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Leptin supplementation in embryo culture medium increases in vivo implantation rates in mice

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Abstract: Leptin is a hormone-like protein consisting of 167 amino acids. The aim of this study is to compare the effects of different leptin concentrations on in vitro and in vivo embryo development rates. In vitro development rates were investigated by embryo culture studies, and in vivo implantation rates and the quality of embryos were assessed by embryo transfers to the recipient mice. The results showed that addition of leptin into the embryo culture medium at 10 and 100 ng/mL doses supported the in vitro development of mouse embryo. Moreover, leptin increased the total cell number of blastocyst, particularly the trophectoderm cells. In vivo assessment showed a significant increase in the proportion of the embryos implanted in 10 and 100 ng/mL groups, compared to the control group. In conclusion, leptin supplement in embryo culture medium increases implantation rates in mice.

Key words: Mouse, embryo culture, leptin, in vitro development, in vivo development

1. Introduction

Leptin, a metabolic hormone, regulates the reproductive functions responding to both nutritional and body condition (1). In female mice, leptin administration before puberty has accelerated the transition to puberty (2,3). Serum leptin levels vary during the menstrual cycle: the highest levels are observed in the preovulation period, while the lowest levels are seen in the luteal phase (4–6). Previous studies revealed that supplementation of the embryo culture medium with leptin promoted the in vitro development of preimplantation mouse embryos, and markedly increased the total cell numbers (7,8). Herrid et al. (9) reported that the development rate of blastocyst significantly increased by media supplemented with 10 and 100 ng/mL, but not with 0.1 and 1 ng/mL of leptin, compared to the control group. In addition, Fedorcsák et al. (10) found that leptin enhanced embryonic development via signal transducer and activator of transcription 3 (STAT3). In another study, Swain et al. (11) investigated the direct effects of leptin on the mouse reproductive system and reported that leptin increased progesterone, estradiol, and testosterone secretion by increasing steroidogenesis. On the other hand, the authors found that leptin did not affect mouse oocyte maturation. Also, Schulz et al. (12) demonstrated that leptin had a role in the human trophoblast cell development and in the formation of placenta in early gestation.

In the literature, there are several studies using farm animals. Craig et al. (13) showed that leptin promoted porcine oocyte maturation prior to implantation and supported embryonic development. It was also demonstrated that leptin supported bovine oocyte maturation and in vitro apoptotic effects decrease in developing blastocyst (9,14). In another study, leptin was found to have a cumulus cell-mediated role in the regulation of oocyte maturation in a horse model (15).

In the literature, there is no publication about the in vivo fetal development of blastocysts developed by in vitro addition of leptin into the culture media. The aim of the study was to investigate the effects of different leptin concentrations on in vitro and in vivo embryo development in mice.

2. Materials and methods

All mice experiments and animal care protocols were approved by Koç University Local Ethics Committee for Animal Experiments (approval number: 2013 - 06). The animals were kept in Koç University, Animal Research Facility of Center for Translational Medicine (KUTTAM) under 12 h light–12 h dark cycle, and a diet of commercial pellet food ad libitum and automatic water containers were provided.

2.1. Embryo collection

CB6F1 (C57BL/6j × BALB/c) female mice (n = 24) were intraperitoneally injected (IP) with 10 IU of equine

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chorionic gonadotropin (eCG, Sigma) and after 48 h, the mice were injected (IP) with 10 IU of human chorionic gonadotropin (hCG; Organon) and CB6F1 female mice were allowed to mate with breeding male mice CB6F1 (n = 6) . Next morning, mated mice were detected by using vaginal plug method. Female mice (n = 16) that showed plugs were, then, sacrificed and pronuclear embryos were collected by rupture of oviductal ampulla. Oocytes were obtained by washing with HEPES buffered-Human Tubal Fluid (HTF, with HEPES) medium + 80 IU/mL hyaluronidase three times and further washing with 4 mg/mL bovine serum albumin (BSA) (16,17). Superovulation treatments used on different days and each experiment was repeated at least four times.

2.2. In vitro culture

In petri dishes, 10 µL of embryo culture drops were formed and drops covered with mineral oil (SAGE) completely to prevent contamination and evaporation and to preserve integrity of drops. At least 2 h prior to embryo collection, the culture media was kept in high humidity incubator at 37 °C in 5% CO_2 for air gassing. Harvested oocytes cultured in Quinn's Advantage cleavage medium supplemented with 4 mg/mL BSA at 37 °C with an atmosphere of 5% CO_2 until two cell stage. The two cell embryos were randomly divided into 3 groups and cultured in Quinn's Advantage blastocyst medium supplemented with 4 mg/mL BSA (Sigma A3311) + 0, 10, and 100 ng/mL of leptin (Leptin Human, Sigma) at 37 °C with an atmosphere of 5% CO_2 until the blastocyst stage. Two cell embryo number of 0, 10, and 100 ng/mL leptin groups were 99, 92, and 113, respectively (Table 1).

2.3. Differential labeling of blastocyst cell number

Blastocysts were incubated with 100 µg/mL propidium iodide (PI) + HTF media + 1% Triton X-100 solution for 10–12 s and then transferred into 100 µg/mL 100% ethanol + 25 µg/mL Hoechst (Sigma 33258) solution for overnight incubation at 4 °C. Next day, blastocysts washed in a 5 µL of glycerol drop and mounted in these drops with coverglass. Stained blastocyst samples were examined under epifluorescence inverted microscope and the numbers of trophectoderm and inner cell mass (ICM) were calculated (18,19). Four blastocysts were used in each group for each experiment.

2.4. Embryo transfer

CD1 female recipient mice mated with vasectomized CD1 males that were observed to have a vaginal plaque were accepted as being at 0–0.5 days and they were used for uterine embryo transfer at 2.5 or 3.5 days that follow. Pseudopregnant mice were intraperitoneally injected (IP) with Xylazine (5–10 mg/kg) + Ketamine (80–100 mg/kg) anesthesia for 30–40 min. Then, an incision line (0.5–1 cm) was made and the uterine horn was taken out from the right fossa paralumalis region. A total of 10 to 15 embryos at the expanded blastocyst stage were transferred into uterine horn of each pseudopregnant mouse for the in vivo development rate. The skin wound was closed by 5.0 number surgery suture. After operation, all the animals were kept in postoperative care station in sterile rodent cage until they woke up. On day 14.5 of pregnancy, the recipient mice were sacrificed and the resorption (absorption site) and living fetuses were detected (16,17). Four recipient mice were used in each group for each experiment.

Table 1. Effect of leptin supplementation on in vitro embryo development rates.

Group	Number of embryos (n)	Blastocyst rate (%) (number of blastocysts)
0 ng/mL	99	83.26 ± 1.69^{a} (82)
10 ng/mL	92	97.37 ± 1.45^{b} (90)
100 ng/mL	113	95.14 ± 1.81^{b} (103)

[ab]: Differences between values marked with different characters in the same column are significant ($P < 0.05$).

2.5. Statistical analysis

Statistical analysis was performed using the SPSS for Windows version 22.0 (IBM Corp., Armonk, NY, USA). Analysis of variance (ANOVA) and Bonferroni's post hoc analysis were performed to analyze significant differences between the groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. In vitro development

The blastocyst development numbers of 0, 10, and 100 ng/mL leptin groups were 82 (83.26%), 90 (97.37%), and 103 (95.14%), respectively (Table 1). There was a significant difference in the in vitro development rates between the control group and leptin groups ($P < 0.05$).

3.2. Differential labeling of blastocyst cell number

According to the differential fluorescence labeling, the mean cell number of each group was calculated as 58.68 ± 2.05, 70.33 ± 6.94, and 78 ± 2.87 in 0, 10 and 100 ng/mL leptin groups, respectively. The mean cell number of trophectoderm of each group was calculated as 46 ± 2.16, 56.67 ± 5.25, and 62.33 ± 2.62 in 0, 10 and 100 ng/mL leptin groups, respectively. The ICM of each group were determined as 12.67 ± 1.70, 13.67 ± 2.62, and 16 ± 1.63, respectively (Table 2). There were significant differences in the total cell number and mean cell number of trophectoderm between leptin and control groups ($P < 0.05$). However, there was no significant difference in the ICM cell number between the leptin and control groups ($P > 0.05$).

3.3. In vivo embryo development rates

After embryo transfer towards evaluation of in vivo development; 5 (13.51%), 19 (48.72%), and 16 (41.03%) implantation areas were detected in 0, 10, and 100 ng/mL leptin groups, respectively, indicating a significant difference between the leptin and control groups ($P < 0.05$) (Table 3). After embryo transfer; the resorption numbers of 0, 10, and 100 ng/mL leptin groups were 4 (10.81%), 12 (30.77%), and 8 (20.52%), respectively. There were no significant differences in the resorption rates between the groups ($P > 0.05$).

4. Discussion

There are several studies in the literature investigating the effects of leptin on mouse embryo culture prior to implantation. Kawamura et al. (7,8) reported that leptin (1, 10, 100, and 1000 ng/mL) supported in vitro growth from two-cell stage to the blastocyst stage. Herrid et al. (9) cultured mouse embryos with 1, 10, 100, or 1000 ng/mL leptin to assess in vitro growth rate until the blastocyst stage and reported that only 10 mg/mL leptin supported in vitro growth. We found that addition of leptin into the in vitro culture media at 10 and 100 ng/mL promoted the in vitro development.

Leptin has been reported to increase the total number of blastocysts, mainly number of trophectoderms in mice (7,8). Jia et al. (20) showed that the addition of 1 or 10 ng/mL leptin significantly increased the total number of blastocyst cells on day 8 of culture and trophectoderm cells group. In the presented study, using differential labeling, we found a statistically significant increase in the total cell number in the leptin groups, compared to the control group. Similarly, the number of trophectoderm cells was found to increase significantly in leptin groups.

The female ob/ob mice have a complete absence of circulating leptin, which results in deficiency of implantation and pregnancy. This can be repaired by the administration of leptin (21). Yang et al. (22) cultured mouse embryos with 0.3, 3, and 300 ng/mL leptin until blastocyst stage and the results showed that 300 ng/mL leptin had the highest implantation rate when blastocysts transferred to in vitro culture model of endometrial coculture. This is the first study on the investigation of in vivo development rate of embryos in the leptin added embryo culture. On the other hand, in our study, we investigated in vivo implantation model by mouse uterus transfer. In this study, the rate of in vivo implantation significantly increased in the 10 and 100 ng/mL leptin groups.

In the present study, embryo culture media supplemented with 10 and 100 ng/mL leptin were found to support in vitro mouse embryo development. Trophectoderm cell lineage plays an important role in development for placenta (23). Leptin significantly enhanced the rate of blastocyst and the number of trophectoderm cells in vitro. In addition, an increase in the implantation rates on day 14.5 was observed in 10 and 100 ng/mL leptin groups compared to the control group. Therefore, leptin promoted in vitro development, embryo quality, and in vivo development in these embryos.

Table 2. Cell number of blastocysts according to differential fluorescence labeling.

Group	Mean of total cell number	Blastocyst number	Means of trophectoderm cell number	Mean of inner cell mass number
0 ng/mL	58.68 ± 2.05[a]	4	46.00 ± 2.16[a]	12.67 ± 1.70[a]
10 ng/mL	70.33 ± 6.94[b]	4	56.67 ± 5.25[b]	13.67 ± 2.62[a]
100 ng/mL	78.00 ± 2.87[b]	4	62.33 ± 2.62[b]	16.00 ± 1.63[a]

[ab]: Differences between values marked with different characters in the same column are significant ($P < 0.05$).

Table 3. In vivo embryo developments rates.

Group	Transferred blastocysts number	Implanted embryo number (%)	Resorptions numbers (%)
0 ng/mL	37	5 (13.51 ± 50) [a]	4 (10.81% ± 40) [a]
10 ng/mL	39	19 (48.72 ± 32) [b]	12 (30.77% ± 22.28) [a]
100 ng/mL	39	16 (41.03 ± 15) [b]	8 (20.52% ± 987) [a]

[ab]: Differences between values marked with different characters in the same column are significant ($P < 0.05$).

Based on the results of the present study, a useful model can be developed for veterinary and human medical sciences. Embryo production is of utmost importance for stockbreeding (i.e. bovine and sheep). Increasing the production of healthy embryos through the use of leptin would contribute to the reclamation and development of national stockbreeding. In the future, leptin-supplemented culture media can be used in several reproductive biotechnology areas, such as somatic cell nuclear transfer, in vitro fertilization, or intracytoplasmic sperm injection. Therefore, we believe that further studies which would employ in vitro and in vivo developmental methods together to identify the effects of leptin on different animal species would contribute to the literature.

In conclusion, leptin supplementation of in vitro culture improved in vivo implantation rate by particularly increased trophectoderm cell numbers and embryo quality.

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