Dietary Administration of Fatty Acids-Enriched Mold Dried Cell Containing γ -Linolenic Acid to Female Pigs Improves Ovulation Rate and Embryo Quality in Summer

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Abstract. The effect of dietary administration with essential unsaturated fatty acids, linoleic acid [18:2 (*n*-6)], a mixture of oleic acid [18:1 (*n*-9)], linoleic acid and γ -linolenic acid [18:3 (*n*-6)], or α -linolenic acid [18:3 (*n*-3)], on early embryo development of pubertal female pigs in summer was examined. Safflower oil was supplemented to a concentrated diet (the control diet) at the rate of 5% (Safflower oil-diet). The concentration of linoleic acid was 3.75 g/100 g in the Safflower oil-diet. Fatty acids-enriched mold dried cell was supplemented to the control diet at the rate of 20% (Mold dried cell-diet). The concentration of γ -linolenic acid was 5.32 g/100 g in the Mold dried cell-diet. Mold dried cell also contains oleic acid and linoleic acid at concentrations of 7.28 g/100 g and 2.98 g/100 g, respectively. Perilla oil was supplemented to the control diet at the rate of 7.5% (Perilla oil-diet). The concentration of α -linolenic acid was 4.125 g/100 g in the Perilla oil-diet. Each diet was given daily to five crossbred pubertal gilts for approximately 4 months. From at least 70 days after the start of the experimental diets, embryo collections were performed. The control embryos were obtained from the same breed, 10 pubertal gilts, given only the control diet. Embryos were collected on day 6 (day 1=the last day of estrus) and assessed morphologically. Embryo collection was repeated twice for each pig fed the diet supplemented with essential unsaturated fatty acids. The mean number of corpora lutea was greater in gilts given Mold dried cell-diet than in gilts given Safflower oil-diet ($P<0.05$) or Perilla oil-diet ($P<0.01$). The mean number of ova recovered in gilts given Mold dried cell-diet was also greater than that in gilts given the control diet ($P<0.05$), Safflower oil-diet ($P<0.05$) or Perilla oil-diet ($P<0.01$). Furthermore, both the mean number and the proportion of embryos showing normal morphology in gilts given Mold dried cell-diet were significantly higher than in gilts given the control diet, Safflower oil-diet or Perilla oil-diet. These results indicate that the unsaturated fatty acids-enriched mold dried cell may be beneficial for improving ovulation rate and embryo quality.

Key words : γ -Linolenic acid, Essential polyunsaturated fatty acid, Pig, Ovulation rate, Embryo quality

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Exposure of most mammals, including pigs, to elevated ambient temperature results in a reduction in reproductive performance. There can be detrimental effects of heat stress directly on the

gametes, the embryo or uterine function, or heat stress may have an indirect effect through alterations of the endocrine system. It has been suggested that heat stress exerts its effect indirectly and that increased glucocorticoids production in a stressed animal blocks or reduces secretion of pituitary gonadotropins [1]. Altered endocrine function in the female could alter gamete and embryo transport, the uterine environment or the conceptus [2]. The greatest fluctuations in plasma estrogen and progesterone occur at estrus and during the first 12 days of gestation in pigs [3]. The alteration in hormone balance during this period plays a critical role in the transport of ova, early blastocyst development, and uterine tissue preparation for the implantation processes. Edwards *et al.* [4] found that gilts exposed to heat stress 1 to 15 days post-breeding had fewer viable embryos at 30 days post-breeding than gilts exposed to the same stress 15 to 30 days post-breeding. The periods of gestation in which high ambient temperatures have the most adverse effects on embryo mortality have been reported to be 0 to 8 days post-breeding in pigs [5]. Tompkins *et al.* [6] also reported fewer viable embryos when gilts were heat-stressed 1 to 5 days post-breeding.

So far, no reliable method has been established to alleviate the reduction in reproductive performance in female pigs during periods of heat stress. It is important to develop effective methods of overcoming heat stress to provide the best environment for the breeding herd with multiple or year-round farrowing.

Essential polyunsaturated fatty acids (PUFA), which must be taken dietetically, play major roles in the structure of all organs and determine some of the properties of all biological membranes. The essential polyunsaturated fatty acids also act as precursors for the eicosanoids, the prostaglandins and leukotrienes. The body requires adequate intakes of linoleic acid and α -linolenic acid but must also be able to metabolize them normally. There is a considerable clinical evidence that γ -linolenic acid, which is a metabolite of linoleic acid through $\Delta 6$ -desaturase, can correct some of the abnormalities in metabolism of essential PUFA and can also lead to simultaneous clinical improvement [7]. Recently, we developed a fatty acids-enriched mold dried cell containing γ -linolenic acid [8]. The aim of the present study was to examine the effect of dietary administration of linoleic acid, γ -linolenic acid, and

α -linolenic acid on early embryo development in pigs.

Materials and Methods

Preparation of experimental diets

Experimental diets were prepared as follows, Safflower oil-diet: Safflower oil (Mitsubishi Trading Co., Tokyo, Japan) containing 75% linoleic acid and 0.12% α -tocopherol (Nisshin Oil, Tokyo, Japan) was supplemented to the control diet at the rate of 5%. The concentrations of linoleic acid and α -tocopherol were 3.75 g/100 g and 600 μ g/g, respectively. Mold dried cell-diet: Fatty acids-enriched mold dried cell (LINOX, US Patent No. 4868001 [8], Idemitsu Materials Co., Ltd., Tokyo, Japan) from fungi (*Mucor circinelloides*) containing 26.6% γ -linolenic acid was supplemented to the control diet at the rate of 20%. The concentration of γ -linolenic acid was 5.32 g/100 g. The mold dried-cell also contains oleic acid [18:1 (*n*-9)] and linoleic acid at the concentration of 7.28 g/100 g and 2.98 g/100 g, respectively. The vitamin composition and fatty acid composition of the mold dried cell are shown in Tables 1 and 2. Perilla oil-diet: Perilla oil (Nippon Oil & Fats Co., Tokyo, Japan) containing 55% α -linolenic acid and 0.25% total tocopherol was supplemented to the control diet at the rate of 7.5%. The concentration of α -linolenic acid and total tocopherol were 4.125 g/100 g and 1,875 μ g/g, respectively.

The experimental diets were manufactured with an electrical mixer once a month. The diets were subdivided into the daily amount consumed by five gilts, packed in vinyl bags and stored at 4 C. Each diet (1.25 to 1.5 kg/head) was given to five gilt embryo donors twice a day. Water was given *ad libitum*. Each mixed diet was formulated to meet

Table 1. Vitamin composition of Mold dried cell

Vitamin	Content (mg/kg)
Thiamin	5.8
Riboflavin	41.5
Pantothenic acid	68.1
Niacin	1,005
Pyridoxine	8.6
Folic acid	4.1
Total carotene	69.1

Nutrient Requirements of Swine by NRC [9].

Analysis of chemical composition of the diets

Chemical composition of the experimental diets were measured by the standard procedures [10]. Fatty acid composition of each diet was determined by the method described as follows. One gram of the diet was dried at 105 C for 5 h to measure the water content. Total lipid was extracted with 100 ml of a mixture (2 : 1 [v/v]) of chloroform and methanol. The extract was dried under a nitrogen gas stream at 65 C. Total lipid extracts from the diet were esterified with 14% boron trifluoride-methanol at 100 C for 2 min. The methyl esters were then extracted with hexane and stored at -20 C until analysis. The composition of the fatty acid methyl esters was determined by gas-liquid chromatography (GC14-A, Shimadzu, Kyoto, Japan) with a flame ionization detector. The chromatograms were recorded on a thermal-pen recorder (Chromatopack CR-4A, Shimadzu). A capillary column was used for the analysis : 30 m long and 0.32 mm i.d. (Omegawax™, Supelco Japan Ltd., Tokyo). Other components were measured by the conventional methods.

Embryo collection

Twenty-five pubertal LWD, which is crossbred between Landrace, Large White and Duroc gilts, aging 6 months old were assigned to four experimental groups (5 for Safflower oil-diet, 5 for Mold dried cell-diet, 5 for Perilla oil-diet and 10 for the control diet). On approximately 70 and 120 days of the feeding trial, embryo collections were performed on each donor during the periods of heat

stress (from July 22 to September 20). Daily atmospheric temperature changes during the period of the present experiment in Tsukuba are shown in Fig. 1. The average maximum temperatures in July, August and September in 1994 were 30.7 C, 32.7 C and 27.4 C, respectively. The average minimum temperature in July, August and September in 1994 were 22.3 C, 22.6 C and 19.4 C, respectively. The temperatures in the summer of 1994 exceeded the past average temperature in each month. Also, the humidity in July, August and September was 83%, 78% and 87%, respectively. The donors were checked for estrus every day. Those exhibiting estrus were inseminated with diluted semen derived from boars of Landrace or Large White as long as they showed estrus at an interval of 24 h. Embryos were surgically collected by flushing both uterine horns on day 6 (day 1 = the last day of estrus). Briefly, in the first embryo collection from each donor, each ovary and uterine horn were exteriorized and the number of corpora lutea was recorded. A balloon catheter (Fr. size=18) was inserted through an incision made at the cervical end of the uterine horn and the whole uterine horn was flushed in a retrograde manner with 50 ml of M2 solution [11] supplemented with 0.1% BSA fraction V (Sigma Chemical, St. Louis, MO, USA) warmed at 38 C into a sterile 100 ml glass tube. The glass tubes containing flushed medium were held in a water bath at 38 C until embryo search-

Table 2. Fatty acid composition of Mold dried cell

Fatty acid	Composition (%)
14:0	1.3
15:0	0.3
16:0	14.6
16:1 <i>n</i> -7	2.2
17:0	0.5
18:0	1.5
18:1 <i>n</i> -9	36.4
18:2 <i>n</i> -6	13.9
18:3 <i>n</i> -6	26.6
20:0	0.7
Others	2.0
Total	100

Fig. 1. Daily atmospheric temperature change between July 22 to September 20, 1994. From the data of Tsukuba Aerological Observatory, Meteorological Agency, the Ministry of Transport. — — : maximum temperature, — — : minimum temperature.

ing started. The uterine horns and ovaries were returned to the peritoneal cavity and the incision was closed with four layers of sutures. For the second embryo collection donor gilts were slaughtered. Three pigs assigned to Perilla oil-diet group were surgically operated upon twice to collect embryos and at the third embryo collection they were slaughtered. The procedures for embryo flushing were similar to those used in the surgical embryo collection. The embryos were recovered with the aid of a stereomicroscope and the developmental stage and morphology were evaluated. The embryos which showed developmental stages corresponding with day 6 after estrus and normal morphologies were declared the embryos showing normal morphology. Those stages were compaction stage to hatched blastocyst. The control embryos were collected from ten pubertal LWD gilts given the control diet, and evaluated by the same procedures as described above. After all donors were slaughtered, the abdominal subcutaneous fat was collected to analyze the fatty acid composition and stored in liquid nitrogen until lipid extraction started.

Analysis of fatty acid compositions of fats

Fatty acids of lipids in the abdominal subcutaneous fat were esterified according to the KOH/methanol method [12]. The esterified sample was subjected to a gas chromatography with a flame ionization detector (GC-17A, Shimadzu). A capillary column was used for the analysis: 30 m long and 0.32 mm i.d. (Shimadzu). The methyl ester of linoleic acid was confirmed by comparing the retention time with an authentic sample.

Statistical analysis

The general linear models procedure of Statistical Analysis System (SAS®) [13] with least square means was used to analyze the effect of diet on the number of corpora lutea, ova recovered and embryo quality, and to analyze the effect of diet on fatty acid composition of lipids in the abdominal subcutaneous fat. The model for analysis of embryo quality was as follows:

$$Y_{ij} = \mu + D_i + P_j + e_{ij}$$

where μ = overall means,
 D_i = effect of diet,
 P_j = effect of pig,
 e_{ij} = residuals.

Other data were analyzed by the χ^2 test. Signifi-

cant difference was declared at $P < 0.05$.

Results

The chemical composition of the experimental diets are shown in Table 3. The diets were not isonitrogenous, in particular the protein content in the Mold dried cell-diet was higher than those in the other three diets, and the protein content in the Safflower and Perilla oil-diets was still higher than that of the control diet. The Perilla oil-diet contained more crude fat than the other three diets, and the crude fat contents in the Safflower oil-diet and the Mold dried cell-diet were more than the control diet. Therefore, energy intake was not held constant among the dietary groups. Food mass intakes did not differ for the pigs fed Safflower oil-diet, Mold dried cell-diet, Perilla oil-diet or the control diet. Through the experimental period, no deleterious effect on the health conditions of the pigs allocated to each experimental group was observed.

The fatty acid composition of lipids in the abdominal subcutaneous fat of gilts given the diets supplemented with PUFA are shown in Table 4. The compositions positively reflected the compositions of the diets. As the control diet contained corn oil, the content of oleic acid [18:1 (*n*-9)] was relatively higher than the Safflower oil-diet and the content of linoleic acid was similar to the Perilla oil-diet and higher than the Mold dried cell-diet. The content of linoleic acid in the Safflower oil-diet was higher than the other three diets. γ -Linolenic acid was detected only in the Mold dried cell-diet. Similarly, α -linolenic acid was detected only in the Perilla oil-diet.

Embryos were obtained from all of the pigs allocated to each experimental group. Least square means ($\pm$ s.e. of lsmean) of numbers of corpora lutea, ova recovered and embryos showing normal morphology in gilts dietetically given PUFA are shown in Table 5. The number of corpora lutea on the ovaries in gilts given Mold dried cell-diet was greater than that in gilts given the Safflower oil-diet ($P < 0.05$) or Perilla oil-diet ($P < 0.01$). The number of corpora lutea in gilts given Perilla oil-diet was less ($P < 0.05$) than that in gilts given the control diet. The number of ova recovered from gilts given Mold dried cell-diet was more than that in gilts given the control diet

($P < 0.05$), Safflower oil-diet ($P < 0.05$) or Perilla oil-diet ($P < 0.01$). Also, the number of embryos

showing normal morphology in gilts given Mold dried cell-diet was more than that in gilts given

Table 3. Chemical compositions (%) of diets used in the present study

	The control diet	Safflower oil-diet	Mold dried cell-diet	Perilla oil oil-diet
Water	13.4	11.9	10.8	11.5
Crude Protein	13.5	14.8	16.9	14.3
Crude Fat	3.4	7.7	7.6	10.0
Crude Fiber	3.3	4.2	4.2	3.7
Crude Ash	4.6	5.1	6.4	4.8
C14:0	ND	ND	1.0	ND
C16:0	13.5	9.8	17.2	8.8
C16:1	0.4	0.3	1.6	ND
C18:0	2.2	2.4	1.8	2.0
C18:1	26.8	15.0	36.8	19.8
C18:2	54.8	70.9	21.6	20.5
C18:3 γ	ND	ND	18.0	ND
C18:3 α	2.0	1.2	0.8	48.3
C20:1	ND	ND	0.6	ND
miscellaneous	0.3	0.4	0.6	0.6

ND = not detected.

Table 4. Fatty acid composition of lipids in the abdominal subcutaneous fat of gilts fed with the diets supplemented with polyunsaturated fatty acids

Fatty acid	The control diet	Gilts fed with		
		Safflower oil-diet	Mold dried cell-diet	Perilla oil-diet
n	10	5	5	5
C14:0	2.74 $\pm$ 0.11	2.52 $\pm$ 0.10	3.51 $\pm$ 0.07*	2.81 $\pm$ 0.10
C16:0	22.79 $\pm$ 0.77 ^a	18.24 $\pm$ 0.69 ^b	23.63 $\pm$ 0.49 ^{ac}	20.04 $\pm$ 0.69 ^{bd}
C16:1	4.54 $\pm$ 0.57 ^e	2.89 $\pm$ 0.51 ^f	4.69 $\pm$ 0.36 ^{eg}	4.01 $\pm$ 0.51
C18:0	18.54 $\pm$ 1.74 ^h	17.32 $\pm$ 1.55	13.63 $\pm$ 1.10 ⁱ	13.58 $\pm$ 1.55 ^{ij}
C18:1	34.52 $\pm$ 1.83 ^{ko}	28.72 $\pm$ 1.63 ^{lp}	35.45 $\pm$ 1.15 ^{km}	31.06 $\pm$ 1.63 ^{nop}
C18:2	16.89 $\pm$ 1.20 ^q	30.30 $\pm$ 1.07 ^r	13.03 $\pm$ 0.76 ^s	13.64 $\pm$ 1.07 ^{qst}
C18:3 γ	ND	ND	6.07 $\pm$ 0.27	ND
C18:3 α	ND	ND	ND	14.85 $\pm$ 0.38

least square mean $\pm$ s.e. of lsmean.

* $P < 0.001$: Mold dried cell-diet vs the control diet, Safflower oil-diet and Perilla oil-diet, a vs b: $P < 0.0005$, b vs ac: $P < 0.0001$, a vs bd: $P < 0.025$, ac vs bd: $P < 0.0005$, e vs f: $P < 0.05$, f vs eg: $P < 0.01$, h vs i: $P < 0.05$, h vs ij: $P < 0.05$, ko vs lp: $P < 0.05$, lp vs km: $P < 0.005$, km vs nop: $P < 0.05$, q vs r: $P < 0.0001$, q vs s: $P < 0.025$, r vs s: $P < 0.0001$, r vs qst: $P < 0.0001$.

Table 5. Least square means ($\pm$ s.e. of lsmean) of numbers of corpora lutea, ova recovered and embryos showing normal morphology in gilts given dietetically polyunsaturated fatty acids

Diet	n	Corpora lutea	Ova recovered (%)	Embryos showing normal morphology (%)
The control diet	10	13.7 $\pm$ 0.71 ^{ab}	11.9 $\pm$ 0.69* (86.9)	9.9 $\pm$ 1.02* (83.2)**
Safflower oil-diet	10	12.6 $\pm$ 0.71 ^{bd}	11.6 $\pm$ 0.69* (92.1)	10.2 $\pm$ 1.02* (87.9)**
Mold dried cell-diet	10	15.0 $\pm$ 0.73 ^a	14.2 $\pm$ 0.71 (93.3)	14.0 $\pm$ 1.04 (98.6)
Perilla oil-diet	13	11.2 $\pm$ 0.64 ^{cd}	10.3 $\pm$ 0.62** (90.5)	8.9 $\pm$ 0.92** (85.8)**

Values in the same column with different superscripts are significantly different: a vs bd: $P < 0.05$, a vs cd: $P < 0.01$, ab vs c: $P < 0.05$. Mold dried cell-diet is higher than the other three diets: * $P < 0.05$, ** $P < 0.01$.

the control diet ($P<0.05$), Safflower oil-diet ($P<0.05$) or Perilla oil-diet ($P<0.01$). The proportion (98.6%, 137/139) of embryos showing normal morphology to embryos recovered from gilts given Mold dried cell-diet was significantly higher ($P<0.01$) than that in gilts given Safflower oil-diet (87.9%, 102/116), Perilla oil-diet (85.8%, 115/134) or the control diet (83.2%, 99/119). Some embryos showed abnormal morphology indicating retardation in development and some degenerative signs, other were unfertilized ova.

Discussion

The Fatty acid composition of lipids in the abdominal subcutaneous fat showed that the relative amounts of ingested unsaturated fatty acids could be absorbed without significant degradation.

To express their biological activities, linoleic acid [18:2 (*n*-6)] and α -linolenic acid [18:3 (*n*-3)] must be desaturated by $\Delta 6$ -desaturase and then further metabolized. Since it was reported that the $\Delta 6$ -desaturase reaction is inhibited by adrenal glucocorticoid [14], the $\Delta 6$ -desaturase reaction may be inhibited by any type of stress. Plasma adrenocorticotrophic hormone as well as adrenal corticoid hormone levels have been shown to be markedly increased in heat-stressed pigs [15, 16]. It may therefore be preferable that metabolites such as γ -linolenic acid [18:3 (*n*-6)], eicosapentaenoic acid [20:5 (*n*-3)] and docosahexaenoic acid [22:5 (*n*-3)] after $\Delta 6$ -desaturase reaction are directly administered to animals affected by stress. However, so far it has been very difficult to apply these three essential PUFA to experiments using livestock because of their high costs. In the present study, the effect of supplementing fatty acids-enriched mold dried cell to the diet of pubertal female gilts was examined under heat stress on ovulation rate and embryo quality, and compared to supplementation with linoleic acid or α -linolenic acid.

When the results obtained from the control group were compared with our results obtained in previous seasons, significant differences were not apparent in ovulation rate, proportions of ova recovered and embryos showing normal morphology. Therefore, it might be possible that the pigs used in the present study received enough stress to produce a deleterious effect on follicle growth, ovulation, fertilization and early embryo

development. However, the ovulation rate, the proportions of ova recovered and embryos showing normal morphology in pigs given Mold dried cell-diet were significantly higher than those in pigs given other diets. Only the Mold dried cell-diet contains γ -linolenic acid among the experimental diets although it has oleic acid and linoleic acid as well. The accurate mechanism by which gilts given Mold dried cell-diet ovulated and produced embryos showing normal morphology at a higher rate in the summer season than gilts given the other diets is not clear. One possible explanation is that the metabolites of PUFA after $\Delta 6$ -desaturase reaction may be essential components in the mechanism of follicle growth, ovulation, fertilization and early embryo development. The beneficial effect during follicle growth, peri-ovulation and early embryo development periods might be attributed to an effect of the dietary supply of γ -linolenic acid by-passing the $\Delta 6$ -desaturase reaction. It might be reasonable to consider that pigs used in the present study were affected by some stress, possibly by heat-stress.

As shown in Tables 1 and 2, the mold dried cell has a variety of vitamins, other fatty acids and amino acids (data not shown). Therefore, the possibility that these other components in the mold dried cell had a beneficial effect on the ovulation rate and embryo quality cannot be denied.

These results indicate that the dietary administration of fatty acids-enriched mold dried cell containing oleic acid, linoleic acid and γ -linolenic acid may be beneficial for improving ovulation rate and embryo quality. Supplementation of the fatty acids-enriched mold dried cell may be a promising candidate for overcoming heat stress. However, for the accurate evaluation of the effect of dietary administration of the fatty acids-enriched mold dried cell on pig reproduction it will be necessary to conduct a more controlled experiment.

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