—Brief Note—

Histochemical Demonstration of Lipids in Cultured Porcine Oocytes and Preimplantation Embryos

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Abstract: *Changes in the number of lipid droplets during meiotic maturation, fertilization and early development were histochemically examined in cultured porcine oocytes and embryos. The oocytes and embryos possessed Sudanophilic lipids composed of small (<2.5 μm), medium (2.5–4.9 μm) and large (≥5.0 μm) droplets. In oocytes soon after collection, the numbers of Sudanophilic lipid droplets with small and medium sizes were few and the number of those with large size was 148 ± 11.36. After being cultured for 22 and 44 hrs, the number of lipid droplets with large size remarkably decreased, while the number of those with small and medium sizes increased. The numbers of lipid droplets of each size in the oocytes 4 and 8 hrs after insemination were similar to those in oocytes 44 hrs after maturation culture. On the other hand, the number of lipid droplets in embryos did not vary greatly between the pronuclear and the 16-cell stages, but gradually decreased after the morula stage. Expanded blastocysts had few small and medium lipid droplets and 11 ± 1.68 large ones. The present findings confirmed that lipid droplets contained in oocytes become smaller in size and larger in number. Since the smaller lipid droplets appear not to be used in the process of fertilization, we presume that they are mainly used as an energy source for the formation and expansion of blastocysts.*

Key words: *Porcine oocyte, Porcine embryo, Lipid droplet, Histochemistry*

It is considered that follicular oocytes and tubal embryos of mammals contain lipid droplets for utilization as an energy source for the growth of oocytes or the development of embryos [1–4]. The amount of such inclusion in oocytes and the fluctuation of lipid content in embryos during early development were found to be different among animal species [1–4]. According to the reports, in which lipids were histochemically detected in the oocytes from antral follicles, oocytes of hamsters [1], Japanese monkeys [1] and mastomys [2] were free of lipids. Oocytes of mice [1], rats [1], rabbits [1] and Japanese field voles [1] contained a small or moderate number of lipid droplets, while those of cattle [1], goats [1], sheep [1], mares [1], dogs [1] and Japanese black bears [3] contained lipids in abundance. It was also reported that early embryos of mastomys [2] contained no lipids from the 1-cell to blastocyst stages, whereas those of Japanese field voles [1], mice [1], guinea pigs [1], rabbits [1] and cattle [4] always contained a small or large amount of lipid droplets. Mongolian gerbil embryos were also rich in lipids at the 1-cell to morula stages, but the number of lipid droplets decreased at the blastocyst stage [1]. In rat [1] and hamster [1] embryos, however, lipids were observed in a strictly limited period.

Histochemistry has revealed that the amount of lipids in porcine oocytes increases with follicular growth, and the oocytes in antral follicles and ovulated oocytes have extremely large amounts of Sudanophilic lipids [5, 6]. It has also been reported that the amount of lipids in the oocytes in antral follicles and oviducts persists until 8-cell embryos, but decreases in blastocysts [5, 6]. Recently, it has been confirmed in porcine oocytes that the number of lipid droplets markedly increases with maturation *in vivo* and *in vitro*, whereas the total amount of lipids does not vary during maturation [7, 8]. However, only changes in the number of lipid droplets have been examined in porcine oocytes during maturation, while the changes in the number of lipid

Received: September 5, 2003
Accepted: October 27, 2003
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droplets in oocytes during fertilization and in preimplantation embryos during the early development have not yet been examined.

In the present study, lipid droplets were histochemically observed in cultured porcine oocytes and embryos to examine the changes in the number of their cytoplasmic inclusion in the processes of maturation and fertilization of oocytes and early development of embryos.

Materials and Methods

Collection and culture of oocytes

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported in saline maintained at 37°C to the laboratory where they were washed in saline containing 200 IU/ml potassium penicillin G (Meiji Seika, Tokyo, Japan). Immature oocytes covered with cumulus cells were aspirated from medium-sized follicles (3–6 mm in diameter) with a 21-gauge needle fixed to a 10-ml disposable syringe. Collected cumulus oocyte complexes (COCs) were washed in phosphate buffered saline (PBS, pH 7.4) [9] and then in a culture medium composed of TCM-199 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) porcine follicular fluid, 10% (v/v) fetal calf serum (Gibco BRL), 10 IU/ml eCG (Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan), 10 IU/ml hCG (Teikoku Hormone Manufacturing Co. Ltd.) and 0.001% (w/v) estradiol-17β (Wako Pure Chemical Industries, Osaka, Japan) [10]. Forty to 50 COCs were transferred into each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 400 μl/well of the culture medium, which had previously been covered with mineral oil (Sigma-Aldrich, St. Louis, MO, USA) and equilibrated in a CO_2 incubator (Asahi Life Science, Tokyo, Japan). For maturation, these COCs were cultured at 39°C in an atmosphere of 5% CO_2 in air.

In vitro fertilization of oocytes

Ejaculated boar semen was treated by the method of Wang *et al.* [11], in order to induce capacitation of spermatozoa. The semen was washed three times in Brackett and Oliphant (BO) [12] medium containing 5 mM caffeine (Sigma-Aldrich) and 0.3% BSA (Sigma-Aldrich). Spermatozoa were resuspended in BO medium containing 5 mM caffeine and 0.3% BSA to give a concentration of 5×10^5 live spermatozoa/ml, and 400 μl of sperm suspension was covered with mineral oil in each well of a Nunc 4-well multidish.

At 44 hrs after maturation culture, COCs were washed twice in BO medium containing 5 mM caffeine and 0.3% BSA. Forty to 50 COCs were introduced into the sperm suspension and cultured at 39°C in a CO_2 incubator (5% CO_2 in air).

Culture of inseminated oocytes

After culture with spermatozoa for 8 hrs, COCs were washed twice in PBS, and then cumulus cells were dispersed from the oocytes by pipetting in PBS containing 0.1% hyaluronidase (Sigma-Aldrich). The denuded oocytes were washed and cultured in NCSU 23 containing 0.4% BSA [13] for 7 days at 39°C in a CO_2 incubator (5% CO_2 in air).

Demonstration of lipid droplets

At 0, 22 and 44 hrs after the onset of maturation culture, and at 4 and 8 hrs after insemination, cumulus cells were dispersed from the oocytes by pipetting in PBS containing 0.1% hyaluronidase (Sigma-Aldrich). In order to demonstrate Sudanophilic lipids, these denuded oocytes and preimplantation embryos at the stages of pronuclear (20 hrs after insemination), 2-cell (2 days), 4-cell (2–3 days), 8-cell (3 days), 16-cell (4 days), morula (5 days), blastocyst (6 days) and expanded blastocyst (7 days) were fixed in PBS containing 10% formalin, and then stained with Sudan IV [14].

After staining, the oocytes and embryos were washed in PBS and placed on glass slides for photography under a light microscope (Nikon, Tokyo, Japan). The same staining procedures were repeated 3 times, using 30 oocytes and embryos in each culture or developmental stage. Degenerated oocytes and embryos were eliminated from the observation.

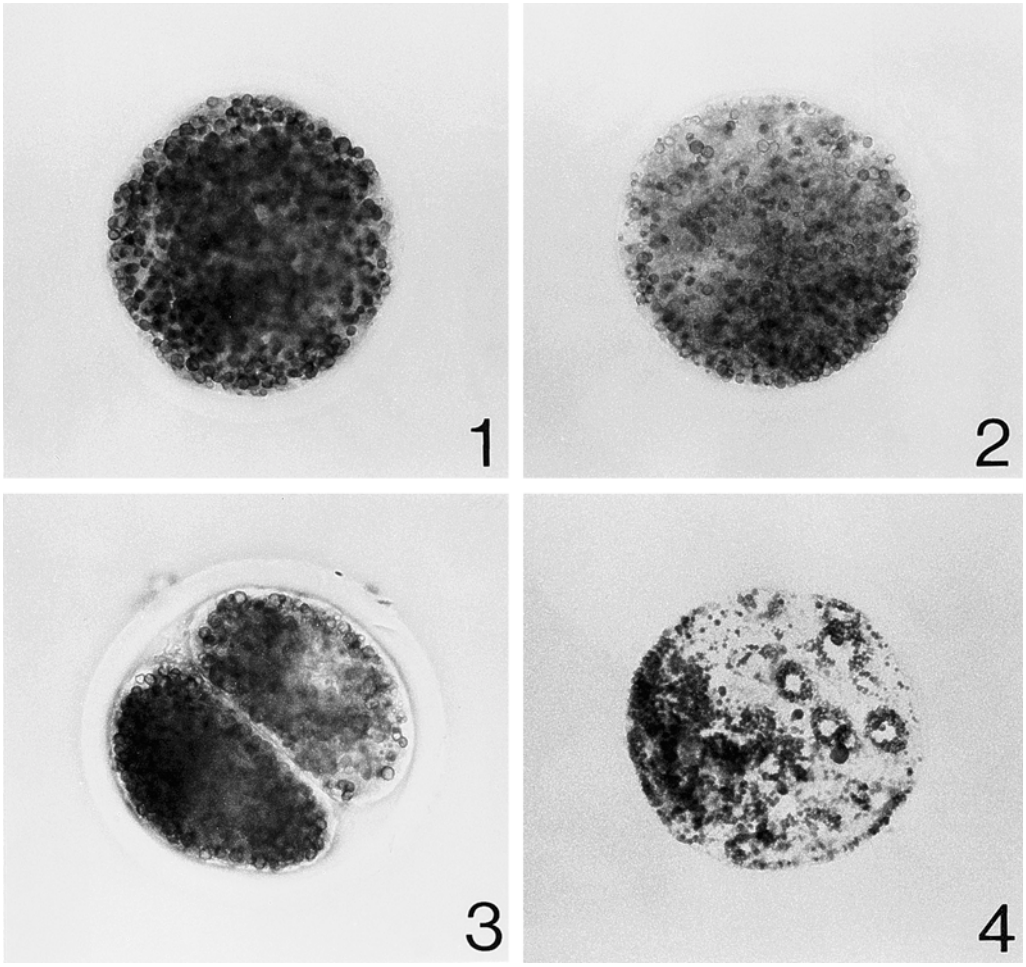
Concerning the oocytes 4 and 8 hrs after insemination and the embryos 20 hrs after insemination, these oocytes and embryos in which lipid droplets had been demonstrated were further stained with Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA) for 5 min at 37°C and then examined for the presence of penetrated sperm(s) or formed pronuclei under an epifluorescent microscope (Nikon, Tokyo, Japan).

Statistical analysis

The number of Sudanophilic lipid droplets of large size was statistically analyzed by one-way analysis of variance.

Results

Sudanophilic lipids were observed as reddish-orange



All the pictures are of cultured porcine oocytes and embryos stained with Sudan IV.

Fig. 1. An oocyte soon after collection. A small number of Sudanophilic lipid droplets of small and medium sizes and a large number of large ones in the cytoplasm. × 400.

Fig. 2. An oocyte 44 hrs after maturation culture. A moderate number of Sudanophilic lipid droplets of small and medium sizes and a small number of large ones in the cytoplasm. × 400.

Fig. 3. A 2-cell embryo. A large number of Sudanophilic lipid droplets of small size, a moderate number of medium ones and a small number of large ones in the cytoplasm of each blastomere. × 400.

Fig. 4. A blastocyst. A small number of Sudanophilic lipid droplets of small and large sizes and a moderate number of medium ones in the cytoplasm of trophoblast and inner-cell-mass cells. The number of lipid droplets of each size is larger in inner-cell-mass cells than in trophoblast cells. × 250.

droplets of different sizes in the cytoplasm when the oocytes and embryos were stained with Sudan IV (Figs. 1–4). We classified these Sudanophilic lipids into three groups using a micrometer under a microscope: small droplets less than 2.5 μm in diameter, medium ones 2.5 to 4.9 μm and large ones more than 5.0 μm. It was impossible to accurately count the number of small and medium lipid droplets because there were so many, so the total was estimated on a 3-point scale: many (+++), moderate (++) and few (+). The number of large lipid droplets was counted under a microscope.

As shown in Table 1, the oocytes soon after collection (Fig. 1) contained few small and medium sized Sudanophilic lipid droplets, and had 148 ± 11.36 large Sudanophilic lipid droplets. In the oocytes cultured for 22 and 44 hrs, although the number of small and medium lipid droplets increased, the number of large ones significantly decreased ($P<0.01$) (Fig. 2). The numbers of lipid droplets of each size in the oocytes 4 and 8 hrs after insemination were similar to those in the

Table 1. Number of Sudanophilic lipid droplets of different sizes in porcine oocytes during *in vitro* maturation (IVM) and *in vitro* fertilization (IVF)

Hours of culture		No. of oocytes examined	Number of lipid droplets		
			Small (<2.5 μm)	Medium (2.5–4.9 μm)	Large (≥5.0 μm)
IVM	0	30	+	+	148 ± 11.36[a]
	22	30	++	++	84 ± 7.12[b]
	44	30	++	++	46 ± 4.24[c]
IVF	4	30	++	++	44 ± 3.78[c]
	8	30	++	++	35 ± 4.34[c]

The symbols + and ++ represent few and moderate, respectively. Data are mean ± S.E. Values with different superscripts in the same column are significantly different (P<0.01).

Table 2. Number of Sudanophilic lipid droplets of different sizes in porcine embryos during *in vitro* development

Developmental stages	No. of embryos examined	Number of lipid droplets		
		Small (<2.5 μm)	Medium (2.5–4.9 μm)	Large (≥5.0 μm)
Pronuclear	30	+++	++	37 ± 3.85[b, c]
2-Cell	30	+++	++	49 ± 4.46
4-Cell	30	+++	++	53 ± 4.30
8-Cell	30	++	++	54 ± 4.35
16-Cell	30	++	++	47 ± 4.09[a, b]
Morula	30	+	++	35 ± 3.83
Blastocyst	30	+	++	25 ± 3.49
Expanded blastocyst	30	+	+	11 ± 1.68

The symbols +, ++ and +++ represent few, moderate and many, respectively. Data are mean ± S.E. Values with different superscripts in the same column are significantly different (P<0.01).

oocytes 44 hrs after maturation culture. These lipid droplets were distributed throughout the cytoplasm of the oocytes.

Changes in the number of Sudanophilic lipid droplets in porcine embryos are shown in Table 2. Pronuclear embryos contained many droplets of small size, a moderate number of medium ones, and 37 ± 3.85 of large ones. The number of lipid droplets in embryos did not vary greatly between the pronuclear and the 16-cell stages (Fig. 3), but gradually decreased after the morula stage. Expanded blastocysts had few small and medium lipid droplets and 11 ± 1.68 large ones. In embryos, Sudanophilic lipid droplets of every size were distributed throughout the cytoplasm, although the number of these droplets differed among blastomeres in some embryos from the 2-cell to morula stages. Furthermore, these lipid droplets were distributed in the cytoplasm of both trophoblast and inner-cell-mass cells of blastocysts (Fig. 4), although the number of lipid droplets was larger in inner-cell-mass cells than in trophoblast cells.

Discussion

It has been histochemically confirmed that the porcine oocytes in antral follicles contain many Sudanophilic lipid droplets in the cytoplasm [5], and that the amount of the lipids does not alter with time in maturation culture, and also that the number of lipid droplets increases with oocyte maturation *in vitro* [8]. This change in lipids was also electron microscopically observed in porcine oocytes matured *in vivo* [7]. Therefore, it was suggested that the large number of smaller lipid droplets accumulated in mature porcine oocytes were an energy source for fertilization and subsequent early embryo development [8].

In the present investigation, the number of small and medium lipid droplets increased with oocyte maturation, while the number of large ones decreased significantly. The changes in the number of lipid droplets in porcine oocytes with maturation were comparable to those examined by Cran [7] and Niimura *et al.* [8]. From these findings, we consider that the increase in the number of smaller lipid droplets is one of the morphological characteristics of matured oocytes. On the other hand,

the number of lipid droplets becoming smaller as maturation progressed did not change during fertilization. Thus we presume that the many small lipid droplets found in matured oocytes are not used as an energy source during fertilization.

Niimura and Ishida [6], who histochemically demonstrated lipid droplets in unfertilized porcine eggs and tubal embryos from the 2-cell to blastocyst stages, reported that the number of lipid droplets was large from the 2-cell until the 8-cell stage, but remarkably decreased in the blastocyst stage. It was also reported that the lipid droplets in blastocysts were exclusively distributed in trophoblast cells and not in inner-cell-mass cells. The present study, performed to demonstrate Sudanophilic lipids in cultured porcine embryos, revealed that the number of lipid droplets of different sizes did not change greatly between the pronuclear and 16-cell stages, but gradually decreased through the morula to blastocyst stages, and that the number of lipid droplets of every size was the smallest at the expanded blastocyst stage. Therefore, we presume that the changes in the number of lipid droplets in cultured porcine embryos during the early development are comparable to those in embryos developed *in vivo*, and that lipids in early embryos are mainly used as an energy source for the formation and expansion of blastocysts. Although the reason was not obvious, most lipids were found to be distributed in inner-cell-mass cells in blastocysts and this was in contrast to blastocysts collected from uteri [6]. Furthermore, porcine oocytes have been biochemically determined to have fatty acids, such as palmitic acid, stearic acid and oleic acid, as well as neutral fats and cholesterols, and the amount of fatty acids has been shown as being larger in porcine oocytes than in bovine and sheep oocytes [15,16]. It seems further study on the changes in amounts of these lipids with maturation and fertilization of oocytes and early development of embryos is required.

References

1) Niimura, S. and Ishida, K. (1989): Morphological and histochemical characteristics of the mammalian egg. In: Progress in Veterinary Science 1989 (Izawa, H. and Shimizu, Y., eds.), pp. 148–168, Kindai Syuppan, Tokyo.
2) Niimura, S., Fujisawa, N., Sato, N.L. and Ishida, K. (1991): Histochemical observations of proteins, polysaccharides and lipids in the oocytes and embryos of mastomys (*Praomys natalensis*) during the folliculogenesis and early development. Jpn. J. Anim. Reprod., 37, 65–69.
3) Niimura, S., Ohtaki, K., Kusuhara, S., Miura, S. and Abe, M. (1998): Histochemical demonstrations of proteins, polysaccharides and lipids in oocytes of Japanese black bears. Jpn. J. Zoo Wildl. Med., 3, 69–74.
4) Niimura, S., Yotsutani, I., Ryoo, Z.R., Matsumoto, H. and Sugawara, S. (1995): Histochemical observations of lipid droplets in cultured bovine eggs and embryos. Bull. Facul. Agr. Niigata Univ., 47, 45–51.
5) Ishida, K. (1960): Histochemical studies of lipids in the ovaries of domestic animals. Arch. Histol. Jap., 19, 547–563.
6) Niimura, S. and Ishida, K. (1980): Histochemical observations of lipid droplets in mammalian eggs during the early development. Jpn. J. Anim. Reprod., 26, 46–49.
7) Cran, D.G. (1985): Qualitative and quantitative structural changes during pig oocyte maturation. J. Reprod. Fert., 74, 237–245.
8) Niimura, S., Takano, H., Onishi, A. and Hosoe, M. (2002): Changes in the amount of proteins, glycogen and lipids in porcine oocytes during *in vitro* meiotic maturation. Anim. Sci. J., 73, 327–332.
9) Dulbecco, R. and Vogt, M. (1954): Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med., 99, 167–174.
10) Yoshida, M., Ishizaki, Y. and Kawagishi, H. (1990): Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured *in vitro*. J. Reprod. Fert., 88, 1–8.
11) Wang, W.H., Abeydeera, L.R., Fraser, L.R. and Niwa, K. (1995): Functional analysis using chlortetracycline fluorescence and *in vitro* fertilization of frozen-thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. J. Reprod. Fert., 104, 305–313.
12) Brackett, B.G. and Oliphant, G. (1975): Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod., 12, 260–274.
13) Wang, W.H., Abeydeera, L.R., Cantley, T.C. and Day, B.N. (1997): Effects of oocyte maturation media on development of pig embryo produced by *in vitro* fertilization. J. Reprod. Fert., 111, 101–108.
14) McManus, J.F.A. and Mowry, R.W. (1964): Staining Methods, pp. 109–123, A Hoeber International Reprint, New York, Evanston, London and Tokyo.
15) Homa, S.T., Racowsky, C. and McGaughey, R.W. (1986): Lipid analysis of immature pig oocytes. J. Reprod. Fert., 77, 425–434.
16) McEvoy, T.G., Coull, G.D., Broadbent, P.J., Hutchinson, S.M. and Speake, B.K. (2000): Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. J. Reprod. Fert., 118, 163–170.