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Effect of high fat diet on artificial oocyte activation following superovulation in mice

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Summary

The aim of the present study was to determine the effects of increased dietary intake and high fat diet (HFD) in mice on artificial oocyte activation by using puromycin or roscovitine. Six-week-old mice were fed as either a control diet group, an increased dietary intake group or an HFD group for 4 weeks. Oocytes were obtained following superovulation and were divided into three treatment groups (no activation treatment, calcium ionophore and puromycin treatment, and calcium ionophore and roscovitine treatment) and were incubated for 4 h. Retrieved oocytes and numbers of oocytes activated as assessed by morphological changes were compared among the three treatment groups. The proportion of degenerated oocytes in HFD mice was significantly higher than that in control diet mice. The rates of activation in oocytes treated with roscovitine were 90.3% in control diet mice, 89.8% in increased dietary intake mice and 67.9% in HFD mice. The rate of activation in oocytes treated with roscovitine in HFD mice was significantly lower than the rates in control diet mice and increased dietary intake mice. The rates of activation in oocytes treated with puromycin were 90.6% in control diet mice, 94.0% in increased dietary intake mice and 71.4% in HFD mice, and the rate of activation in oocytes treated with puromycin in HFD mice was significantly lower than the rates in control diet mice and increased dietary intake mice. HFD-induced obesity deteriorated induction of oocyte activation by roscovitine or puromycin in mice.

Keywords: High fat diet, Oocyte activation, Puromycin, Roscovitine

Introduction

Oocyte activation has been induced by various types of physical and chemical stimulation, including electric pulses (Prochazka *et al.*, 1993), ethanol (Kubiak, 1989), calcium ionophore (Ware *et al.*, 1989), inhibitors of protein synthesis (Fulka *et al.*, 1991) and puromycin (Nakasaka *et al.*, 2000; Nakagawa *et al.*, 2001a). In humans, oocyte activation with calcium ionophore and puromycin has been reported to result in successful pregnancy in patients with repeated failed fertilization following intracytoplasmic sperm injection (ICSI) (Murase *et al.*, 2004). Roscovitine has also been used to arrest meiotic activation in various animals (Jimenez-Macedo *et al.*, 2006; da Silvia Rascado *et al.*, 2010), and oocyte activation by combined treatment with calcium ionophore and roscovitine has been reported in cats and mice (da Silvia Rascado *et al.*, 2010, Iba *et al.*, 2011).

Reproductive function in females is affected by obesity at various stages including ovulation, oocyte development, embryo development, endometrial development, embryo implantation and fetal development (Brewer & Balen, 2010). There are several

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lines of evidence for negative effects of excessive metabolites, such as glucose (Bermejo-Alvarez *et al.*, 2012), lipids (Leroy *et al.*, 2010; Wonnacott *et al.*, 2010) and leptin (Arias-Alvarez *et al.*, 2011), on oocyte and embryo development in animal models. In high fat diet (HFD)-induced mice, it has been reported that ovulation rate, embryo development, placental function, ovarian function and mitochondrial function in mice were affected by an HFD (Minge *et al.*, 2008; Igosheva *et al.*, 2010; Jungheim *et al.*, 2010; Cardozo *et al.*, 2011). However, Bermejo-Alvarez *et al.* (2012) reported that the number of oocytes in HFD-fed mice was the same as that in control diet-fed mice and that embryo development was normal in HFD-fed mice. In humans, inconsistent findings regarding the effects of obesity on oocytes and embryos have also been reported. Dokras *et al.* (2006) reported that retrieved oocytes and metaphase II oocytes in obese women were significantly fewer than those in normal-weight women despite the fact that the numbers of developing oocytes were the same. However, Carrell (2001) reported that difference in the numbers of retrieved oocytes and metaphase II oocytes between obese women and normal-weight women were not statistically significant. It has been reported that the quality of embryos in obese women was significantly poorer than that in lean women (Carrell, 2001), but another group found this difference only in women less than 35 years of age (Metwally *et al.*, 2007). Several groups have also demonstrated that there was no significant correlation between embryo quality and BMI (Spandorfer *et al.*, 2004; Shalom-Paz *et al.*, 2011).

Therefore, the effects of obesity on oocytes and the embryo have been controversial in animal models and humans. In addition, to the best of our knowledge, the influence of obesity on artificial activation of oocytes in mice with HFD-induced obesity has not been reported. The aim of the present study was to determine the effects of increased dietary intake and HFD on artificial oocyte activation by using puromycin or roscovitine.

Materials and methods

Induction of obesity and breeding

All animal experiments were conducted in accordance with the ethical standards of the Animal Care and Use Committee of the University of Tokushima Graduate School. Six-week-old female B6C3F1 mice (Japan SLC Inc., Shizuoka, Japan) were randomly divided into a control diet group (12 mice), increased dietary intake group (12 mice) and HFD group (12 mice). Mice were fed 35 g/cage/day and 120 g/cage/day of a low-dose irradiation diet (Oriental Yeast Co. Ltd, Tokyo, Japan) for 4 weeks as control diet group and increased dietary intake group, respectively. Mice in the HFD group were fed 110 g/cage/day of HFD-60 (Oriental Yeast Co. Ltd, Tokyo, Japan) for 4 weeks.

Oocytes

After 4 weeks, cumulus-enclosed oocytes were recovered from the oviducts of mice that had received an intraperitoneal injection of 10 IU of pregnant mare's serum gonadotropin (Sigma, St. Louis, MO, USA) followed 48 h later by 10 IU of human chorionic gonadotropin (Wako, Osaka, Japan) at 14 h prior to sacrifice by cervical dislocation. The cumulus masses were dispersed in 0.1% hyaluronidase (Sigma, St. Louis, MO, USA) in modified human tubal fluid (mHTF; Nippon Medical & Chemical Instruments Co., Ltd. Osaka, Japan) medium at 37°C in an atmosphere of 5% CO_2 in air for 8–10 min. The cumulus-free oocytes were collected and rinsed twice in fresh mHTF medium prior to use. The numbers and morphological appearance of recovered oocytes were compared among the three groups.

Chemicals

Stock solutions of 1 mg/ml calcium ionophore A23187 (Sigma-Aldrich, Tokyo, Japan), 10 mg/ml puromycin (Sigma, St. Louis, MO, USA) and 10 mg/ml roscovitine (Sigma, St. Louis, MO, ISA) were stored at –20°C. Prior to each experiment, these chemicals were diluted with mHTF containing 0.4% bovine serum albumin (BSA) to the following concentrations: 5 μM calcium ionophore A23187, 10 μg/ml puromycin and 50 μM roscovitine.

Oocyte activation

Oocyte activation was performed in accordance with a previous report (Nakasaka *et al.*, 2000). After cumulus-free oocytes had been treated with 5 μM calcium ionophore in mHTF medium for 5 mins, the oocytes were divided into the following three groups: (1) a no activation treatment group, in which oocytes were cultured in mHTF medium with 0.4% BSA for 4 h; (2) a calcium ionophore and puromycin group, in which oocytes were cultured in mHTF medium with 0.4% BSA containing 5 μM calcium ionophore and 10 μg/ml puromycin for 4 h; and (3) a calcium ionophore and roscovitine group, in which oocytes were cultured in mHTF medium with 0.4% BSA containing 5 μM calcium ionophore and 50 μM roscovitine for 4 h. Oocytes were cultured at 37°C in an atmosphere of 5% CO_2 in air. Oocyte activation was observed at ×400 magnification under an inverted microscope (IMT2–31 Olympus Diaphot, Tokyo, Japan) equipped with Nomarski differential interference contrast. Activated oocytes were defined as oocytes with at least one pronucleus. The rates of

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Table 1 Oocytes in control diet, increased dietary intake and HFD mice

	Body weight (g)	Number of retrieved oocytes	Number of degenerated oocytes (%)	Number of M-phase oocytes (%)
Control diet mice	31.7 ± 3.6	285	33 (11.6)	252 (88.4)
Increased dietary intake mice	32.4 ± 3.6	266	24 (9.0)	242 (91.0)
HFD mice	43.4 ± 4.7[a,c]	271	48 (17.7)[a,b]	223 (82.3)[a,b]

HFD: high fat diet. Body weight: mean ± standard deviation.
[a]$P < 0.05$ versus control diet mice. [b]$P < 0.01$ versus increased dietary intake mice. [c]$P < 0.05$ versus increased dietary intake mice.

Table 2 Serum levels of glucose, triglyceride, free fatty acid and total cholesterol in control diet, increased dietary intake and HFD mice

	Glucose (mg/dl)	Triglyceride (mg/dl)	Free fatty acid (μEO/l)	Total cholesterol (mg/dl)
Control diet mice	119.2 ± 31.9	90.2 ± 59.4	1114.0 ± 197.7	101.2 ± 14.2
Increased dietary intake mice	122.8 ± 24.6	87.8 ± 24.0	1092.7 ± 200.0	85.0 ± 18.1
HFD mice	201.8 ± 48.5[a]	61.3 ± 9.2	717.8 ± 152.7[b]	112.5 ± 9.1[c]

HFD: high fat diet. Values are mean ± standard deviation.
[a]$P < 0.05$ versus control diet mice. [b]$P < 0.05$ versus control diet mice and increased dietary mice. [c]$P < 0.01$ versus increased dietary mice.

oocyte activation were compared among control diet mice, increased dietary intake mice and HFD mice.

Assessment of blood glucose and lipid profiles

Blood samples for measurements of glucose and lipid profiles were taken from fasting mice on the day before the experiment. Samples obtained were frozen at −40°C until used for analysis. Serum levels of glucose, triglyceride, free fatty acid and total cholesterol were measured.

Statistical analysis

Rates of oocyte activation were evaluated using the chi-squared test. Differences in body weights and serum parameters among control diet mice, increased dietary intake mice and HFD mice were determined by analysis of variance (ANOVA). Results with *P*-values less than 0.05 were considered to be statistically significant.

Results

Body weights

As can be seen in Table 1, the mean body weight of HFD mice was significantly greater than the mean body weights of control diet mice and increased dietary intake mice ($P < 0.05$).

Numbers of oocytes and proportions of metaphase II oocytes following superovulation

As can be seen in Table 1, total numbers of superovulated oocytes in control diet mice, increased dietary intake mice and HFD mice were 285, 266 and 271, respectively. There were no significant differences in total number of superovulated oocytes among the three groups. The proportions of metaphase II oocytes following superovulation in the three groups were 88.4% (252/285), 91.0% (242/266) and 82.3% (223/271), respectively. The proportions of degenerated oocytes following superovulation in the three groups were 11.6% (33/285), 9.0% (24/266) and 17.7% (48/271), respectively. The proportions of degenerated oocytes following superovulation in HFD mice were significantly higher than those in control diet mice and increased dietary intake mice ($P = 0.04$ and $P = 0.003$, respectively).

Serum levels of glucose, triglyceride, free fatty acid and total cholesterol

The mean glucose level in HFD mice was significantly higher than those in control diet mice and increased dietary intake mice ($P = 0.019$ and $P = 0.032$, respectively) (Table 2). Total cholesterol level in HFD mice was significantly higher than that in increased dietary intake mice ($P = 0.008$). Free fatty acid level in HFD mice was significantly lower than those in control diet mice and increased dietary intake mice ($P = 0.002$ and $P = 0.017$, respectively).

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Table 3 Proportions of oocytes activated in control diet, increased dietary intake and HFD mice

	Percentage of oocytes activated (*n*)		
	No treatment	Roscovitine	Puromycin
Control diet mice	0 (0/60)	90.3 (56/62)	90.6 (58/64)
Increased dietary intake mice	0 (0/51)	89.8 (44/49)	94.0 (47/50)
HFD mice	0 (0/52)	67.9 (36/53)[a,b]	71.4 (40/56)[a,b]

HFD: high fat diet,
[a]$P < 0.01$ versus control diet mice. [b]$P < 0.01$ versus increased dietary intake mice.

Appearance of activated oocytes

As can be seen in Table 3, oocytes with only calcium ionophore treatment were not activated during the 4-h incubation period. The rates of activation in superovulated oocytes treated with calcium ionophore and roscovitine were 90.3% (56/62) in control diet mice, 89.8% (44/49) in increased dietary intake mice and 67.9% (36/53) in HFD mice. The rate of activation in calcium ionophore and roscovitine-treated oocytes in HFD mice was significantly lower than the rates in control diet mice and increased dietary intake mice ($P = 0.003$ and $P = 0.007$, respectively). The rates of activation in superovulated oocytes treated with calcium ionophore and puromycin were 90.6% (58/64) in control diet mice, 94.0% (47/50) in increased dietary intake mice and 71.4% (40/56) in HFD mice. The rate of activation in calcium ionophore and puromycin-treated oocytes in HFD mice was significantly lower than the rates in control diet mice and increased dietary intake mice ($P = 0.007$ and $P = 0.002$, respectively). The degree of suppression of activation in roscovitine-treated oocytes and that in puromycin-treated oocytes in HFD mice were not significantly different.

Discussion

In the present study, the rates of degenerated oocytes in HFD mice were significantly higher than that in control diet mice, although there was no significant difference in total numbers of retrieved oocytes. Results of previous studies on the effects of obesity on follicle development and ovulation are conflicting. It has been reported that ovarian follicles following superovulation were significantly more apoptotic and that mature oocytes following superovulation were smaller and fewer in obese mice than in control diet mice (Cardozo *et al.*, 2011; Jungheim *et al.*, 2010). The oocytes following superovulation in mice with diet-induced obesity showed increases in mitochondrial DNA content, generation of reactive oxygen species and an oxidated redox state (Igosheva *et al.*, 2010). However, another study showed that diet-induced obesity did not affect the number of oocytes that naturally ovulated (Bermejo-Alvarez *et al.*, 2012). Minge *et al.* (2008) reported that the natural ovulation rate in HFD mice was increased compared to that in control diet mice. A difference in the degrees of obesity due to diets including HFD in mice might be involved in the difference in these results. Also, the discrepancy of these results may be due to naturally ovulated oocytes and superovulated oocytes. In humans, the effect of obesity on oocyte maturity is also controversial. Retrieved oocytes and metaphase II oocytes in obese women were significantly fewer than those in normal-weight women despite the fact that the numbers of developing follicles were the same in the IVF cycles (Dokras *et al.*, 2006). Carrell (2001) reported that the number of metaphase II oocytes retrieved in obese women undergoing IVF was decreased, but the difference was not statistically significant. A large prospective study did not demonstrate a weight-related reduction in the number and maturity of oocytes retrieved in the IVF-ICSI cycles (Bellver *et al.*, 2010).

To date, the influence of HFD on oocyte activation has not been reported. We firstly found that the number of activated oocytes treated with calcium ionophore and roscovitine or puromycin in HFD mice was significantly smaller than the numbers in control diet mice and increased dietary intake mice, suggesting that HFD deteriorates induction of artificial oocyte activation. HFD may influence various reproductive stages including fertilization and embryo development. In previous studies, fertilization rates in naturally ovulated oocytes in HFD mice were shown to be similar to those in control diet mice (Robker, 2008; Cardozo *et al.*, 2011). It has been reported that naturally ovulated oocytes from HFD mice exhibited slower development of the embryo (Minge *et al.*, 2008; Cardozo *et al.*, 2011). In humans, it has been reported that fertilization rates of the IVF cycles in overweight and obese women were lower than those in normal-weight women (Salha *et al.*, 2001; van Swieten *et al.*, 2005), while other studies did not show a weight-related reduction in fertilization rate of the IVF cycles (Dokras *et al.*, 2006; Bellver *et al.*, 2010). Also, results of studies regarding the

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effect of obesity on embryo quality were inconsistent. While Carrell (2001) showed that the quality of the embryo was significantly decreased in obese women undergoing IVF, other studies showed that there were no significant differences in quality of the embryo among the BMI strata in patients undergoing *in vitro* maturation and IVF (Shalom-Paz *et al.*, 2011; Spandorfer *et al.*, 2004).

The mechanism for suppression of oocyte activation by roscovitine and puromycin in HFD mice has not been clarified. The arrest of meiosis at metaphase II in mammals is maintained by high levels of M-phase-promoting factor (MPF) and a cytoplasmic factor that stabilizes MPF activity in metaphase II oocytes. Oocyte activation is characterized by an increase in pulsatile of intracellular calcium due to binding of spermatozoa with oocytes and by decreases in activities of MPF and MPF kinase. MPF is a heterodimer composed of a cdc2 kinase subunit and a regulatory cyclin B subunit. Roscovitine, which is a specific inhibitor of cyclin-dependent protein kinase, prevents p34cdc2 dephosphorylation and inhibits MPF kinase activity (Meijer & Kim, 1997). Conversely, puromycin, which is a protein synthesis inhibitor, is responsible for reducing cytostatic factor synthesis and thus indirectly decreasing MPF activity, although the action of puromycin in oocyte activation has not been fully clarified. Thus, both roscovitine and puromycin are associated with a decrease in MPF activity. In HFD mice, suppression of MPF activity may be attenuated by roscovitine or puromycin. Further study on the effect of roscovitine or puromycin on MPF activity in HFD mice may be needed.

We speculated that increases in circulating lipidemic factors might have adverse effects on degeneration of oocytes and oocyte activation in HFD mice. However, contrary to our expectation, serum triglyceride and free fatty acid levels in HFD mice were lower than those in the other two groups in this study. Our results are supported by the results of a previous study showing that the existence of ovaries, i.e. presence of ovarian steroid hormones, prevents aggravation of HFD-induced dyslipidemia in female mice (Ludgero-Correia *et al.*, 2012). Thus, other unexamined factors, particularly omega-3 and omega-6 polyunsaturated fatty acids, induced by HFD might have been involved in the increase in degeneration of oocytes and decrease in oocyte activation in this study. Oocyte activation by roscovitine and puromycin might be involved in glucose metabolism rather than lipid metabolism. Further study on the difference in oocyte activation by insulin resistance may be needed.

If unfertilized oocytes after ICSI are properly activated, they may form two pronuclei with extrusion of the second polar body and then cleave and develop normally. It has been demonstrated that the combination of calcium ionophore and puromycin could effectively salvage unfertilized oocytes after ICSI (Nakagawa et al 2001a; Lu *et al.*, 2006) and that the use of this combination was an effective method for producing human haploid parthenogenones (Nakagawa, *et al.*, 2001b). Murase *et al.* (2004) reported that a successful pregnancy outcome was achieved by oocyte activation with calcium ionophore and puromycin in patients with previous repeated failed fertilization following ICSI. Obesity due to intake of high fat food in women may influence oocyte activation by using puromycin at ICSI.

There are some limitations in this study. First, we could not follow up the parthenotes to the blastocyst stage. A study for further stages of oocytes may be needed. Second, to date, results of studies regarding the effects of omega-3 and omega-6 polyunsaturated fatty acids on ovarian function and oocyte quality have been inconsistent (Santos *et al.*, 2008; Wonnacott *et al.*, 2010), and their effects on artificial oocyte activation have not been reported. A study regarding artificial oocyte activation by using omega-3 and omega-6 polyunsaturated fatty acids is needed. In addition, the mechanism by which a HFD affects the process of oocyte activation is needed. Recently, Ge *et al.* (2013) reported that DNA methylation of imprinted genes in oocytes was not altered in HFD mice, while DNA methylation of metabolism-related genes was changed (Ge *et al.*, 2013).

In conclusion, HFD-induced obesity deteriorated induction of oocyte activation by roscovitine and puromycin in mice.

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Statement of interest

The authors hereby that there are no conflicting interests.

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