Reprod Med Biol (2015) 14:117–121
DOI 10.1007/s12522-015-0203-y

ORIGINAL ARTICLE

# In vitro fertilization embryo development from caffeine-treated murine sperm

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Received: 17 June 2014 / Accepted: 13 February 2015 / Published online: 27 February 2015
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**Abstract**
*Purpose* To evaluate the effect of long-term caffeine administration on murine sperm and subsequent in vitro fertilization (IVF).
*Methods* Male mice were injected with various doses (0, 0.2 and 1.0 mg/mouse/day) of caffeine for 1 month. After sperm collection, the IVF rate and embryo development to the blastocyst stage were evaluated.
*Results* The mean body weight significantly decreased in the 1.0 mg/day treatment group compared to the control group ($P < 0.01$). Testicular weight and histological features did not differ, and total blood testosterone was no different in spite of the difference between 0.2 and 1.0 mg/day of caffeine. The IVF rate differed significantly between the control group [100/105 (95.2 %)] and 0.2 mg/day group [106/121 (87.6 %)] ($P < 0.05$). Furthermore, blastocyst formation was significantly and dose-dependently lower with higher caffeine levels: control group: 85/100 (85.0 %); 0.2 mg/day group: 84/106 (79.2 %) ($P < 0.05$); 1.0 mg/day group: 64/102 (62.7 %) ($P < 0.001$).
*Conclusions* Caffeine treatment affected body weight of male mice. However, testicular weight, histological features and total blood testosterone concentration were not statistically different. In addition, following IVF using sperm from these mice, blastocyst formation decreased in a dose-dependent manner. These findings suggest that embryo development from oocytes fertilized with sperm from caffeine-administered male mice is negatively affected.

**Keywords** Blastocyst formation · Caffeine · Hatching · In vitro fertilization · Mouse sperm

## Introduction

The consumption of caffeine (1,3,7-trimethylxanthine), which is found in coffee, tea, chocolate and some soft drinks, particularly cola-containing beverages, is high in the industrialized world [1–3]. Caffeine intake has been associated with increased risk of spontaneous abortions and some studies have reported a decrease in female fertility [4–8], although both these associations remain controversial.

On the other hand, sperm motility is enhanced by adding caffeine in vitro [9, 10], and no negative reports on male fertility have been found to date [11–13]. Previous studies of caffeine intake on semen quality have yielded contradictory results [14–17]. One interesting study found an association between male caffeine intake and embryo reduction in utero; and while caffeine intake had no impact on semen quality, it was associated with an increase in serum testosterone [13]. To date, no studies have investigated associations between in vitro fertilization (IVF) and sperm from caffeine-treated mice.

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We therefore investigated embryo development following IVF using the sperm of caffeine-treated male mice.

## Materials and methods

### Caffeine treatment of mice

Caffeine-(trimethyl-13$C_3$) (99 % pure) was purchased from Wako Chemical Industries (Osaka, Japan). The caffeine was dissolved into 0, 2 and 10 mg/ml saline solutions, which were then aliquoted and stored at −20 °C. Prior to injection, the tubes were thawed at room temperature. The treatment consisted of a 0.2 ml intraperitoneal injection every other day; a 2-day dose was therefore injected at each concentration, which resulted in an average of 0, 0.2 and 1.0 mg per mouse per day.

### Sperm collection and testicular weight and histophysiological examination

Mature Jcl:ICR strain 8-week-old male mice received an intraperitoneal injection of caffeine every other day (2-day dose per injection) for 1 month. The thirty mice were divided into three equal groups, according to caffeine dosage: 0 mg/mouse (control), 0.2 mg/mouse/day and 1.0 mg/mouse/day (as described above). Each caffeine dose was injected on the terminal preparation day. After cervical spine dislocation, laparotomies were immediately performed to the testis and epididymis. The cauda epididymis was cut and semen removed using a small glass stick. The stick was placed into medium until sperm swam freely under microscopic observation. Percent motility was measured using the Makler chamber (Polymedco Inc., Yorktown, NY, USA) under microscopy.

The testes were removed, weighed and fixed into Bouin's solution for histopathological examination. We evaluated average sperm motility from 4 of 10 mice. We then chose tissue from two different mice, and these specimens were outsourced and observed under microscopy.

### Hormonal assay

We used the same dosing (0, 0.2 and 1.0 mg/mouse/day) of caffeine and performed an intraperitoneal injection for 1 month in mature Jcl:ICR strain 8-week-old male mice (6 mice per group). At the end of the month we injected pentobarbital intramuscularly. Once the mice were anesthetized, we extracted blood from the jugular vein into coated heparin tubes and centrifuged it at 750*g* for 20 min. The upper plasma was collected by pipette and cryopreserved at −20 °C until assay. Follicle-stimulating hormone (FSH), luteinizing hormone (LH) and total testosterone (T–T) were evaluated by chemiluminescence immunoassay (CLIA) by an outsourcing company (BML Ltd., Tokyo, Japan).

### Oocyte collection and IVF

Oocytes from mature Jcl:ICR strain 8-week-old female mice were used. Superovulation was induced via intraperitoneal injection with 10 IU pregnant mare serum gonadotropin (PMSG), and 5 IU human chorionic gonadotropin (hCG) was administered 48 h later. Fourteen hours afterwards, oocytes were collected; oocytes surrounded by a cumulus complex were inseminated with mouse sperm in HFF99 (Fuso Pharmaceutical Industries, Osaka, Japan) and supplemented with 10 % (v/v) synthetic serum substance (SSS; Irvine Scientific, Santa Ana, CA, USA). After fertilization, these embryos were incubated in HFF99 at 37 °C under 5 % $CO_2$, 5 % $O_2$ and 90 % $N_2$ until blastocyst formation or hatching.

Statistical analysis of the different groups was conducted by the chi-squared test or *t* test. $P < 0.05$ was considered statistically significant.

We obtained certification for the mouse study from the institutional review board of our hospital.

## Results

Following caffeine injection, mean (± standard deviation) body weights were 46.8 ± 3.6, 44.4 ± 3.4 and 42.1 ± 2.9 g in the control (0 mg, saline only), 0.2 mg and 1.0 mg/day groups, respectively. Mean body weight was significantly decreased in the 1.0 mg/day treatment group compared to the controls (42.1 vs 46.8 g) ($P < 0.01$). Testicular weight did not differ with caffeine dose (control group: 0.215 ± 0.074 g; 0.2 mg/day: 0.219 ± 0.075 g; 1.0 mg/day: 0.209 ± 0.096 g) (Table 1). No inhibition of spermatogenesis was histopathologically evident in the seminiferous tubules of 0.2 and 1.0 mg/day groups compared to the control group (Fig. 1).

FSH and LH were not detected because the lower limitation for the assay was not specific enough. T–T was 481.2 ± 445.4, 709.0 ± 514.0 and 899.0 ± 560.9 ng/dl in

**Table 1** Body weight and testes weight with different caffeine doses in mice ($n = 10$ per group)

| Caffeine dose (mg/mouse/day) | Average body weight (g) | Average testes weight (g) |
|---|---|---|
| 0 | 46.8 ± 3.6[a] | 0.215 ± 0.074 |
| 0.2 | 44.4 ± 3.4 | 0.219 ± 0.075 |
| 1.0 | 42.1 ± 2.9[a] | 0.209 ± 0.096 |

[a] Same letter indicates $P < 0.01$

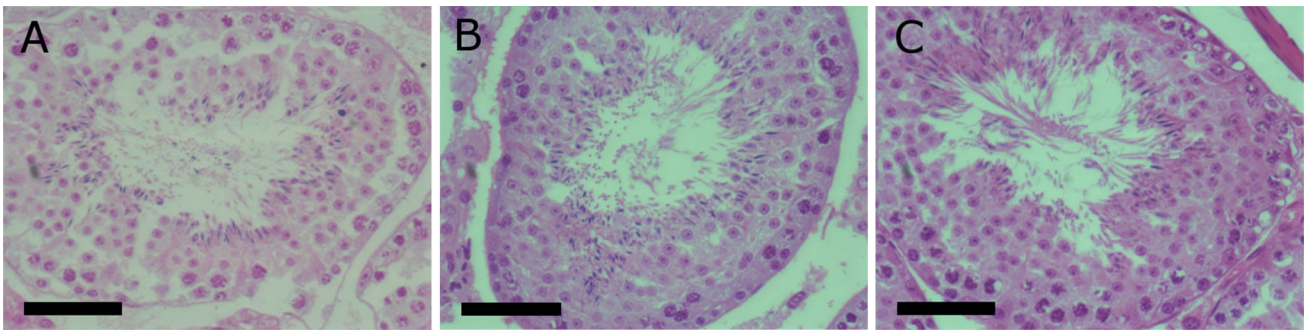



**Fig. 1** Histopathological features of seminiferous tubule tissue. **a** 0 mg/day (control group), **b** 0.2 mg/day group, **c** 1.0 mg/day group. *Bar* 50 μm

**Table 2** Total blood testosterone concentration with different caffeine doses in mice

| Caffeine dose (mg/mouse/day) | Number of mice | Total testosterone (ng/dl), mean ± SD |
|---|---|---|
| 0 | 6 | 481.2 ± 445.4 |
| 0.2 | 6 | 709.0 ± 514.0 |
| 1.0 | 5 | 899.0 ± 560.9 |

**Table 3** Sperm motility ratio with different caffeine doses in mice

| Caffeine dose (mg/mouse/day) | Number of mice | Sperm motility (%), mean ± SD |
|---|---|---|
| 0 | 4 | 75.4 ± 17.2 |
| 0.2 | 4 | 84.3 ± 8.1 |
| 1.0 | 4 | 80.0 ± 11.5 |

**Table 4** In vitro fertilization rate, blastocyst and hatched blastocyst formation with different caffeine doses in mice

| | Caffeine dose (mg/mouse/day) | | |
|---|---|---|---|
| | 0 | 0.2 | 1 |
| Number of normal oocytes after insemination | 105 | 121 | 110 |
| Number of fertilized oocytes 2PN formation (%) | 100 (95.2 %)[a] | 106 (87.6 %)[b] | 102 (92.7 %) |
| Blastocyst formation 96 h after insemination | 85 (85.0 %)[c] | 84 (79.2 %)[d] | 64 (62.7 %)[e] |
| Blastocyst formation 120 h after insemination | 90 (90.0 %)[f] | 91 (85.8 %)[g] | 69 (67.6 %)[h] |
| Hatched blastocyst formation 120 h after insemination | 15 (15.0 %)[i] | 2 (1.9 %)[j] | 10 (9.8 %)[k] |

a–b, c–d, f–g, f–h, g–h, i–j, j–k indicates $P < 0.05$; c–e, f–h, i–k indicates $P < 0.001$

*2PN* two pronuclei

the 0, 0.2 and 1.0 mg/day groups, respectively. There was no significant difference between the three groups (Table 2). Average sperm motility was 75.4 ± 17.2, 84.3 ± 8.1 and 80.0 ± 11.5 %, in the 0, 0.2 and 1.0 mg/day groups, respectively, with no significant differences (Table 3).

The IVF rate was significantly different between the control [100/105 (95.2 %)] and 0.2 mg/ml group [106/121 (87.6 %)] ($P < 0.05$). Furthermore, blastocyst formation 96 h after insemination was significantly and dose-dependently lower with higher caffeine levels: [0.2 mg/day group: 84/106 (79.2 %) ($P < 0.05$); 1.0 mg/day group: 64/102 (62.7 %) ($P < 0.001$); control group: 85/100 (85.0 %)]. Similarly, blastocyst formation at 120 h differed in a dose-dependent manner [0.2 mg/day group: 91/106 (85.8 %) ($P < 0.05$); 1.0 mg/day group: 69/102 (67.6 %) ($P < 0.001$); control group: 90/100 (90.0 %)]. Furthermore, the hatched blastocyst rate at 120 h significantly decreased between the 0.2 and 1.0 mg/day groups [0.2 mg/day group: 2/106 (1.9 %); 1.0 mg/day group: 10/102 (9.8 %)] ($P < 0.05$) (Table 4).

## Discussion

The testes weights, T–T hormone, sperm motility and testicular tissue histopathological features were not significantly different. However, fertilization and blastocyst formation rates significantly decreased with caffeine treatment. We are unable to explain why the high concentration group did not have significantly different T–T hormone levels, spermatogenesis features or sperm motility. The only evidence of caffeine-treated sperm was in embryo growth. It might be that caffeine affects the developing embryo function after fertilization; however, we have no clear explanation for this.

Further study is needed to determine whether daily caffeine injections are a suitable way to mimic human

consumption and effects. Treatment with 0.2 mg caffeine per day is the equivalent of 300 mg caffeine per day for a human, approximately the equivalent of three cups of coffee. Therefore, we administered caffeine levels of 0.2 and 1.0 mg per mouse per day by intraperitoneal injection. Wang et al. [18] reported no difference in serum concentrations between intraperitoneal and oral caffeine administration.

In our previous study, female mice were administered long-term continuous caffeine for at least 1 month, and the retrieved oocytes had decreased fertility. The IVF procedure resulted in a significantly decreased fertilization rate and blastocyst formation rate [19]. Therefore, it is hypothesized that caffeine may also affect sperm quality.

In the present study, fertilization and blastocyst formation rates decreased following caffeine administration (Table 4), suggesting that caffeine negatively impacts embryonic development despite fertilization with sperm from male mice given caffeine for 1 month.

Most animal studies are conducted in pregnant rats and mice, and are designed to produce teratogenic malformations. For example, caffeine causes limb and palate malformations in rodents [20–23].

To date, there are numerous studies investigating the effects of caffeine on female animals, while only a few have looked at males. One reason is that it is difficult to evaluate sperm quality. In general, even if total semen quality decreases, it is difficult to precisely judge an individual's sperm quality, and thus we have no suitable animal model for humans. When we collected sperm from the cauda epididymis, it was impossible to determine tissue volume and precisely determine sperm concentration. However, we could evaluate percent motile sperm and the result was no difference in numbers. Furthermore, it is possible that spermatogenesis may not be inhibited in seminiferous tubules following high caffeine treatment (1.0 mg/day) as observed under microscopy (Fig. 1). It is speculated that spermatogenesis is not stimulated following 1 month of caffeine. Again, T–T gradually increased following caffeine treatment despite no significant relationship (Table 2); therefore, it is speculated that long-term caffeine administration might affect both spermatogenesis and sperm motility.

Although the most common reason for infertility in females is decreased ovarian function due to advancing age, the treatment of infertile males is also on the rise. Dietary habits are changing in both men and women, which could also contribute to infertility. More specifically, increased caffeine consumption, including coffee, tea, chocolate and some soft drinks, particularly cola, needs to be considered.

The molecular structure of caffeine is very similar to adenosine. Therefore, it can be theorized that caffeine acts as an adenosine antagonist [23]. Adenosine plays a role in the fundamental ATP-related energy metabolism and is necessary for RNA and DNA synthesis. Thus, if caffeine adversely impacts spermatogenesis, sperm quality will decrease in vivo.

In the present study, fertilization rate decreased with 0.2 mg caffeine, while the 1.0 mg treatment was not significantly different from the control group. Blastocyst formation was influenced by dose-dependent caffeine treatment. Caffeine might also influence sperm quality [23], although we have not found any direct evidence for this. We also have no current understanding regarding the underlying mechanisms whereby caffeine impacts sperm quality. Further animal studies are necessary.

We speculated that the testicular testosterone level differs in a similar way to ovarian follicle concentration following caffeine treatment [24], but more basic human studies are necessary.

The current literature contains only a handful of reports addressing the relationship between caffeine and IVF in rodents. Therefore, we attempted to confirm whether caffeine administered to mice would have a similar impact on male sperm and female function.

In conclusion, caffeine administration prior to sperm collection impacts spermatogenesis, resulting in reduced blastocyst formation rate. Thus, our findings suggest that caffeine not only affects females in vivo but embryo development as well. Further studies are needed to elucidate the mechanisms responsible for these effects.

**Conflict of interest** Yoshimasa Yokota, Hidemi Yokota, Mikako Yokota, Yasuyuki Araki and Yasuhisa Araki declare that they have no conflict of interest.

**Human rights** This article does not contain any studies with human subjects performed by any of the authors.

**Animal studies** All institutional and national guidelines for the care and use of laboratory animals were followed.

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