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—Review—

Lipid in Mammalian Embryo Development

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Bovine *in vitro* matured (IVM) and *in vitro* fertilized (IVF) oocytes have generally been cultured up to the blastocyst stage.

It has been reported that the transfer of *in vitro* derived embryos often results in the production of bovine calves with abnormalities such as heavier than normal birth weight and a high incidence of dystocia [1]. It has also been reported that ovine embryos developed in serum-supplemented medium are morphologically different from those cultured in serum-free medium; the latter containing abundant cytoplasmic lipid droplets [2].

In vitro produced embryos were viable after cryopreservation and the transfer of frozen-thawed embryos resulted in the birth of calves [3], but *in vitro* derived bovine embryos were more sensitive to standard cryopreservation procedures than *in vivo* derived embryos [4]. Bovine IVM/IVF embryo tolerance of cryopreservation was increased after the removal of their cytoplasmic lipid droplets [5]. Therefore, intracellular lipids may have a strong influence on the greater sensitivity of embryos to cryopreservation. From these points of view, cytoplasmic lipid accumulation of *in vitro*-derived embryos developed in serum-supplemented medium may be a possible cause of the production of overweight offspring after embryo transfer and high sensitivity of embryos to cryopreservation. Therefore, it is very important to undertake metabolism of lipids and biochemical analysis of the intracellular lipids of mammalian embryos.

Fatty Acid Composition in Reproductive Tract

Lipids in the mammalian organism, which are of metabolic significance, include triglycerides (neutral fat), phospholipids and steroids, together with products of their metabolism such as long-chain fatty acids (free fatty acids), glycerol and ketone bodies.

The lipid metabolism in embryos, on the other hand, has not been studied so much. Lipids not only serve as a source of energy for the cell, but also play a vital role in constructing the physical properties and functions of biological membranes [6], and they have potential effects on cell-cell interactions, cell proliferation and intra- and inter-cellular transport [7]. Moreover, the function of membrane proteins depends on their immediate lipid environment, and the ability of hormone receptor complexes on the membrane to combine with the effector molecules may be modified by the hydrophilic head group and the fatty acid composition of membrane lipids [8, 9]. The importance of fatty acids in the maintenance of normal cell function is obvious.

We [10–14] reported that fatty acid composition of lipids in different reproductive samples of rats, rabbits, pigs and cows was analyzed by gas chromatography (Table 1). Eight fatty acids, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and arachidonic acids were commonly detected together with some other compounds in some cases. In the most cases, oleic acid was the most abundant and palmitic or arachidonic acids followed. Generally, myristic, palmitoleic, stearic, arachidic and lignoceric acids were minor components, and undetected in some samples at some stages.

There was a difference in the percentage of fatty acid content but the fact that the number of fatty acids remains almost constant in all samples among from four animals, may be due to the similar fatty acid requirement for oocyte maturation, fertilization and subsequent embryo development. Proportions of various fatty acids in lipids and their changes during the embryonic developmental stage differed greatly among different samples. Killian *et al.* [15] reported that lipid composition in the fluid recovered from the lumen of the bovine oviduct

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Table 1. Fatty acid constituents and relative compositions of different bovine reproductive samples

Fatty acid	Relative percentage of fatty in methylated samples			
	Oocytes	Follicular fluid	Oviductal fluid	Uterine fluid
Myristic	UD	3.4 ± 0.10	0.9 ± 0.08	1.0 ± 0.19
Palmitic	24.1 ± 0.34	28.1 ± 0.14	18.6 ± 0.86	21.3 ± 0.29
Palmitoleic	UD	2.5 ± 0.05	12.9 ± 0.54	13.4 ± 0.33
Stearic	10.9 ± 0.03	14.4 ± 1.34	20.4 ± 0.41	17.8 ± 0.64
Oleic	33.2 ± 0.33	32.3 ± 2.85	21.9 ± 0.30	27.0 ± 0.44
Linoleic	16.8 ± 0.08	13.3 ± 2.99	14.1 ± 0.18	7.6 ± 0.27
Linolenic	UD	1.6 ± 0.08	1.6 ± 0.12	2.1 ± 0.11
Arachidonic	15.1 ± 0.09	4.5 ± 0.67	9.7 ± 0.58	9.8 ± 0.93

Values are the means SEM of three analyses and are expressed as a % of the total fatty acid. UD=Undetectable.

differed considerably from that in blood serum. These differences must be ascribed to those in the process of synthesis, secretion and transudation of lipids and fatty acids in various organs and tissues, even between the oviduct and uterus [16–21]. The proportions of secreting cells in the tissue and their capacity to synthesize lipids in the oviduct and uterus [19] differ.

In most cases, the higher proportions of oleic, palmitic, linoleic and arachidonic fatty acids in the lipid of reproductive samples of all four animals may play special roles in embryo development. Among these four fatty acids, oleic and palmitic acids are especially important as the starting material of long chain desaturated fatty acid in human lymphocytes [22] and mammalian liver [23]. Their special importance for *in vitro* embryo development was also reported in mice [24].

Among four poly-unsaturated fatty acids, linoleic, linolenic, arachidonic and docosahexaenoic acids, which were found in embryos and oviductal and uterine fluids of rats and rabbits, three other than docosahexaenoic acids were also found in oocytes and ovarian follicular, oviductal and uterine fluids of pig and cow. Linoleic, linolenic and arachidonic acids are regulated as essential fatty acids [25]. It is necessary for these fatty acids to be supplied from outside and function as the precursor of some local hormones such as prostaglandins, leukotriens, thromboxanes and lipoxins. Arachidonic acid is the precursor for the biosynthesis of prostaglandins in ovary and uterus [26, 27] and the prostaglandin is postulated to act as one of the regulators of events concerning ovulation [28, 29] and as the second messenger [30]. In relation to such kinds of functions of prostaglandins, high arachidonic acid content in embryo, uterine fluid and blood serum is interesting. The metabolic function of docosahexaenoic acid has not been sufficiently clarified, but it was suggested that in Y-79 retinoblastoma cells the acid plays a part in intracellular storage of precursor in the formation of 20:5 fatty acids, which are necessary for the synthesis of phosphatidylinositol and phosphatidylserine [31].

A relatively low level or absence of myristic, palmitoleic, stearic, arachidic and lignoceric acids in embryo and reproductive tract secretions are common to almost all species. This finding appears to indicate the lesser importance of these fatty acids in the process of embryo development.

Metabolism of Exogenous Fatty Acids by Preimplantation Embryos

Embryo development: The culture experiments [32, 33] were conducted to evaluate the effect of fatty acids bound with bovine serum albumin (BSA), in Brinster medium ovum culture-III (BMOC-III), with or without the addition of carbohydrate (glucose, pyruvate and lactate) substrates, on 8-cell rat embryo development. Good embryo development was maintained in the medium with BSA F-V even in the absence of carbohydrate supplement. The rate of embryo development in the BSA FAF medium was kept almost equal to that in BSA F-V medium when carbohydrate substrates were added, but decreased largely in the absence of carbohydrates. In the PVA medium, the embryo development ceased completely, unless the carbohydrate supplement was given (Table 2). Linoleic acid was identified to be the major fatty acid (54.6%) bound with BSA, followed by oleic (25.8%), stearic (12.3%) and linoleic (7.4%) acids. BSA binding fatty acids had a considerable effect on both mouse and rat embryo development in general but they provided only the rat embryo with energy.

Table 2. Effect of fatty acids bound to BSA on rat embryo development from the 8-cell stage onwards during 48 h culture in media lacking carbohydrate substrates

Medium*	Carbohydrate	Development % (mean ± SE)	
		Morula	Blastocyst
BSA F-V	—	88.0 ± 3.7[a]	64.0 ± 4.0[a]
BSA FAF (<0.02)	—	6.0 ± 5.1[b]	22.0 ± 4.9[b]
BSA FAF (<0.005)	—	2.0 ± 3.7[c]	16.0 ± 4.0[b]
PVA	—	0.0[d]	0.0[c]

*BSA F-V (5 mg/ml) in BMOC-medium was replaced with BSA FAF (5 mg/ml) containing less than 0.02 or 0.005% of fatty acids or PVA (1 mg/ml). Culture of 10 embryos was repeated 4–5 times for each medium. Significant differences were found between the means having different letters in their superscripts in the same column ($p<0.05$ in FPLSD-test).

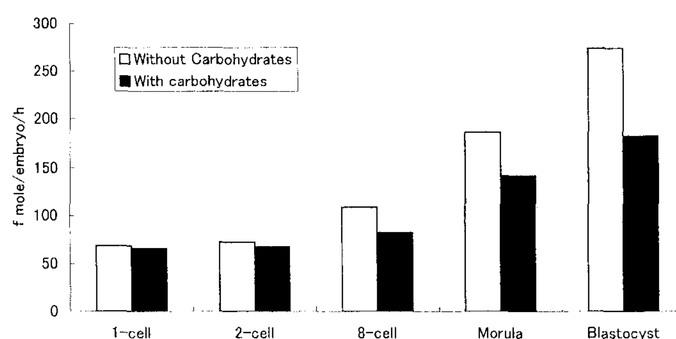

Fig. 1. Comparison of the oxidation rates of exogenous ^{14}C-palmitic acid in labeled M16 media without and with carbohydrates.

Incorporation of Fatty Acids

As yet no attempt has been made to characterize and compare the differences in incorporation of individual fatty acids into embryo lipids and the metabolic abilities of the incorporated fatty acids in preimplantation rabbit [34], rat [35] and mouse [36, 37] embryos developed *in vitro* to different cell stages.

The rate of fatty acid oxidation was relatively constant in the 1-cell and 2-cell stages, and continued to increase up to the blastocyst stage of rabbit, rat and mouse (Fig. 1). Incorporation of ^{3}H-palmitic acid into rabbit, rat and mouse embryo lipids was examined by culturing morulae for 2 h. Lipids were extracted and fractionated by thin-layer chromatography. Radioactivity was recovered in various neutral and polar lipid classes. Among the neutral lipid species, about 50% of the radioactivity was recovered in triacylglycerol whereas the proportion recovered in fatty alcohols and diacylglycerol was 19.0%, and 16.5%, respectively. Among the polar lipids, most of the radioactivity was recovered in choline phosphatides (77.5%). Radioactivity was also recovered in other neutral and polar lipid fractions.

The oxidation of exogenous palmitic acid by mouse embryos was maintained steadily in culture medium with carbohydrates, but significantly lower than those in the medium without carbohydrates from the 8-cell to the blastocyst stage ($p<0.05$). In the presence of the same fatty acid content in the medium, the incorporation rates for double ^{3}H-palmitic and ^{14}C-oleic acids were significantly lower than those for single fatty acid at the same cell stage ($P<0.05$) (Fig. 1).

In the presence of single fatty acid, 64.4 to 78.3% of ^{3}H-palmitic acid in neutral lipids was distributed in triacylglycerols, whereas only 31.6 to 42.8% of ^{14}C-oleic acids in triacylglycerols, and large amounts of ^{14}C-oleic acids were widely distributed in fatty alcohol and other classes. The amounts of ^{3}H-palmitic acids in the labeled polar lipids were mainly found in choline phosphatides and ethanolamine phosphatides, whereas

Table 3. Rates of incorporation of single palmitic and oleic acids into embryo lipids from 1-cell to blastocyst stage

Cell stage	*f* mol/embryo/h					
	In embryo lipids		In neutral lipids		In polar lipids	
	Palmitic	Oleic	Palmitic	Oleic	Palmitic	Oleic
One-cell	183.5 ± 7.2[a]	88.0 ± 9.4[a]	145.0 ± 15.3[a]	72.0 ± 6.4[a]	38.5 ± 2.9[a]	16.0 ± 1.2[a]
Two-cell	238.4 ± 8.5[b]	104.4 ± 8.9[ab]	191.4 ± 11.8[b]	80.7 ± 13.5[a]	47.0 ± 3.2[b]	16.1 ± 2.3[a]
Eight-cell	261.9 ± 9.3[c]	240.5 ± 8.2[c]	218.4 ± 13.2[bc]	136.4 ± 10.6[b]	43.5 ± 6.2[b]	47.9 ± 3.0[b]
Morula	286.6 ± 6.3[d]	240.5 ± 8.2[c]	220.7 ± 10.9[bc]	188.3 ± 12.6[c]	66.0 ± 5.8[c]	52.2 ± 2.4[b]
Blastocyst	294.9 ± 8.4[d]	408.3 ± 9.8[d]	235.9 ± 9.8[cd]	328.3 ± 14.8[d]	50.9 ± 4.3[d]	80.0 ± 2.6[c]

a~d: Superscript values within the same column differ significantly from each other. Data are expressed as the mean ± SEM. The results were obtained from four determinations in two independent experiments on about 150 labeled embryos in each.

Table 4. Rates of incorporation of double palmitic and oleic acids into embryo lipids from one-cell to blastocyst stage

Cell stage	f mol/embryo/h					
	In embryo lipids		In neutral lipids		In polar lipids	
	Palmitic	Oleic	Palmitic	Oleic	Palmitic	Oleic
One-cell	58.5 ± 4.1[a]	61.1 ± 5.7[a]	42.2 ± 3.4[a]	49.2 ± 2.1[a]	16.3 ± 3.2[a]	12.0 ± 2.6[a]
Two-cell	61.1 ± 5.2[a]	57.0 ± 4.3[a]	41.1 ± 4.3[a]	44.9 ± 3.9[ab]	20.0 ± 1.5[a]	12.8 ± 2.0[a]
Eight-cell	78.0 ± 3.0[b]	63.7 ± 3.2[a]	53.1 ± 3.7[ab]	53.1 ± 4.3[bc]	24.9 ± 2.8[ab]	10.6 ± 2.8[a]
Morula	113.1 ± 3.6[c]	97.5 ± 4.6[b]	83.1 ± 2.9[c]	75.0 ± 3.2[d]	30.0 ± 2.7[c]	19.5 ± 2.8[b]
Blastocyst	223.6 ± 8.7[d]	170.3 ± 6.7[c]	168.4 ± 3.2[d]	138.3 ± 3.6[e]	55.2 ± 2.3[d]	32.0 ± 3.0[c]

a~e: Superscript values within the same column differ significantly from each other. Data are expressed as the mean ± SEM. The results were obtained from four determinations in two independent experiments on about 150 embryos each.

smaller amounts of ^{14}C-oleic acid than those of ^{3}H-palmitic acid were found in choline and ethanolamine phosphatides ($P<0.05$). Large amounts of ^{14}C-oleic acids were found in the mixtures of inosital and serine phosphatides from the 1-cell to the blastocyst stage (Table 3).

In the experiment on double ^{3}H-palmitic and ^{14}C-oleic acids, the radioactivity in ^{3}H-palmitic acid recovered in triacylglycerols, monoacyldiglycerols and diacylglycerols was significantly higher than that in ^{14}C-oleic acid, whereas that in ^{3}H-palmitic acid in monoacylglycerols and fatty alcohols was significantly lower than that in ^{14}C-oleic acid at most cell stages ($P<0.05$). The radioactivity in ^{3}H-palmitic acid in spingomyecins, choline and ethanolamine phosphatides was significantly higher than that in ^{14}C-oleic acid, but significantly lower than that in ^{14}C-oleic acid in inositol or serine phosphatides, lysophosphatidylcholines and monoglycosylglycerides at most corresponding cell stages ($P<0.05$) (Table 4). These results indicate that fatty acids are certainly utilized for energy production and for the synthesis of various lipids through β-oxidation, the TCA cycle and lipogenesis in embryos at the preimplantation stage.

Lipid Synthesis from Glucose

The pattern of utilization of glucose from the 1-cell to the blastocyst stage in rat embryos indicates a substantial increase in the capacity to oxidize this substrate with development. This increasing CO_2 production of [U-^{14}C] glucose could arise from the increased pentose phosphate pathway (PPP) activity, increased TCA cycle processing, or a combination of both. The measurement of PPP activity in mouse embryo indicated that activity in this pathway increases with development [39].

At the blastocyst stage, embryonic metabolism switches from using oxidative phosphorylation of pyruvate to glycolysis of glucose [40]. This energy switch is believed to occur in preparation for implantation and a temporary anaerobic existence and to afford synthesis of macromolecules from glycolytic intermediates [41, 42].

As a result, glucose consumption increases significantly, with the greatest consumption occurring in the inner cell mass [43].

Waugh and Wales [44] reported that the major incorporation of glucose carbon by cattle embryos occurs in the acid-soluble, non-glucose pool, and approximately 70% of total incorporation is found in this fraction with most of the remainder in non-glycogen macromolecules. Our data indicated that preimplantation rat embryos can incorporate the labels from [U-^{14}C] glucose into lipids [45]. In close agreement with mouse embryos studies [46–48], glucose carbon in rat embryo lipogenesis is through glucose-3-phosphate into triacylglycerols.

A major biological role of triacylglycerols is to serve as a storage form of metabolic energy. Since preimplantation embryos readily utilize other compounds (e.g. lactate, pyruvate, glucose) as energy sources during preimplantation development [49], it is possible that triacylglycerols are synthesized and stored by these embryos for a later period of development when they may not be capable of utilizing exogenous substrates. The triacylglycerols have a higher energy content per unit mass than glycogen. The accumulation of triacylglycerols during the early stages of rat embryogenesis may also have developmental significance since the accumulation of triacylglycerols also occurs during the early development of other vertebrates [49].

Hormone Regulation of Lipid Metabolism

Insulin promotes anabolic processes and inhibits catabolic ones in muscle, liver and adipose tissue. Specifically, insulin increases the rate of synthesis of glycogen, fatty acids and proteins and also stimulates glycolysis. Insulin inhibits catabolic processes such as the breakdown of glycogen and fat. It also decreases gluconeogenesis by lowering the level of enzymes such as pyruvate carboxylase and fructose 1,6-diphosphatase [50].

Simultaneous with this increase in glucose consumption, the blastocyst leaves the fallopian tube and enters the uterine cavity, at which time in normal physiology insulin and IGF-1 levels both rise [51]. Carayannopoulos *et al.* [52] suggested that an insulin/IGF-1 regulated glucose transporter responds by translocating to the apical plasma membranes of the blastocyst to maintain glucose homeostasis despite this major shift in energy consumption and glucose utilization.

The influence of insulin of glucose metabolism was utilized for oxidation and the synthesis of embryos lipids, blastocysts were cultured for 5 h in medium containing [U-^{14}C] glucose, and total lipids extracted from the embryo were separated into various neutral lipids and phospholipids by thin layer chromatography and the radioactivity of these lipid fractions. We [53] found that insulin promotes the oxidation of glucose and inhibits its incorporation from glucose into lipids, specifically triacylglycerols, in rat blastocyst. From these points of view, the *in vitro* development of zygotes to blastocysts cultured in medium supplemented with insulin may decrease the cytoplasmic lipid accumulated *in vitro* derived embryos.

Moley *et al.* [54] have shown that high glucose-induced apoptosis causing increased apoptosis at the blastocyst stage results in higher resorption rates in the mouse. Therefore, an increase in the apoptotic pathway may protect the early embryo by eliminating abnormal cells, but loss of balance in this tissue remodeling may lead to developmental arrest and demise.

Serum-free Medium of Embryos Culture

Ovine embryos developed in serum-supplemented medium are morphologically different from those cultured in serum-free medium; the latter contains abundant cytoplasmic lipid droplets [55]. The inclusion in serum in the embryo culture medium leads to the production of larger than normal offspring. It has been suggested that the addition of serum to the culture medium results in different morphology, biochemical composition, and rate of development of embryos. This factor may contribute to the production of offspring with abnormally high birth weight.

A serum-free culture system was used to allow determination of the fatty acid composition of bovine embryos during *in vitro* development. The proportion of embryos developing to the blastocyst stage in serum-free medium (TCM199 supplemented with bovine serum albumin, insulin, apotransferrin and transforming growth factor-α; BITTα) was as high as in serum-supplemented medium (TCM199+5% calf serum). Bovine embryos which develop in serum-supplemented medium contain abundant cytoplasmic lipid droplets. The fatty acid composition of immature bovine oocytes, and embryos at the 2-cell and blastocyst stages cultured in serum-free or serum-supplemented medium was determined by capillary column gas chromatography. The fatty acid composition of calf serum alone was also determined. These results show that bovine embryos grown in serum-supplemented medium contained a large amount of cytoplasmic lipid droplets and have a fatty acid composition when compared to those cultured in serum-free

medium [56]. The post-thaw viability of bovine blastocysts developed in serum-supplemented medium was significantly lower than that of those developed in serum-free medium.

Conclusion

Much fatty acid exists in the oviduct and uterus fluid. The serum and serum albumin, which contained much fatty acid, used for the culture of embryos. These fatty acids are incorporated into the preimplantation embryos. Development of the embryo deteriorates after removal of these fatty acids from bovine serum albumin. Fatty acids incorporated into the embryo are used as a source of energy, and the surplus is accumulated as triacylglycerols of the lipid. A change to the lipid from glucose takes place in the preimplantation embryo. Moreover, after addition of insulin the metabolism of glucose becomes active, and accumulation of the lipid in embryonic cytoplasm decreases.

It is advisable to use culture medium which does not contain serum or serum albumin in the culture of the preimplantation embryo. Lipid droplets decrease in the embryo when it is cultivated in serum-free culture system. These factors will contribute to the production of embryos, which are resistant to freezing and the thawing.

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