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Caffeine consumption during early pregnancy impairs oviductal embryo transport, embryonic development and uterine receptivity in mice†

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ABSTRACT

Caffeine consumption has been widely used as a central nervous system stimulant. Epidemiological studies, however, have suggested that maternal caffeine exposure during pregnancy is associated with increased abnormalities, including decreased fertility, delayed conception, early spontaneous abortions, and low birth weight. The mechanisms underlying the negative outcomes of caffeine consumption, particularly during early pregnancy, remain unclear. In present study, we found that pregnant mice treated with moderate (5 mg/kg) or high (30 mg/kg) dosage of caffeine (intraperitoneally or orally) during preimplantation resulted in retention of early embryos in the oviduct, defective embryonic development, and impaired embryo implantation. Transferring normal blastocysts into the uteri of caffeine-treated pseudopregnant females also showed abnormal embryo implantation, thus indicating impaired uterine receptivity by caffeine administration. The remaining embryos that managed to implant after caffeine treatment also showed increased embryo resorption rate and abnormal development at mid-term stage, and decreased weight at birth. In addition to a dose-dependent effect, significant variations between individual mice under the same caffeine dosage were also observed, suggesting different sensitivities to caffeine, similar to that observed in human populations. Collectively, our data revealed that caffeine exposure during early pregnancy impaired oviductal embryo transport, embryonic development, and uterine receptivity, which are responsible for abnormal implantation and pregnancy loss. The study raises the concern of caffeine consumption during early stages of pregnancy.

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Summary Sentence

Caffeine exposure during early pregnancy disrupts oviductal embryo transport, embryonic development and uterine receptivity, leading to aberrant implantation and compromised pregnancy.

Key words: embryo implantation, embryo transport, embryonic development, uterine receptivity, caffeine, pregnancy outcome.

Introduction

Embryonic implantation is a gateway for establishment of pregnancy [1], and 75% of pregnancy losses are rooted in the peri-implantation period [2, 3]. After sperm fertilizes the egg in the ampulla region of the oviduct, the zygote undergoes several rounds of cleavage to form a morula while being transported through the isthmus region of the oviduct [1]. Synchronous development of the implantation-competent blastocyst and receptive uterus is prerequisite for implantation initiation, and another indispensable event is timely transport of the embryos from the oviduct into the uterus [4, 5]. Previous studies have demonstrated that dysregulation of cannabinoid/endocannabinoid signaling or hydrogen sulfide metabolism derailed oviductal embryo transport, which delayed embryo implantation and caused pregnancy loss at the end of gestation [6–8].

Caffeine (1,3,7-trimethylxanthine) is the most frequently used psychoactive substance in the world [9, 10]. Approximately 80% of women in the United States ingest caffeinated beverages daily [11], and based on data of Continuing Survey of Food Intakes by Individuals, 68% of women continue daily caffeine consumption during pregnancy [12]. Epidemiological studies have shown that maternal caffeine consumption is closely correlated with delayed conception [13–15], pregnancy loss [16–19], and low birth weight [20–23] in humans. In mice, maternal caffeine exposure before and during pregnancy was also found to reduce conception rates and increase the risks of intrauterine growth restriction [24]. Embryonic caffeine exposure at mid-term stages has been shown to change fetal cardiac DNA methylation patterns, leading to adult cardiac defects [25–27]. Although it is known that consumption of caffeine has negative impacts on embryonic development, it is not known whether and how caffeine consumption during early pregnancy influences pregnancy establishment. It has been reported that caffeine exposure in rats prior to embryonic implantation does not affect blastocyst glucose utilization and cell number; however, these conclusions were formed after excluding any degraded embryos from the study [28]. Studies using mice blastocysts in an in vitro culture system have shown that blastocyst formation was sensitive to caffeine exposure [29, 30]. Despite the significance of these early studies, when and how caffeine consumption can affect early embryonic development and subsequent pregnancy events in vivo remain unclear. In the present study, we show that maternal caffeine exposure during preimplantation severely impaired embryo transport through the oviduct, disrupted embryonic development and uterine receptivity, resulting in compromised embryo implantation and negative outcomes of pregnancy in mice.

Materials and methods

Mice

Mature CD1 7- 8-week old (female) and 10-week-old (male) mice were purchased from Charles River Laboratories China Inc. All animal experiments were approved by the animal ethic committee and the Institutional Animal Care and Use Committee, Institute of Zoology, Chinese Academy of Sciences. All mice were housed on a 12 h light/12 h dark cycle (light on 07:00AM-19:00PM) with free water and food supply at a temperature of 23°C. Virgin females were mated with males overnight and the morning (08:00 AM) finding of the vaginal plug was designated as day 1 of pregnancy. After vaginal plugs were identified, pregnant mice were sacrificed on day 4 (09:00 AM) of pregnancy. Uterus and oviduct of each animal were flushed separately with sterile saline, then the number and morphology of the embryos were examined under a dissecting microscope. To detect implantation sites on day 5 and day 6 (09:00 AM), the mice were euthanized 10 min after tail-vein injections of 0.1 ml 1% Chicago blue dye (Sigma) in saline [31, 32]. To check the midterm pregnancy status on day 12, uteri were fixed in Bouin solution overnight and then implantation sites and fetuses were isolated and weighed separately as previously described [32].

Drug delivery

Vaginal plug-positive mice were treated with caffeine (Sigma-Aldrich, No. 93784) intraperitoneally (IP) or orally (PO) during day 1–3 of pregnancy, followed by detecting embryo distribution and morphology in oviducts/uteri on day 4. Mice treated with caffeine from day 1 to day 4 were sacrificed to detect embryo implantation/development on day 5, 6, and 12 of pregnancy. Caffeine was delivered at the dosages of 2 mg/kg body weight (BW) (as a low dosage group), 5 mg/kg BW (as a moderate dosage group), or 30 mg/kg BW (as a high dosage group), and delivered three times per day at 08:30 AM, 13:00 PM, and 17:30 PM. Vehicle-treated mice received sterile saline in a similar manner. Previous studies have demonstrated that the effects of caffeine are dependent on plasma caffeine concentrations [9, 33–35]. According to plasma caffeine concentrations [9, 34, 35], the concentrations of caffeine used on mice in the present study i.e. 2 mg/kg three times, 5 mg/kg three times, or 30 mg/kg three times daily, respectively, is equivalent to consuming less than 1 cup, 1 to 2 cups, or 6 to 10 cups of coffee (containing 50–100 mg caffeine per serving) for a typical women weighing 60 kg [25, 36].

Hematoxylin-eosin staining

After caffeine treatment, pregnant mice were sacrificed on day 4 at 09:00 AM and oviducts were subsequently fixed in Bouin solution for 24 h. The fixed oviducts were embedded in paraffin and cut into sections 5 μm in thickness. These sections were later stained with hematoxylin solution for 5 min and eosin solution for 10 s.

In vitro culture of embryos

Virgin females received (IP) 7.5 IU pregnant mare serum gonadotropin, and 7.5 IU human chorionic gonadotropin 48 h later for superovulation, females were then mated with fertile males overnight. On day 2 11:00 AM, pregnant mice were sacrificed to retrieve 2-cell stage embryos by flushing oviducts with equilibrated M2 medium (Sigma-Aldrich, No. M7167). Caffeine was added to

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the culture medium KSOM (Millipore, No. MR-107-D) at concentrations of 1, 2, 3, 4, and 5 mM, according to previous studies [29, 30]. Embryos were cultured in an incubator with 5% CO_2 at 37°C for 72 h, and photomicrographs collected every 12 h to monitor the developmental status of the embryo.

Blastocyst cell number counting

For in vitro blastocyst cell number counting, embryos (flushed from oviducts at 2-cell stage) were collected after culturing for 60 h; for in vivo blastocyst cell number counting, embryos were flushed from the uteri of vehicle or caffeine-treated mice at day 4 10:00 AM. Collected embryos were fixed with 4% paraformaldehyde for 30 min at room temperature and incubated with Hoechst (1:500 dilution) for 1 h. Photographs of blastocyst were obtained using a Leica SP8 and cell number counted with Imaris (Version 9.0.2) software.

Immunohistochemistry

Immunohistochemistry was performed as previously described [37]. Day 5 and day 6 uteri were fixed in 4% paraformaldehyde, and examination of implantation sites was performed in 5 μm-thick, paraffin-embedded sections by using antibody against COX2 (1:200 dilution; product no. 12282S; CST, Inc.). Hematoxylin solution was further used for nuclear staining. Images were captured by Leica Aperio VERSA8.

Quantitative real-time PCR

RT-PCR was performed as previously described [38]. Total RNA of uterus was extracted using TRIzol reagent (Invitrogen) following the manufacturer's procedural guide. A total of 4 μg RNA was used to synthesize cDNA with oligo dT primers. Expression of all implantation-related genes was normalized against *Gapdh*. All PCR primers were listed in Supplemental Table S1.

Embryo transfer

For the pseudopregnant mice preparation, we followed the protocol in previous study [39]. Adult virgin female mice were mated with vasectomized male. The morning of finding a vaginal plug was designated day 1 of pseudopregnancy. Then pseudopregnant mice received 5 mg/kg BW or 30 mg/kg BW caffeine from day 1 to day 4 to examine caffeine's effects on uterine receptivity; or received 30 mg/kg BW caffeine from day 3 to day 4 or only day 4 to examine effects of periimplantation caffeine exposure on uterus. All recipient females received caffeine three shots daily (08:30 AM, 13:00 PM, and 17:30 PM) or only one shot (08:30 AM) on day 4. Sixteen normal day 4 blastocysts were transferred into uteri of day 4 pseudopregnant recipients (eight blastocysts for each uterine horn). The number of implantation sites was examined by intravenous blue dye method 48 h after transfer. In the mice without blue bands, the ones with recovered embryos were considered as abnormal/failed implantation mice and others without recovered blastocysts were excluded from the experiment.

Statistical analysis

Percentage of embryos recovered from oviduct and uterus in mice was analyzed by using the Fisher exact test, embryo development was analyzed by using the Chi square test, and one-way ANOVA was used when comparing multiple groups, and two-tailed unpaired Student t-test was used when comparing two groups. Statistical analyses were performed with GraphPad (Version 6.0c). Data were presented as mean ± SEM.

Results

Caffeine treatment at early pregnancy causes oviductal embryo retention

Our previous studies have shown that caffeine can hyperpolarize membrane potential of oviduct myosalpinx, inhibit spontaneous electrical activity of oviductal pacemaker cells, and disrupt myosalpinx contractions [40]. Considering the potential importance of oviduct myosalpinx contractions for embryo transport in vivo, we further examined whether caffeine treatment during preimplantation (day 1–3 of pregnancy) affects normal oviductal embryo transport. As shown in Figure 1A, in the morning (09:00 AM) of day 4 pregnancy, all embryos in vehicle and low-dosage (2 mg/kg) groups had entered into the uteri. However, early embryos were retrieved from the oviducts of 18% of moderate-dosage (5 mg/kg) and 21% of high-dosage (30 mg/kg) treated mice (Figure 1A). Among the mice with embryos retained in the oviduct, 22% of embryos in high-dosage group and 31% of embryos in moderate-dosage group were found in the oviducts (Figure 1B). We also found comparable blastocyst rates between the embryos that are trapped in the oviducts and those entered uteri (57% vs 68% in moderate-dosage group; 44% vs 40% in high-dosage group, respectively). These results suggest that caffeine's effect on oviductal transport is an additional factor independent of its effect on embryo development. By performing microscopy on paraffin embedded transverse sections of the oviduct, we found that different stages of early embryos (including morula and blastocyst) were "trapped" in the isthmus (Figure 1C). These data clearly show that in vivo caffeine treatment can affect on-time oviductal transport of embryos during early pregnancy, presumably by affecting the pattern of oviductal peristalsis.

Caffeine treatment impairs development of embryos in vivo and in vitro

In addition to the oviductal embryo retention, we also noticed that embryos recovered from mice treated with moderate and high dosages of caffeine showed asynchronous and abnormal embryo development. As shown in Figure 2A and B, in the morning of day 4, the majority of embryos (91% or 89%) in vehicle or low caffeine dosage group can develop to the blastocyst stage. However, in the moderate dosage group, 68% of embryos reached blastocyst stage (Figure 2A and B); and in the high dosage group, only 40% of embryos can develop to blastocysts and a considerable number (31%) of embryos displayed severely uneven cleavage and degeneration (Figure 2A and B). In addition, cell number in the blastocysts from high caffeine dosage group was also significantly decreased (Figure 2C). These results demonstrated that caffeine, in a dose-dependent manner, impaired embryonic development in vivo.

The adverse effect of caffeine on embryonic development was further demonstrated in 2-cell embryos culture in vitro (Figure 2D and E, Supplemental Figure S1). Embryos in all groups developed normally in the first 12 h (2-cell to 4-cell stage), while in caffeine exposure groups, morphologically signs of abnormality were first observed after 36 h culturing, around the compaction stage (Figure 2D). Uncompacted embryos displayed severely scattered or localized fragmentation in 3 mM or higher caffeine groups, and the observed defects became more severe later (Supplemental Figure S1A and B). After culturing for 72 h, most of embryos in vehicle and low

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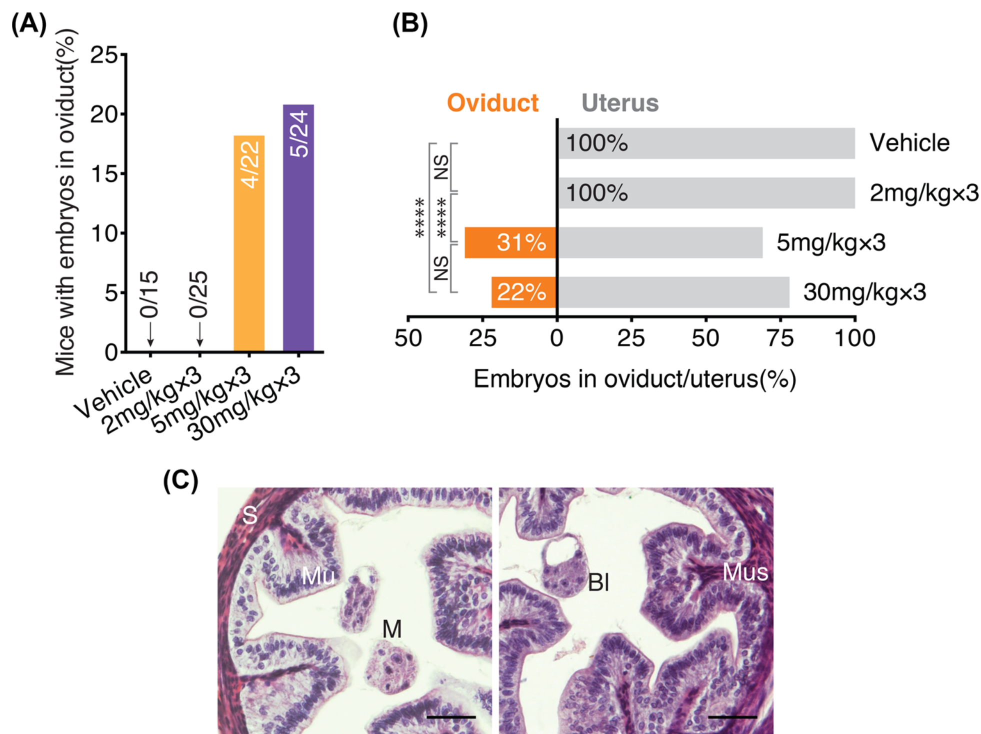

Figure 1. Caffeine exposure during early pregnancy leads to oviductal retention of preimplantation embryos. (A) Percentages of mice with oviductal embryo retention on day 4 (09:00 AM) of pregnancy. Different dosages of caffeine were delivered (IP) from day 1 to day 3, three shots per day at 08:30 AM, 13:00 PM, and 17:30 PM. Numbers within the bars represent the number of mice used for each treatment. (B) Percentages of embryos recovered from oviduct/uterus in mice with oviductal embryo retention. Fisher's exact test, NS > 0.05, $^{****}P < 0.0001$. (C) Representative histological images showing trapped embryos at the isthmus region of the oviduct on day 4 (10:00 AM). Bar = 100 μm. Bl, blastocyst; M, morula; Mu, mucosa; Mus, muscularis; S, serosa.

caffeine (1 mM) groups had developed into blastocysts and started zona pellucida dissolution; however, caffeine dose-dependently inhibited blastocyst formation with a great number of embryos showed severe fragmentation and degeneration (Figure 2D and Supplemental Figure S1B). Moreover, caffeine also notably decreased blastocyst cell number in 1 mM concentration group (Figure 2E), which was consistent with in vivo results. Collectively, early embryos exposed to high caffeine concentrations showed impaired blastocyst formation both in vivo and in vitro, which could be due to either a direct effect on embryos or through a secondary effect via oviductal environment or both.

Since caffeine intake by humans is mainly through consumption of beverages, we asked whether oral caffeine treatment has similar effects compared with intraperitoneal delivery. Indeed, oviductal embryo retention (Supplemental Figure S2A and B) and deferred embryo development (Supplemental Figure S2C) were similarly observed by oral caffeine treatment (both moderate and high caffeine groups) compared with intraperitoneal delivery, demonstrating that intraperitoneal or oral administration of caffeine has similar effects for early pregnancy.

Caffeine consumption causes dose-dependent defects in embryo implantation, accompanied with compromised uterine receptivity

The above results led us to further investigate embryo implantation after caffeine treatment. We found that both intraperitoneal and oral administrations of caffeine impaired embryo implantation rates in the morning of day 5 and day 6 of pregnancy, showing increased number of mice without implantation sites or with abnormal implantation sites (Figure 3A and B, Supplemental Figures S3A, B and S4). Unimplanted embryos could be recovered from the uteri of mice without implantation sites, and some of them have reached the stage of blastocyst (Supplemental Figure S3C and D, and Supplemental Table S2). This suggests that caffeine treatment may negatively affect uterine receptivity as well.

In support of this idea, weak blue bands (suggestive of abnormal implantation sites) were observed on day 5 from both moderate and high dosages of caffeine-treated mice (Figure 3A and C), and some of the implantation sites showed "catch-up" in moderate dosage group on day 6 regarding gross morphology (Figure 3D), suggesting delayed implantation due to impaired uterine receptivity. Indeed, further tissue section and COX2 staining of the implantation sites further confirmed the defective embryo implantation after caffeine treatment (Figure 3E). Very interestingly, the pattern of COX2 expression became abnormal after moderate and high dosages of caffeine treatments, showing ectopic expression in the luminal epithelium, but decreased in subepithelial stromal cells at the anti-mesometrial pole of implantation chamber (Figure 3E). In some high-dosage caffeine-treated mice, the embryos were floating in the uteri without attachment reaction and no COX2 expression was found in the subepithelial stromal cell on both day 5 and day 6 (Figure 3E, Supplemental Figure S3 and Supplemental Table S1). In addition to COX2 expression, other uterine receptivity related genes also became abnormal (Figure 3F). For example, estrogen responsive genes including leukemia inhibitory factor (*Lif*), mucin 1 (*Muc1*), and lactoferrin (*Ltf*) were all dysregulated in high caffeine group, and *Lif* and *Muc1* were also severely changed in moderate group.

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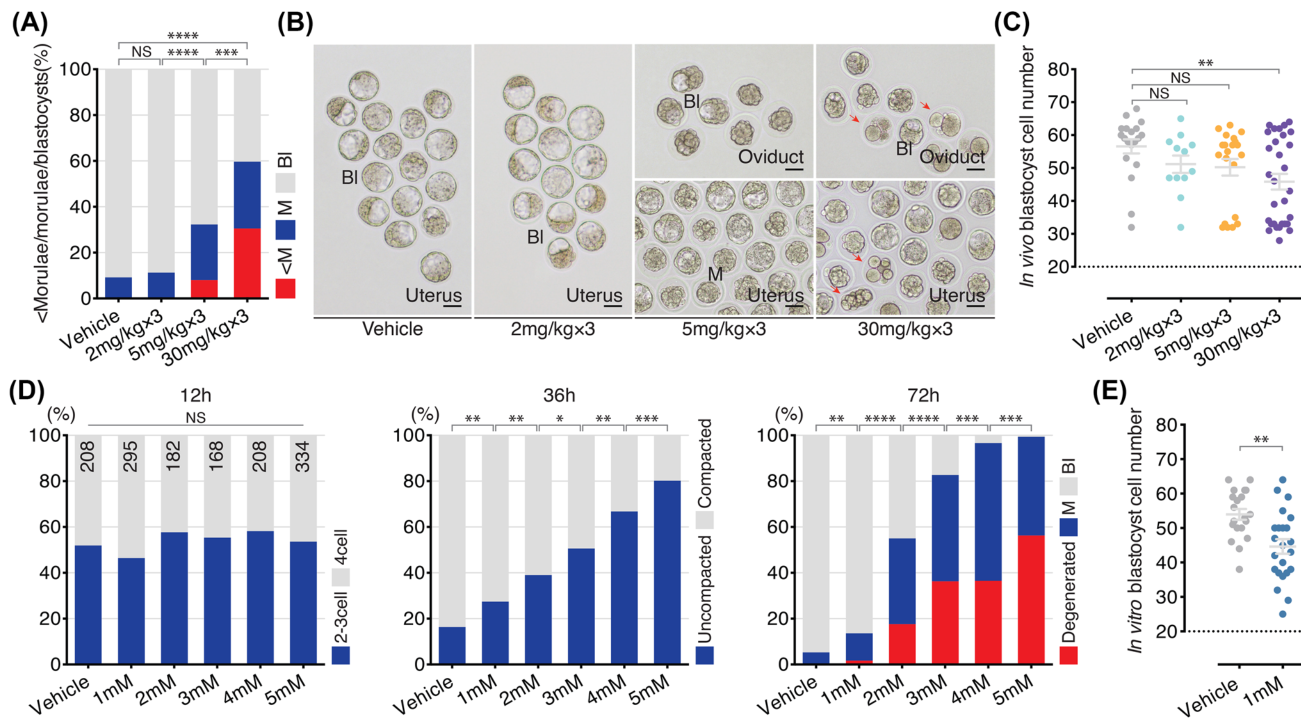

Figure 2. Caffeine exposure impairs in vivo and in vitro early embryo development. (A) Stages of embryos collected from oviducts/uteri on day 4 (09:00 AM). In vehicle control and 2 mg/kg caffeine groups, no embryo was recovered from oviduct. Embryo stages were classified as "Before morulae" (<M), "Morulae" (M), or "Blastocysts" (Bl). Chi-square test, $^{***}P < 0.001$, $^{****}P < 0.0001$. (B) Morphology of embryos flushed out from oviducts/uteri. Red arrowheads indicate morphologically distinct aberrantly fragmented and degenerated embryos. Bar = 50 μm. Bl, blastocyst; M, morula. (C) In vivo blastocyst cell number. One-way ANOVA, $^{**}P < 0.01$ was considered as statistically significant. (D) Embryo statuses which were examined every 12 h in vitro. Numbers within the bars represent 2-cell embryos used in each group. Chi-square test, NS > 0.05 $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (E) In vitro blastocyst cell number. Student *t*-test, $^{**}P < 0.01$ was considered as statistically significant.

And progesterone responsive genes, such as epithelial amphiregulin (*Areg*), were also inhibited (Figure 3F). These results indicate that caffeine disrupts uterine epithelium response to steroid hormones, which may result in abnormal uterine receptivity.

To further examine the direct effects of caffeine on uterine receptivity, we transferred normal blastocysts into the day 4 uteri of caffeine-treated pseudopregnant females (treatment from day 1–4, day 3–4, or day 4), followed by examining implantation status 48 h later (Figure 3G). The result showed that while the implantation rate in the vehicle group is 74%, the moderate or high caffeine dosage treatment severely decreased the implantation rate (Figure 3H). The abnormally smaller blue bands were observed in moderate transfer group, and unimplanted embryos could be recovered from moderate or high caffeine-treated mice showing no implantation site (Figure 3I). Furthermore, implantation rates were also significantly reduced after day 3–4 or only day 4 high dosage of caffeine treatment (Figure 3J). These data strongly indicate that caffeine treatment can directly impair uterine receptivity and thus compromise embryo implantation.

Caffeine exposure during early pregnancy leads to poor pregnancy outcomes

Since some of the embryos still manage to implant after moderate or high dosage of caffeine treatment, we further examined their development status on day 12 of pregnancy. In addition to a substantial proportion of mice showing no implantation site (Figure 4A), the mice with implantation sites showed increased resorption rates (Figure 4B) and decreased average weights of surviving embryos (Figure 4C).

At the termination of pregnancy, fetuses born from both moderate and high dosages of caffeine groups showed lower birth weights (Figure 4D), accompanied with decreased litter sizes (Figure 4E). This result may provide a possible explanation for the low body weights of fetuses delivered by women who consumed caffeine during pregnancy [20–22].

Notably, despite the fact that the majority of mice failed to achieve pregnancy when exposed to high dosage of caffeine, 23% of pregnant mothers delivered pups and showed comparable litter sizes as vehicle control or low dosage of caffeine group (Figure 4E). These data would suggest that there are strong individual variations in the susceptibility to caffeine exposure.

Discussion

Use of caffeine-containing products by women during pregnancy is very common, and the negative effects for early pregnancy and gestation outcomes have a high medical social impact. In the present study, we demonstrated that exposure to both moderate and high caffeine concentrations during early pregnancy can impair oviductal embryo transport and uterine receptivity, while exposure to high concentration of caffeine also causes significant disruption in embryonic development, leading to pregnancy loss, decreased litter size, and low birth weight.

Oviductal retention of preimplantation embryos has been reported to be an important cause for implantation failures, and this

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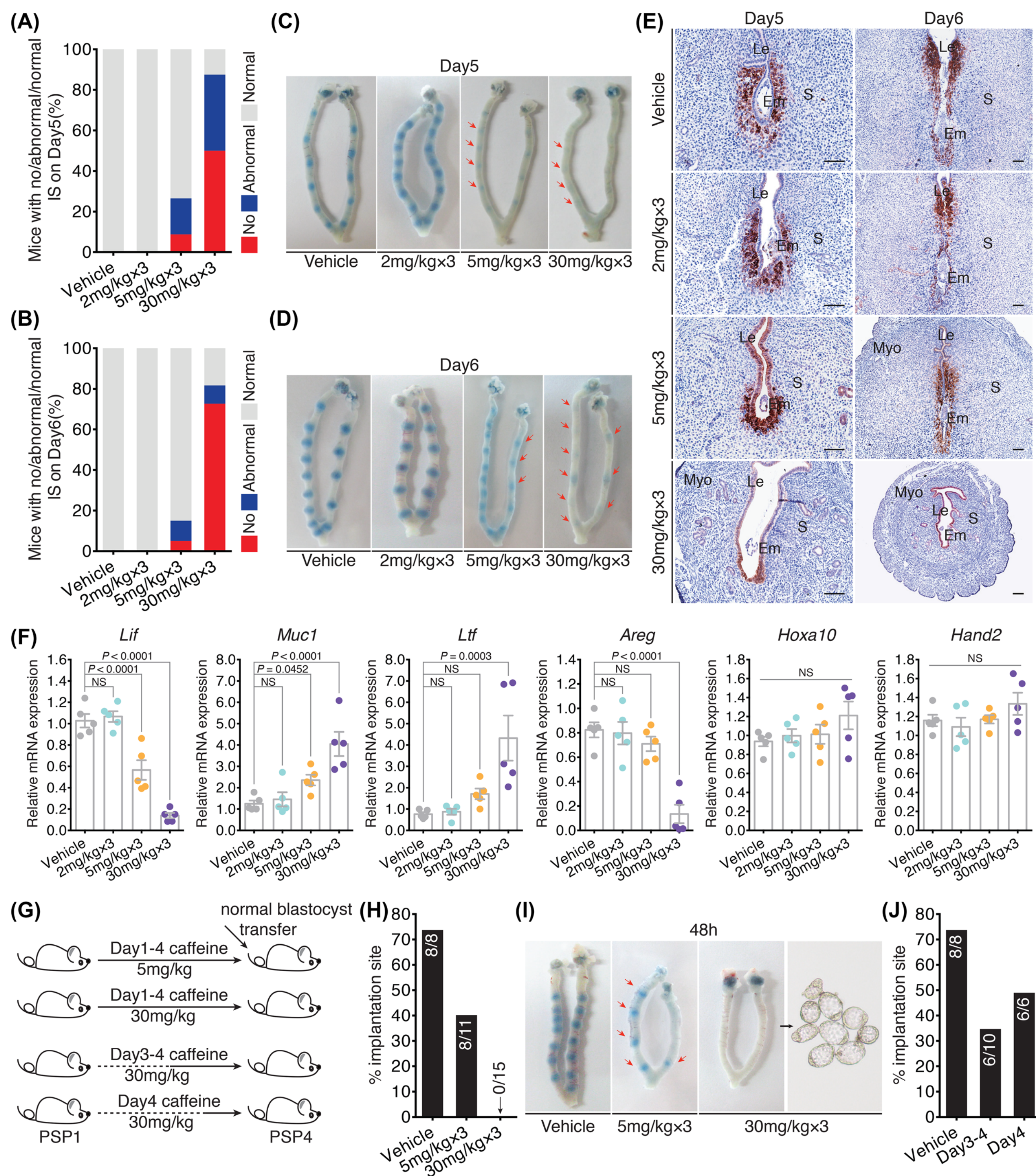

Figure 3. Preimplantation caffeine exposure causes defective embryo implantation, accompanied with compromised uterine receptivity. (A and B) Percentages of mice without implantation sites/with weak or small implantation sites/with normal implantation sites on day 5 (A) or day 6 (B) of pregnancy. Different dosages of caffeine were delivered (IP) during day 1–4. (C and D) Representative pictures of embryo implantation in vehicle and caffeine-treated mice on day 5 (C) or day 6 (D) of pregnancy. Each blue spot indicates a site of implantation. Red arrowheads indicate seriously weak and small implantation sites. (E) Immunohistochemistry of COX2 in implantation sites of vehicle and caffeine-treated mice on both day 5 and day 6. Bar = 100 μm. Em, embryo; S, stroma; Le, luminal epithelium; Myo, myometrium. (F) Quantitative real-time PCR analyses of uterine receptivity marker genes in vehicle and caffeine-treated mice on day 4 of pregnancy. One-way ANOVA, NS > 0.05 was considered as no significance. Error bars represent SEM (n = 5). (G) Experimental procedure of embryo transfer to examine the effects of preimplantation caffeine treatment (day 1–4 or day 3–4) on uterine receptivity. PSP, pseudopregnancy. (H) Implantation rates of pseudopregnant mice receiving moderate and high dosages of caffeine from day 1 to day 4. Numbers within the bars represent number of mice used for each treatment. (I) Representative pictures of implantation sites 48 h after embryo transfer in moderate or high dosage of caffeine-treated mice. Note the red arrowheads indicating smaller implantation sites in the moderate transfer group. (J) Implantation rates of pseudopregnant mice receiving high dosage of caffeine exclusively on day 3–4 or day 4. Numbers within the bars represent number of mice used for each treatment.

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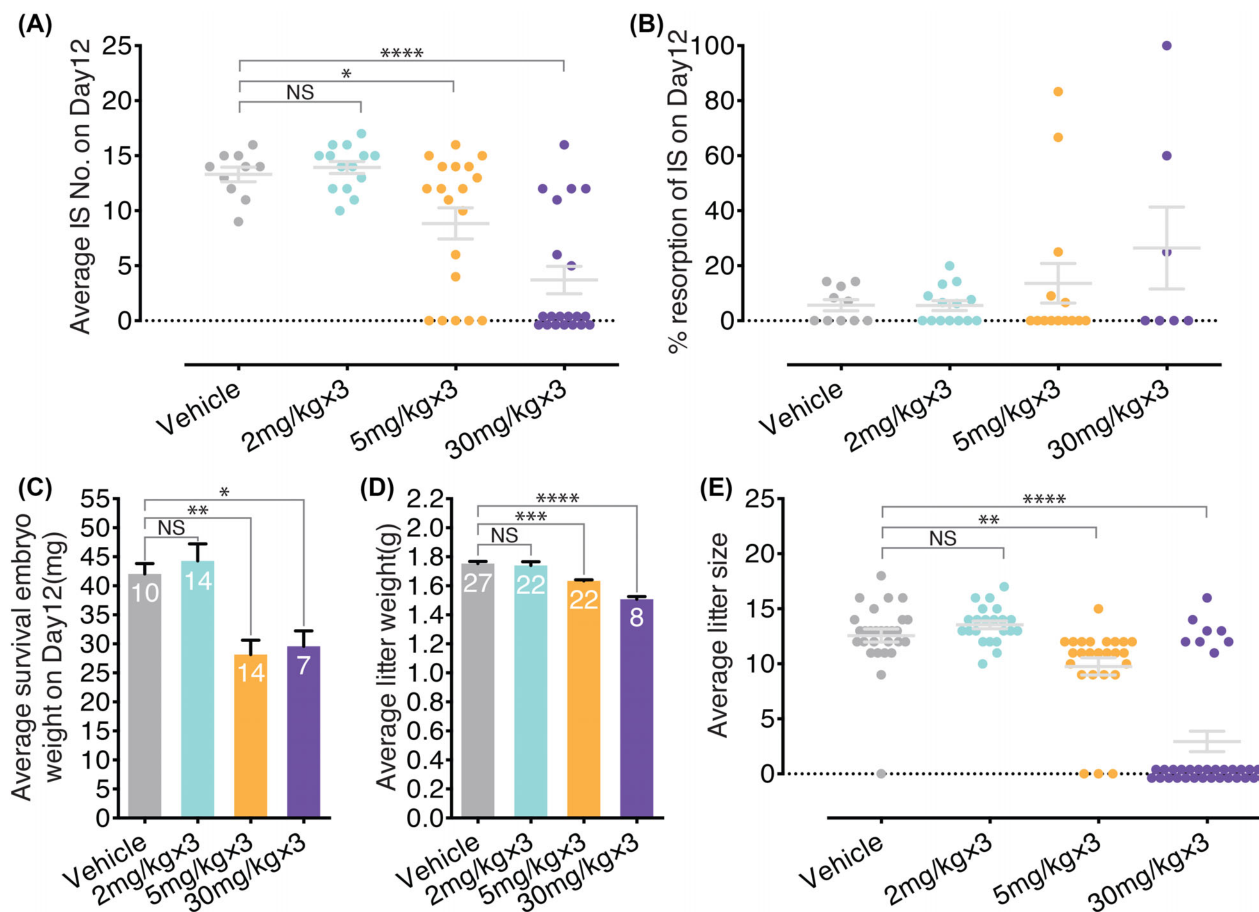

Figure 4. Caffeine exposure during early pregnancy impairs embryonic development and leads to pregnancy loss. (A) Average number of implantation sites on day 12 of pregnancy. One-way ANOVA, $^{*}P < 0.05$, $^{****}P < 0.0001$ were considered as statistically significant. (B) Embryo resorption rates on day 12 of pregnancy. (C) Average weight of surviving embryos on day 12. One-way ANOVA, NS > 0.05 was considered as no significance, $^{*}P < 0.05$, $^{**}P < 0.01$ were considered as statistically significant. (D) Average litter weights at birth. Numbers within the bars indicate the number of mice used for each treatment. One-way ANOVA, $^{***}P < 0.001$, $^{****}P < 0.0001$ were considered as statistically significant. (E) Average litter sizes at birth. Numbers within the bars indicate the number of mice used for each treatment. One-way ANOVA, $^{**}P < 0.01$, $^{****}P < 0.0001$ were considered as statistically significant. Error bars represent SEM.

can generate adverse ripple effects for subsequent gestations by missing the "window of uterine receptivity" [6, 7, 41–43]. It is thought that the incessant beating of cilia is the major driving force for the transport of embryos along the ampulla, while embryonic passage through the isthmus is predominantly driven by rhythmic myosalpinx contractions and relaxations [4]. Slow waves generated by interstitial cells of Cajal (ICC-OVI), the oviduct pacemaker cells, have been reported to drive concerted contractions and relaxations of oviduct smooth muscle or myosalpinx [44–47]. Importantly, caffeine exposure in vitro abolished slow wave pacemaker activity in the oviduct and consequently inhibited rhythmic myosalpinx contractility via a cAMP-dependent mechanism [40], demonstrating potential effects of caffeine on impeding myosalpinx contractions and embryo transport along the oviduct. Further, as an antagonist of adenosine receptors, caffeine consumption alters plasma adenosine concentrations in a dose-dependent and saturable manner [48], and adenosine has been reported to coordinate spontaneous contractile activity of human fallopian tubes [49], suggesting the possible negative influences of caffeine on embryonic transport in humans. Herein, we provided the in vivo evidence of caffeine-triggered embryonic retention in the isthmus region of the oviduct, demonstrating the adverse effects of caffeine exposure for oviductal embryo transfer, and supporting previous mechanistic studies conducted in vitro.

Clinical trials have revealed that fragmented human embryos usually suffer aberrant chromosome or multinucleation because of the disrupted cytokinesis, and these embryos possess poor implantation potential and can lead to compromised pregnancy outcomes after embryo transfer [50–54]. But the origin and formation of the fragmented embryos are still unclear. In our study, we found that exposure to high caffeine concentration during early pregnancy impaired embryonic compaction through asymmetric cell division and extensive fragmentation in the cleaving stage, this could be among the causes for compromised embryo quality in human early pregnancy.

We also revealed the adverse effects of caffeine on uterine receptivity. Evidences from molecular and genetic studies indicate that numerous signaling molecules from uterine epithelial and stromal cells, together with steroid hormones, coordinate the intricate establishment of uterine receptivity. The abnormal expression pattern of COX2 after caffeine exposure revealed here is similar with its expression in *Lif*(–/–) mice [55], and interestingly, we also found significantly decreased expression of *Lif* in both moderate and high

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caffeine dosage-treated mice, suggesting caffeine may disrupt the expression of important implantation molecules thus impair uterine receptivity. Moreover, *Muc1*, *Ltf,* and *Areg*, which localized in epithelium, were also dysregulated after caffeine treatment, suggesting caffeine exposure may also impair the function of epithelial cells and affect uterine hormone response. While the influence of caffeine in human uterine receptivity remains unknown, our results observed from mice may provide a clue for further detailed investigations.

Importantly, we observed similar effects via intraperitoneal and oral caffeine administrations. Indeed, it has been reported that caffeine has similar pharmacokinetics and behavioral effects via the intraperitoneal or oral administration [56], and there is no pronounced hepatic first-pass effect for caffeine [57–59]. Because of the similar metabolic pathway of caffeine in mice and human [9, 59], our results may help to understand caffeine's impact on human reproductive health.

Another interesting finding from the present study is the individual susceptible variations in responses to caffeine. Under physiological conditions, as a nonselective adenosine receptor antagonist, caffeine exerts its effects predominantly via blockade of two adenosine receptor subtypes (i.e. A_1 receptor and A_{2a} receptor) [60, 61], and is metabolized by cytochrome-450 oxidase enzyme system in hepatic microsomes, principally by CYP1A2, which is demethylated and hydroxylated to uracil and uric acid [9]. Genetic polymorphisms of adenosine receptors and CYP1A2 may be the reason for different caffeine susceptibilities between individuals [62–64], for example, specific genetic polymorphism of A_{2a} receptor contributes to various anxiety/sleep responses to caffeine in humans [65–67]. Cohort studies on women who consumed caffeine during pregnancy, even at amounts considered regular, confirmed that females with high CYP1A2 activity were correlated with increased risks of spontaneous abortions and intrauterine growth restrictions [68, 69]. Moreover, significant correlations between maternal caffeine intake and decreased fetal birth size or recurrent pregnancy loss were also reported among women with specific CYP1A2 genotypes [70, 71]. These results indicate that genetic polymorphisms of caffeine metabolism genes determine the effects of its exposure, suggesting pregnancy outcomes after caffeine exposure are not only dependent on the dosage, but are also related to the metabolism rate of a particular individual.

Taken together, our data have demonstrated that caffeine consumption during early pregnancy impairs normal transport of the embryo in the oviduct, its subsequent development, and uterine receptivity, leading to defective embryo implantation and pregnancy loss in mice. It is important to note that the time of caffeine exposure in our study is before embryo implantation, emphasizing the importance of restricting caffeine intake for women who are preparing to conceive, this would be particularly important for those susceptible to the effects of caffeine even at normal consumption rates.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Caffeine exposure leads to fragmentation and defective compaction of early embryos in vitro. (A) After 60 h of culture, developmental statuses of embryos were classified as "Degenerated embryos," "Fragmented embryos," "Morulae," and "Blastocysts." Chi-square test, NS > 0.05, $^{****}P < 0.0001$. (B) Representative photomicrographs of embryos every 12 h in culture. Fragmented embryos were firstly detected in 3 and 5 mM caffeine concentration groups after 36 h in culture. Red arrowheads indicate fragmented embryos. Bar = 50 μm.

Supplemental Figure S2. Oral caffeine administration impairs oviductal transport and development of embryos. (A) Percentages of mice with oviductal embryo retention on day 4 (09:00 AM) of pregnancy. Mice treated with caffeine orally from day 1 to day 3. Numbers within the bars represent number of mice used for each treatment. (B) Percentages of embryos recovered from oviduct/uterus in mice with oviductal embryo retention. Fisher's exact test, NS > 0.05, $^{***}P < 0.001$, $^{****}P < 0.0001$. (C) Stages of embryos collected from oviducts/uteri on day 4. In vehicle control group, no embryo was recovered from oviduct. Embryo stages were classified as "Before morulae" (<M), "Morulae" (M), or "Blastocysts" (Bl). Chi-square test, $^{****}P < 0.0001$.

Supplemental Figure S3. Preimplantation caffeine administration (IP) causes decreased implantation rate and implantation failure. (A and B) Average numbers of implantation sites (IS) on day 5 (A) and day 6 (B) (09.00 AM). One-way ANOVA, $^{***}P < 0.001$ was considered as statistically significant. (C and D) Representative photos of embryos recovered from mice not showing blue bands on day 5 (C) and day 6 (D) after 30 mg/kg caffeine treatment. Both zona pellucida-encased (red arrowheads) and free (black arrowheads) embryos were found.

Supplemental Figure S4. Oral administration of caffeine during preimplantation impairs embryo implantation. (A) Percentages of mice without implantation sites on day 5 (09:00 AM) of pregnancy. Mice treated with oral administration of caffeine from day 1 to day 4 of pregnancy. (B) Representative pictures of embryo implantations in vehicle control and caffeine groups on day 5. Red arrowheads indicate indistinct implantation sites. (C) Percentages of mice without implantation sites on day 6 (09:00 AM) of pregnancy.

Supplemental Table S1. Primers for quantitative real-time PCR.

Supplemental Table S2. Moderate and high caffeine treatments cause failed implantation. Pregnant mice treated with different dosages of caffeine during day 1–4 of pregnancy and implantation were examined by intravenous blue dye injections. Uteri without showing implantation sites were flushed to collect unimplanted embryos. The mice which could not collect embryos were excluded from the experiment. N/A, not applicable.

Author contributions

YZ and ED designed the project, YZ and JQ designed and performed experiments, JQ, YZ, and YQ detected embryo implantation and oviductal embryo transfer, JS, XZ, and LZ helped to establish in vitro embryonic culture system, SL bred the mice, BHK and SJH provided technical assistance, TZ, QC, and SMW helped to revise the paper.

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