

Research Article

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Treatment of mice with maternal intermittent fasting to improve the fertilization rate and reproduction

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Summary

Maternal intermittent fasting (MIF) can have significant effects on several tissue and organ systems of the body, but there is a lack of research on the effects on the reproductive system. So, the aim of our study was to analyze the effects of MIF on fertility. B6C3F1CrI (C57BL/6N × C3H/HeN) male and female mice were selected for the first part of the experiments and were analyzed for body weight and fat weight after administration of the MIF intervention, followed by analysis of sperm counts and activation and embryo numbers. Subsequently, two strains of mice, C57BL/6NCrI and BALB/cJrJ, were selected and administered MIF to observe the presence or absence of vaginal plugs for the purposes of mating success, sperm and oocyte quality, pregnancy outcome, fertility status and *in vitro* fertilization (IVF). Our results showed a significant reduction in body weight and fat content in mice receiving MIF intervention in B6C3F1CrI mice. Comparing the reproduction of the two strains of mice. However, the number of litters was increased in all MIF interventions in C57BL/6NCrI, but not statistically significant. In BALB/cJrJ, there was a significant increase in the number of pregnant females as well as litter size in the MIF treatment group, as well as vaginal plugs, and IVF. There was also an increase in sperm activation and embryo number and the MIF intervention significantly increased sperm count and activation. Our results suggest that MIF interventions may be beneficial for reproduction in mice.

Introduction

Infertility is an observed health problem in 10–15% of couples (Gnoth *et al.*, 2005; Garolla *et al.*, 2020). Previously, most reproductive failures were thought to be caused by problems with the female partner, but the current view is increasingly that it is a two-pronged cause, with 30–50% of infertility being caused by male factors (Inhorn and Patrizio, 2015). In contrast, advances in reproductive assistive technology have had a marked improvement in the symptoms of the disease (Matzuk and Lamb, 2008). Most infertile couples worldwide do not have access to IVF due to its absence, inaccessibility, or unaffordability. In some countries, IVF clinics are lacking, while in many others, the high cost *in vitro* fertilization is a barrier. In response, clinicians have initiated efforts to introduce 'low-cost *in vitro* fertilization' (LCIVF) in resource-poor settings (Inhorn and Patrizio, 2015). Therefore, there is still a lack of effective treatments for specific diseases, so it is important to find and realize the clinical translation of new treatments for infertility treatment (Snow *et al.*, 2022).

Sperm cryopreservation and *in vitro* fertilization (IVF) of oocytes are essential procedures in contemporary mouse facilities (Takeo *et al.*, 2022). Sperm cells are intricate, and their quality and fertilization capacity can be affected by various biochemical, physiological, and morphological factors (Jeyendran *et al.*, 2019). The coordination of follicular development and ovulation involves gonadotropins, growth factors, transcription factors, and signalling pathways. While cautioning against the negative effect of phytoestrogens and environmental estrogens on follicular development, it is also suggested that preserving the size of the primordial follicular pool could potentially extend ovarian function lifespan and mitigate the effects of menopause-associated diseases such as osteoporosis, cardiovascular disease, and gynaecologic cancers (Dey, 2010; Hellemans *et al.*, 2019; Rix and Cutler, 2021).

Energy restriction, as a novel means of intervening in the metabolic state of an organism, has been widely used in a variety of diseases (Madeo *et al.*, 2019), including the promotion of weight loss, the improvement of organismal health, and the extension of animal lifespan, and intermittent fasting has attracted much attention as a solution for the realization of energy restriction (Pifferi and Aujard, 2019; Alidadi *et al.*, 2021; Rhoads and Anderson, 2022). Despite the interest in intermittent fasting as a lifestyle proposal for humans, the real potential lies in understanding the mechanisms and translating them (Rhoads and Anderson, 2022).

Intermittent fasting interventions have also been found to play an important role in the regulation of the diseases of ageing (Hofer *et al.*, 2022). Therefore, the role and mechanisms of intermittent fasting in a wide range of diseases require further study.

Intermittent fasting as a means of energy restriction also has significant effects on reproduction (Vasim *et al.*, 2022). In the 1930s, McCay *et al.* first found that intermittent fasting increased the lifespan in rats with restricted food intake at weaning or 2 weeks postweaning (McCay *et al.*, 1989). To date, intermittent fasting is generally recognized as extending mean and maximum lifespan and delaying age-associated deleterious alternations in strains ranging from yeast to mammals (Xiang *et al.*, 2012). There is still significant controversy over the effects of CR on reproduction, with some studies suggesting that it is detrimental to female reproduction, but others suggesting that it is beneficial (Moatt *et al.*, 2016). Selesniemi *et al.* reported that adult-onset CR was able to maintain the activity of the reproductive axis in aged female mice. The current view is that it is mainly beneficial for follicular maturation (Selesniemi *et al.*, 2008). However, considering the possible hazards of high-intensity intermittent fasting to the organism, this study used maternal-intensity intermittent fasting to study the effects on reproduction (Papakonstantinou *et al.*, 2022; Günbatar *et al.*, 2023).

CR is often reported to be associated with delayed puberty and reduced fertility (McShane and Wise, 1996; Bordone and Guarente, 2005; Mao *et al.*, 2021). This CR setting is generally between 30% and 60% (Weindruch and Sohal, 1997), so we hypothesized that a more maternal CR setting (20%) might segregate reproductive effects (Weindruch and Sohal, 1997), that is, effects that might improve reproductive function.

Materials and methods

Animals

The animals were maintained and used in compliance with Chinese animal welfare legislation. The local Advisory Committee for Institutional Animal Care and Research approved all experiments, which were also licensed by the Office for Consumer Protection and Food Safety of the Animal Welfare Service of the State of Lower Saxony (reference number: FSSD2–2332).

C57BL/6NCrl and B6C3F1Crl (C57BL/6N × C3H/HeN) mice were purchased from Charles River, Sulzfeld, Germany, while BALB/cJrj mice were purchased from Janvier, Le Genest-Saint-Isle, France, at 4–6 weeks of age. B6C3F1Crl female mice were used as mating partners for treated male mice. The mice were allowed to acclimatize to the new conditions for 2 weeks and were housed at the Central Animal Facility of the Hannover Medical School in individually ventilated cage systems in type 25 cages (Bioregion, Ramsgate, Kent, UK) under standard conditions (mean temperature of $22 \pm 2^\circ\text{C}$; $50 \pm 10\%$ relative humidity; 14 h:10 h, light:dark cycle) on sterile aspenwood pellet beds (AsBe-wood GmbH, Ahrensfeld, Germany). They received a commercial pellet diet (Altromin 1314 TPF, Altromin GmbH, Lage, Germany) and *ad libitum* water from an automated watering system. The mice were kept in sibling groups or in threes (one male with two females).

The animals had been tested according to Federation of European Laboratory Animal Science Associations (FELASA) recommendations and were free of listed microorganisms. Body condition scores were monitored to determine the health status of the animals. Pain or distress was identified using the Rat Ghostface Scale, while normal behaviours (respiratory rate, grooming,

nesting, response to environmental stimuli) were observed. Control animals had free access to food and water throughout the procedure. The 24-h food and water intake of the two groups of dams was calculated by weighing the food and water containers simultaneously each day; consumption was calculated as the difference in weight. The pairing method is as follows: MIF female (20 mice) × wt male (10 mice), wt female (20 mice) × MIF male (10 mice), MIF female (20 mice) × MIF male (10 mice) and wt female (20 mice) × male (10 mice). The minimum number of pairs of paired mice in each group was 10.

Every other day intermittent fasting protocol: 4-week-old female mice were fasted every other day for 12 weeks. These 16-week-old female mice were then mated and resumed normal feeding (MIF group). Control female mice were fed *ad libitum* (Control group). At the end of the experiment, the following tissue samples were harvested from offspring: plasma, intestine and its digesta, liver, retroperitoneal white adipose tissue (rWAT), epididymal white adipose tissue (eWAT) or parametrial white adipose tissue (pWAT), and subcutaneous white adipose tissue (sWAT).

Sperm evaluation

The normal diet group ($n = 10$) and the MIF diet group ($n = 10$) underwent a 12-week intervention. After execution, the epididymis was prepared and transferred to a medium containing $1 \mu\text{l}$ human tubal fluid (HTF; Chemicon MR-500-D; Hofheim, Germany). The epididymis was cut into pieces with fine scissors. Sperm were allowed to swim out of the tissue for 7–15 min at room temperature. The supernatant was used for sperm counting.

Preparation of overactivated spermatozoa

Spermatozoa were collected from the epididymal tail of male mice; overactivated spermatozoa were prepared with modifications as previously described (Fujinoki *et al.*, 2006). The capacitation medium used was modified Tyrode's lactate albumin pyruvate (mTALP) medium. A drop of spermatozoa was obtained by puncturing the epididymis using a 26 g (0.45-mm) needle (Terumo Co., Ltd., Tokyo, Japan) (Maleszewski *et al.*, 1995). Approximately $3 \mu\text{l}$ of epididymal tail spermatozoa was placed on a 35-mm diameter Petri dish (Iwaki, Asahi Glass Co., Ltd, Tokyo, Japan) and 3 ml of culture medium was added. The spermatozoa were incubated at 5°C for 37 min to activate and the supernatant containing the activated spermatozoa was placed in a new Petri dish containing the vector or RU486. After 5 min of incubation, the supernatant was transferred to a dish and sperm were incubated at 4°C and $37\% \text{CO}_2$ for 5 h to induce hyperactivation. The maximum concentration of carrier (ethanol and purified water) used for all experiments was 0.2%.

Measurement of motility and hyperactivation

Motility and hyperactivation were measured as described previously (Sugiyama *et al.*, 2019). The motility of active spermatozoa was recorded on a DVD recorder (RDR-HX50; Sony Corp., Tokyo, Japan) using a microscope attached to a microscope (IX3; Olympus Corp., Tokyo, Japan) in a small carbon dioxide incubator (MI-IBC; Olympus). Visual analysis of the films consisted of manual counting of total number, motility number, and 10 different fields of overactivated spermatozoa. For all experiments, visual analysis was performed in a blinded manner. Motile sperm exhibiting asymmetric and flagellar motility were

considered to be overactivated (Fujinoki *et al.*, 2016; Sugiyama *et al.*, 2019). The percentage of motile and motile overactivation was defined as motile sperm/total sperm count \times 100 and overactivated sperm/total sperm count \times 100, respectively. Each experiment was repeated four times with four mice. If the percentage of motile sperm was \leq 80%, the experiment was repeated.

Viability kinetics using the Sperm Viability Analysis System (SMAS)

Sperm viability kinetics were assessed using the SMAS. Spermatozoa were hyperactivated in the presence or absence of P 'Preparation of hyperactivated spermatozoa'. After 2 h of incubation, the suspension containing motile spermatozoa (15 μ l) was transferred to an observation chamber (0.1 mm deep, 18 mm in width, 18 mm long) made of repair tape attached in two parallel strips to a slide covered with a coverslip (Sugiyama *et al.*, 2019). Sperm motility was recorded on a rigid for 1 s using a high-speed digital camera (HAS-L2; Ditect) attached to a microscope (ECLIPSE E2000; Nikon Corporation, Tokyo, Japan) with phase contrast illumination, a 650 nm band-pass filter, and a warming plate (MP10DM; Kitasato Co., Ltd, Shizuoka, Japan). SMAS analyzed 10 consecutive images obtained from a single field of view at \times 150 magnification. SMAS automatically calculated linear velocity (VSL; μ m/s), curvilinear velocity (VCL; μ m/s), mean path velocity (VAP; μ m/s), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH; μ m), and beat-crossing frequency (BCF; Hz) using the manually computed wobble coefficient (WOB; defined as VAP/VCL)²⁹. The SMAS analysis was repeated four times using four different mice and more than 300 spermatozoa were detected. Only active spermatozoa were judged significance was analyzed (Mortimer, 1997).

Embryo collection and *in vitro* culture

For embryo collection and *in vitro* culture, oviducts of PEG-positive females were excised on day 0.5 of pregnancy and placed in a Petri dish (ϕ 30 mm; Nunc GmbH + Co. KG, Wiesbaden, Germany) containing 500 μ l of phosphate-buffered medium (PB1). Oviduct–oocyte complexes were prepared from juxtapositions as described by Nagy *et al.* (see Brownstein, 2003). The oviducts were then incubated in M16 culture medium (Sigma M7292; Whittingham, 1971; Brownstein, 2003).

Oocytes were counted and kept in M16 medium (Sigma M7292) for further *in vitro* development. They were incubated at 37°C, 5% CO₂ and 50 \pm 5% humidity in a four-well Petri dish (Nunc). The number of fertilized oocytes was assessed by counting two-cell embryos the next day.

Oestrous cycle continuity

Oestrous cycle continuity was evaluated by vaginal smears. Vaginal washes with 50 ml of isotonic solution were performed, and samples were immediately placed on slides to determine the stage of the cycle microscopically using the following guidelines: pro-oestrus was indicated by predominating nucleated epithelial cells; oestrus by predominating non-nucleated cornified cells; metoestrus by a mixture of nucleated epithelial cells, cornified cells, and leukocytes; and dioestrus by predominating leukocytes (Frick *et al.*, 2000). These observations were carried out daily throughout the whole experimental period.

Number of fetuses

The number of fetuses was determined on the 15th day of pregnancy by quantifying the fetuses present in both horns of the uterus in treated females and those housed with treated males.

Number of fetuses and number of corpora lutea

After sacrifice, both ovaries were obtained from each female and the number of corpora lutea (CL) was quantified by watching them under a stereoscopic magnifying glass (Olympus SZ30, Tokyo, Japan). This number was then compared with the number of fetuses present in the uterus, and the ratio was expressed as a percentage of fertilized ova (number of fetuses in the uterus \times 100, divided by the number of CL).

Statistics and reproducibility

Animal experiments were independently repeated at least twice with consistent results. Plots and statistical analyses were performed using Prism software, and experimental results are shown as standard error of the mean (SEM) \pm mean. Statistical significance of differences between groups was analyzed using *t*-test or one-way/two-way analysis of variance (ANOVA) followed by Tukey's multivariate post hoc analysis. A *P*-value $<$ 0.05 indicates statistical significance.

Results

The role of MIF in mice metabolism

Compared with the control group, MIF-intervened mice showed a significant reduction in body weight and food intake. Additionally, there was a slight reduction in the amount of fat in perirenal white adipose tissue (rWAT), parawombic white adipose tissue (pWAT), and subcutaneous white adipose tissue (sWAT). However, there was no significant change in liver weight (Figure 1A–F).

The effect of MIF on pregnancy

In adult females treated with MIF, there was a significant reduction the number of corpora lutea in the ovaries and the number of fetuses in the uterus of each animal compared with control females injected with isotonic solution for the same period (Table 1).

Pregnancy index was assessed in Figure 1. MIF administration also significantly improved the derived from 11-week-old mice compared with the control group (55.3% vs. 39.3%, *P* = 0.0024). After MIF treatment from 9-week-old mice, the percentage of 2-cell embryos was 51.26 % in the MIF-treated group and 35.64% in the control group (*P* $<$ 0.0001). For 11-week-old mice, to evaluate the possible effects of the treatment with MIF on the reproductive capability of mice, different reproductive parameters were analyzed in Figure 2. The chronic administration of MIF to adult male mice during a period that included a spermatogenic cycle and epididymal migration, in addition to the period that they were housed with females until mating, provoked a clear downward tendency in the ability of these animals to impregnate females (per cent effectiveness). MIF administered to adult females for 30 days did not affect the number or duration of each phase of the oestrous cycles that occurred during the treatment period.

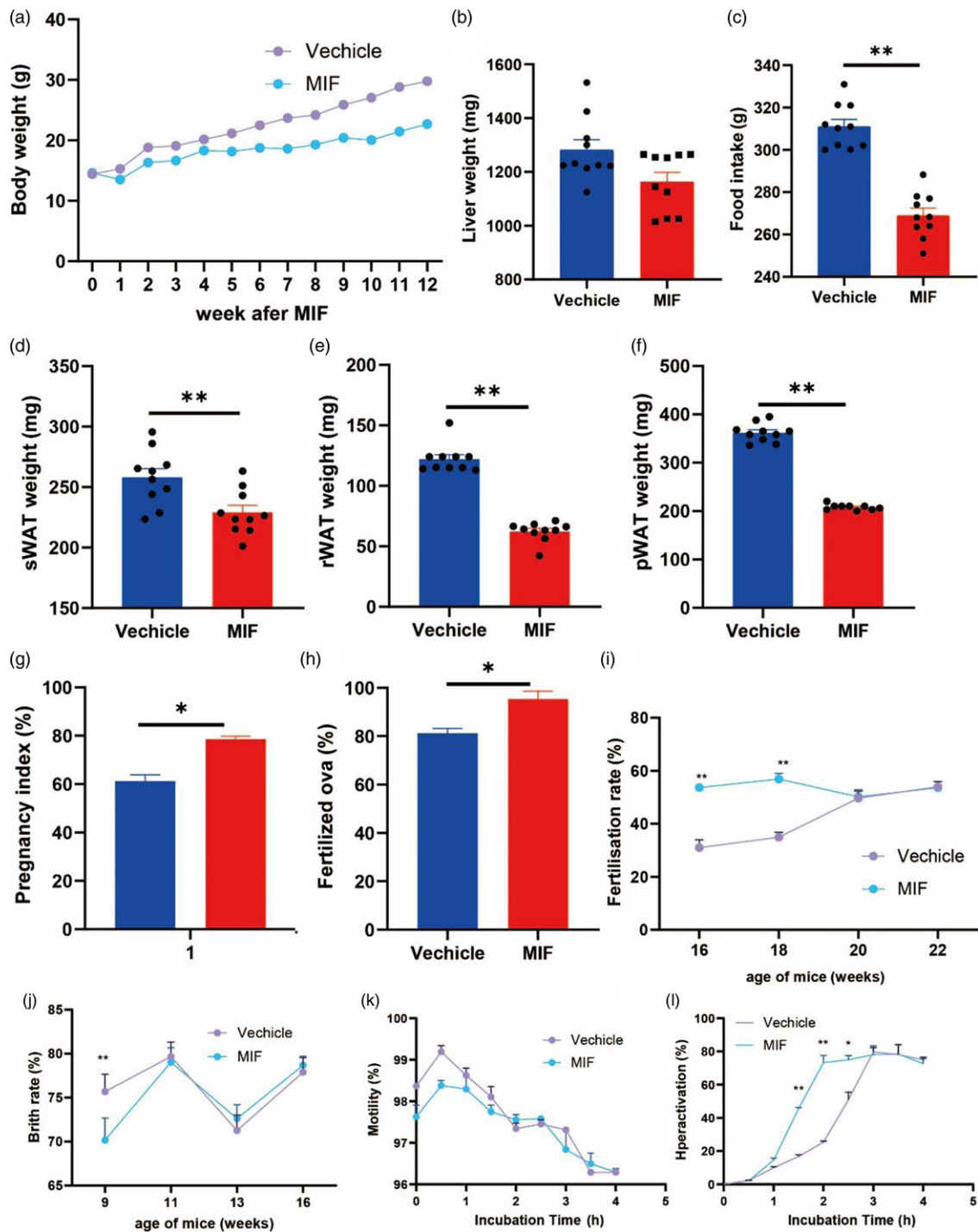


Figure 1. MIF interferes with metabolic status in mice. (A) Body weight; (B) liver weight; (C) food intake; (D) sWAT weight; (E) rWAT weight; (F) pWAT weight; (G) pregnancy index; (H) fertilized ova; (I) fertilization rate in female mice that ended the MIF intervention and were initiated for pairing; (J) birth rate; (K) motility; (L) hyperactivation. eWAT, epididymal white adipose tissue; MIF, maternal intermittent fasting; rWAT, retroperitoneal white adipose tissue; pWAT, parametrial white adipose tissue; sWAT, subcutaneous white adipose tissue. * $P < 0.05$, ** $P < 0.01$. Note: Body weight measurements were taken from the first week of the start of the MIF intervention and continued for 12 weeks. Changes in the pairing results of MIF-intervened male and female mice versus normal-dieted male and female mice are depicted in (A–H), respectively. (I, J) depicts mice at 16 weeks of age after the start of the pairing.

The effect of MIF on pregnancy weaning and pups in female mice of different strains

We analyzed synchronized reproductive cycles in BALB/cJrj females. Four days later, they were in oestrus and paired with MIF-treated or control males for 1 week. Each male ($n = 10$ /

treatment group) was matched with two females. The number of pregnant females was significantly increased in C57BL/6N male MIF treatment. However, there was no statistically significant increase in litter size for all MIF interventions (Tables 2 and 3).

Table 1. Effects of MIF administration on cyclicity and duration of the estrous cycles and maternal metabolism in mice

	Control (10)	MIF (10)	P-value
Number of estrous cycles	4.65 ± 0.52	4.35 ± 0.35	0.35
Length of estrous cycles (days)	6.38 ± 0.31	7.22 ± 0.75	0.02
Length of estrous phase (days)	2.15 ± 0.12	1.27 ± 0.13	0.01
Length estrous/length cycles (%)	35.62 ± 3.58	26.35 ± 4.25	0.03

The effect of MIF on fertilization rate, and reproduction

We further analyzed fertilization rates, reproductive behaviour, and competence by matching each male with two females and checking the females for vaginal plugs (VP) the next morning. In the MIF intervention group, the number of presenting apparent VP was significantly higher. The number of VP-positive females increased if males and females were from the same strains. BALB/cJrj females and C57BL/6N had higher VP occurrence after MIF treatment. Females with VP were executed to collect oocytes. Oocytes were kept in culture overnight to assess their developmental competence. In C57BL/6N mice, treatment with either hormone increased the number of fertilized oocytes, independent of the genetic background of the female mating partner. MIF treatment of BALB/cJrj males resulted in an increase in fertilization rate if mated to BALB/cJrj females but a decrease in fertilization rate if mated to BALB/cJrj females. MIF treatment of BALB/cJrj males resulted in an increase in fertilization rate independent of female stress; however, the difference was not significant (Table 4).

The effect of MIF on sperm status

To evaluate the effects of MIF on reproduction and sperm hyperactivation in mice, we analyzed different reproductive parameters in male mice. Our results showed a significant increase in sperm counts for C57BL/6NCrl after MIF intervention; however, there was no significant effect on sperm counts for BALB/cJrj mice. We also analyzed the effect of sperm viability and showed a significant increase in the number and percentage of motile spermatozoa in C57BL/6NCrl and BALB/cJrj mice after MIF intervention treatment (Tables 5 and 6).⁷

Discussion

MIF has received much attention as a weight loss programme within the last decade (Varady *et al.*, 2021, 2022). Intermittent fasting has been shown to have a significant effect in reducing body weight, improving insulin sensitivity, blood pressure and oxidative stress in obese adults (Sutton *et al.*, 2018). However, there is a lack of research on reproductive health. In our study, we first found that MIF had a significant effect on body weight and adiposity in mice and, second, sperm count and motility were significantly increased by MIF intervention. For VP, pregnancy rates were significantly increased, but C57BL/6N mice showed no significant change in litter size, while BALB/cJrj showed no significant difference in reproductive rate.

The effect on the body's metabolic state is currently considered the most important function of intermittent fasting, but this is also

associated with the form of intermittent fasting (Masoro, 2005). Previous studies have found that 20% energy restriction does not have a significant effect on body weight, and that weight loss is often proportional to energy intake (Li *et al.*, 2023; Yin *et al.*, 2023). Over a 12-month period, the alternate-day fasting group and the daily calorie restriction group were 7% below their baseline weight at 6 months, while the control group remained near their baseline weight (Trepanowski *et al.*, 2017; Vasim *et al.*, 2022). Intermittent fasting also improves cardiometabolic risk factors such as blood pressure, cholesterol levels, insulin resistance, and HbA1c (Varady *et al.*, 2022). Intermittent fasting protocols are hypothesized to influence metabolic regulation through effects on (i) circadian rhythm biology, (ii) the gut microbiome, and (iii) modifiable lifestyle behaviours (e.g. sleep) (Patterson and Sears, 2017; Fanti *et al.*, 2021). Our study confirmed that MIF significantly improved body weight and adiposity in mice, consistent with previous findings (Bartke *et al.*, 2002; Bartke, 2005).

MIF is energy restricted in a way that mimics the way Muslim women eat during the month of Ramadan (Yin *et al.*, 2023). They found that MIF led to a significant increase in the incidence of low-birth-weight babies, impaired placental efficiency, as well as upregulation of gene expression for placental amino acid transport (Alkhalefah *et al.*, 2021). This type of eating has the potential to lead to fetal growth restriction *in utero* (Alkhalefah *et al.*, 2021). Yin *et al.* found that maternal MIF was also found to significantly affect hepatic DNA methylation in offspring (Yin *et al.*, 2021). Jayet *et al.* (2010) found that prolonged IF prior to pregnancy disrupts gut homeostasis in the offspring, subsequently leading to disturbances in glucose and lipid metabolism. This is accomplished by reducing beneficial microbiota (e.g. *Lactobacillus intestinalis*) (Liang *et al.*, 2023). Maternal IF does not affect resting cardiovascular, metabolic, or renal function; however, when challenged by dietary salt load, male IF offspring are more susceptible to kidney injury (Alkhalefah *et al.*, 2022). Possible causes are associated with circadian rhythm disruption, maternal sleep deprivation and stress, changes in feeding patterns, or dehydration. However, this study focused on the effects of pregnancy and offspring, and there is less research on the effects of MIF on fertility (Darmaun, 2021).

Sperm hyperactivation is crucial for fertilization, facilitating zona pellucida penetration and sperm movement through the oviductal lumen (Sharif *et al.*, 2022; Takei, 2022). Hyperactivation may also facilitate the release of sperm from the oviductal reservoir and may propel sperm through the oviductal lumen and mucus in the stroma of the oviduct (Zhu *et al.*, 1994; Chang and Suarez, 2010). Hyperactivation is characterized by high amplitude, asymmetric flagellar bending (Suarez, 2008; Zaferani *et al.*, 2021). In our study to investigate the effect on reproduction, considering that hyperactivation is associated with fertilization energy acquisition (Sherrer *et al.*, 2004; Coy and Avilés, 2010), we first analyzed the relationship of MIF on hyperactivation, and found that MIF enhanced sperm hyperactivation. In addition, hyperactivation tends to be associated with IVF success in C57BL/6NCrl mice. However, hyperactivation induced by MIF intervention tends not to affect *in vitro* fertilization success in BALB/cJrj mice. In addition, the MIF intervention also significantly improved sperm count and motility in mice of different strains. For example, BALB/cJrj mice showed no significant difference in reproductive rate. We observed a large number of unfertilized oocytes, while the number of oocytes was within the expected range, so we concluded that a significant decrease in the number or quality of spermatozoa produced was associated with the high

Table 2. Percentage of females with a visible copulatory plug on the morning after mating

Treatment	B6 × B6	B6 × F1	C × C	C × F1
Control (10)	52.36 ± 2.68	76.95 ± 11.25	31.28 ± 11.26	36.95 ± 14.62
MIF (10)	66.95 ± 6.35	76.82 ± 11.36	53.62 ± 3.62	71.09 ± 13.76

Table 3. Fertilization ability after treatment with MIF

Treatment	B6 × B6	B6 × F1	C × C	C × F1
Control (10)	44.614 ± 4.89	77.98 ± 4.89	18.64 ± 3.66	35.84 ± 13.65
MIF (10)	76.98 ± 8.65	79.65 ± 11.08	17.65 ± 6.25	44.25 ± 6.35

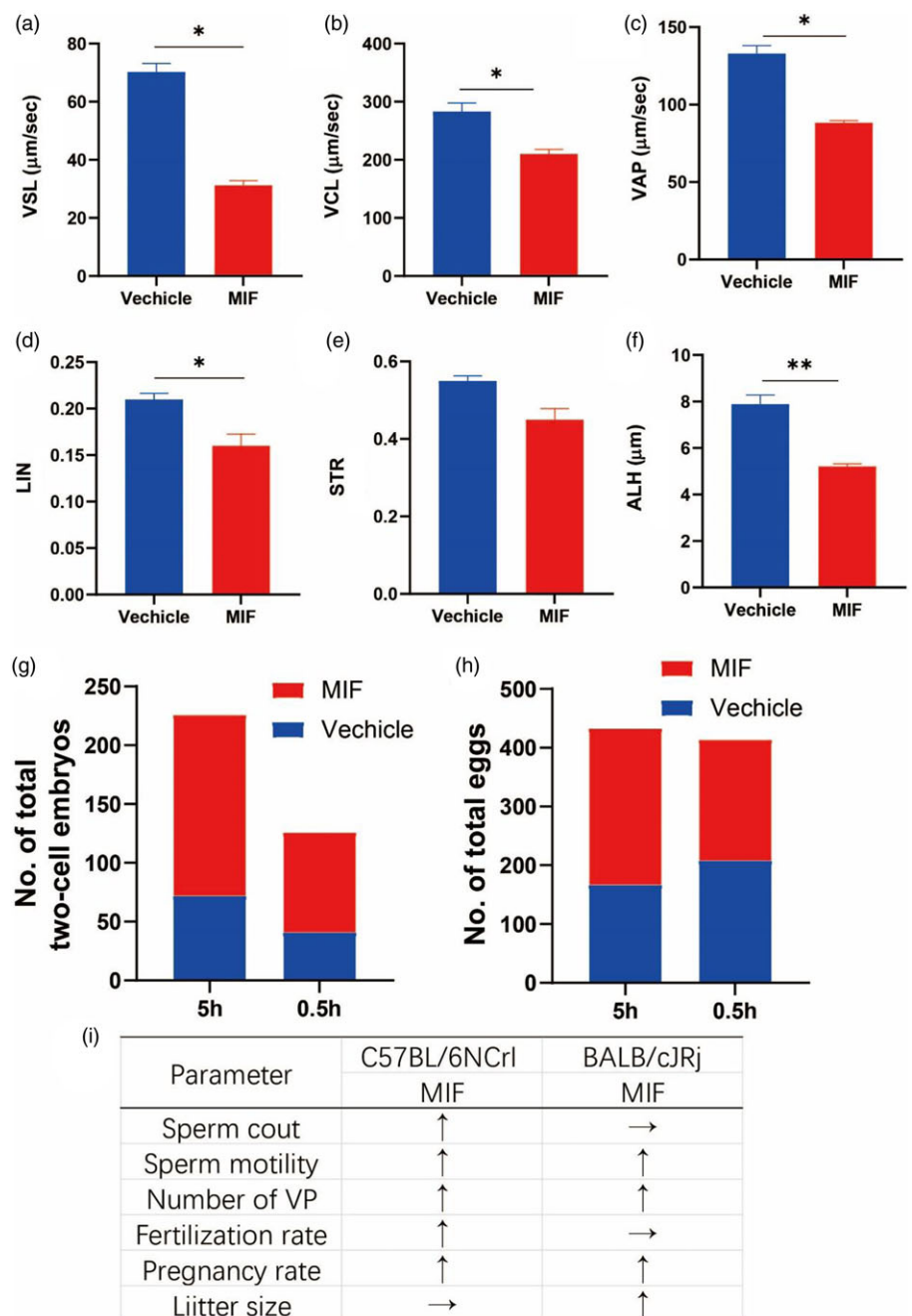


Figure 2. The role of MIF in development of pregnancy. (A) VSL; (B) VCL; (C) VAP; (D) LIN; (E) STR; (F) ALH. (G) Number of two-cell embryos; (H) Number of total eggs. (I) Summary of fertilization rate and reproduction indicator. ALH, amplitude of lateral head displacement; LIN, linearity; STR, straightness; VAP, mean path velocity; VCL, curvilinear velocity; VSL, linear velocity. **P* < 0.05, ***P* < 0.01.

Table 4. Litter size after treatment with MIF

Treatment	C57BL/6NCrI	BALB/cJRj
Control (10)	4.68 ± 1.15	9.36 ± 0.56
MIF (10)	4.99 ± 1.36	12.39 ± 1.36
P-value	0.056	0.025

Table 5. Sperm count after MIF treatment

Treatment	C57BL/6NCrI	BALB/cJRj
Control (10)	21.22 ± 4.69	16.75 ± 5.67
MIF (10)	28.69 ± 1.99	16.02 ± 1.99
P-value	0.035	0.095

Table 6. Sperm motility after MIF treatment

Treatment	C57BL/6NCrI		BALB/cJRj	
	Motile sperm ($\times 10^3$)	Percentage of motile sperm (%)	Motile sperm ($\times 10^3$)	Percentage of motile sperm (%)
Control (10)	5.88 ± 1.42	26.35 ± 2.65	7.44 ± 1.36	51.36 ± 1.36
MIF (10)	11.36 ± 2.62	36.58 ± 2.26	9.36 ± 0.85	66.51 ± 2.68
P-value	0.032	0.042	0.025	0.044

number of spermatozoa abnormalities in BALB/cJRj mice (Ohta *et al.*, 2009; Vasudevan *et al.*, 2010).

Two strains of mice were used in our study and the main differences were in genetic background and variability in melanin. Gautier *et al.* (2021) found that melanins are pigments used by fungi to withstand various stresses and to strengthen vegetative and reproductive structures. Melanosome plays a key role in the light wavelength-dependent response of melanophores, flexibly mediating the transmission of light wavelength information between photoreceptors and melanophores (Mizusawa *et al.*, 2018). Wu *et al.* (2009) also found that the role of melanin-concentrating hormone (MCH) in regulating energy balance and of GnRH and kisspeptin in triggering puberty and maintaining fertility, MCH may provide a critical link between energy balance and reproduction directly at the level of the kisspeptin-activated vGluT2-GnRH neuron (Wu *et al.*, 2009). Increasing melanin in the diet can also affect reproductive outcomes (Scheu and Simmerling, 2004). MCH gene expression varies based on the reproductive cycle, which might be associated with the long-term regulation of MCH in energy balance (Xu *et al.*, 2019).

MIF can help improve reproductive ageing (Carey and Molleman, 2010). Previous studies on naturally ageing mice, and studying the role of MIF in reproduction, found that fasting every other day for 1 month was sufficient to improve oocyte quality. MIF not only increased sinus follicle number and ovulation, but also enhanced oocyte meiotic competence and embryonic development by improving nuclear and cytoplasmic maturation of maternally aged oocytes (Chico-Sordo *et al.*, 2021; Secomandi *et al.*, 2022; Liu and Gao, 2023). Beneficial effects of MIF have been demonstrated by attenuating structural

abnormalities of spindle and chromosome segregation errors and maintaining correct cytoplasmic organelle reorganization (Zeydan, 2021). In addition, single-cell transcriptome analyses indicated that restoration of nicotinamide adenine dinucleotide (NAD⁺)/Sirt1-mediated antioxidant defences mediated the positive effects of MIF on aged oocytes, resulting in elimination of excess accumulation of ROS to inhibit DNA damage and apoptosis (Li *et al.*, 2023). Our study found that, for MIF, there was a significant improvement in fertility. This is also consistent with previous studies (Martin *et al.*, 2009).

There are some limitations in our study. The first limitation is the variability in metabolic rate between mice and humans. The general opinion is that the metabolic rate of mice is seven times higher than that of humans (Perlman, 2016). So there is an effect of different metabolic states on the organism and the response to fasting. In future studies we may add clinical studies to further confirm the effects of maternal fasting during pregnancy on reproduction.

In conclusion, our study found that MIF intervention had a significant ameliorative effect on fertility and sperm viability in mice. This improvement was different in different strains of mice and, for C57BL/6N mice, except that litter size was not affected by MIF intervention. Whereas sperm count and fertilization rate were not affected in BALB/cJRj mice.

Data availability statement. The data used to support the findings of this study are included in the article.

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Author contributions. Conceptualization: YNW; XL; RTG; YZ; Data curation: XL; RTG; YZ; Formal analysis: XL; RTG; YZ; Funding acquisition: XL; RTG; YZ; Investigation: XL; RTG; YZ; Methodology: XL; RTG; YZ; Project administration: XL; RTG; YZ; Resources: RTG; YZ; Software: RTG; YZ; Supervision: RTG; YZ; Validation: RTG; YZ; Visualization: RTG; YZ; Writing – original draft: RTG; YZ; Writing – review & editing: RTG; YZ.

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Ethics approval and consent to participate. No application

Consent for publication. We all agree to publication.

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