in medium with BSA-V and

FAF. This is supported by Kane [14], who found that 1-cell rabbit embryos would grow to blastocysts in a complex medium containing 1.5% BSA, amino acid and vitamins, but without carbohydrate type of energy sources. The development of mouse and rat embryos is dependent on a sufficient supply of fatty acids from lipid concentrate (Tables 4 and 5). Without carbohydrate type of energy supplement, rat embryo development were higher in both BSA-V and BSA-FAF with lipid concentrate than control (Table 4). In mouse this was not found. Rat embryo development was not evident under PVA, might be due to lacking of bond formation of PVA with fatty acids. In complete medium, both mouse and rat embryo developed to blastocyst with lipid concentrate (Table 5). The results of our experiment are supported by many workers. Earlier studies of Nodijeka and Hillman [23] have demonstrated that mouse embryos can take up labelled fatty acids from the culture medium. Preimplantation mouse embryos can take up and utilize exogenous fatty acids both for oxidation to carbon dioxide [8]. Fatty acids are necessary components for lipid synthesis and it is possible that preimplantation embryos synthesize and store triacylglycerols in preparation for a specific developmental event such as the hatching of the blastocysts [8]. Mills and Brinster [21] have reported that oxygen consumption by preimplantation mouse embryos remains relatively constant up to the 8-cell stage, but then undergoes a highly significant increase between the 8-cell and morulae stage. Thus these appears to be a correlation between changes in the rates of oxygen uptake and fatty acid oxidation in these embryos. Flynn and Hillman [8] stated that mouse embryo can oxidize palmitic acid to carbondioxide and also stated that preimplantation mouse embryos can reduce fatty acids to the corresponding fatty alcohols and incorporate them into ether lipids. Lipid concentrate is a combination of different fatty acid mixture. The combined effect of different fatty acid is usually more effective and this is supported by Quinn and Whittingham [26]. They found effective combined effect of palmitic and oleic acid on mouse embryo development. Embryo development in complete media of mouse were higher than that of rat in the same culture conditions (Tables 2 and 5). This may be due to species difference and we may speculate that the embryo development pattern in different species is different.

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マウスおよびラット胚の発生に及ぼすBSA結合脂肪酸の影響

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ウシ血清アルブミンfraction-V（BSA-V）の脂肪酸含量を分析し，マウスおよびラット胚の発生に及ぼすBSA結合脂肪酸の影響を検討した．脂肪酸分析のためにガスクロマトグラフィーを行ない，脂肪酸の影響を検討するためにマウス4細胞期胚およびラット8細胞期胚を用いて培養実験を行なった．linoleic acid（54.55%）が脂肪酸含量の過半数を占め，以下，oleic（25.8%），stearic（12.27%）およびlinolenic（7.36%）acidであった．BSA結合脂肪酸はマウスおよびラット胚両発生に影響を及ぼし，ラットにおいてエネルギー的効果も示した．異なる脂肪酸（chemically defined lipid concentrate）の複合的な影響はマウスおよびラット胚両発生においてより効果的であった．

キーワード：マウス・ラット胚，BSA，脂肪酸，エネルギー不含培地，化学限定脂質濃縮液．