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Antifertility effectiveness of a novel polymer matrix composite and its influence on the endometrium in rhesus macaques (*Macaca mulatta*)

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ABSTRACT

Objective(s): To explored the antifertility effectiveness and influence on the endometrium of a micro-copper/low-density polyethylene/methyl vinyl silicone rubber (Cu/LDPE/MVQ) composite in rhesus macaques.
Study design: Healthy reproductive aged female rhesus macaques underwent abdominal hysterotomy for surgical placement of either the experimental Cu/LDPE/MVQ composite (Cu/LDPE/MVQ, $n=5$), bare copper wire (Cu, $n=5$), or hysterotomy only sham-operation group [(SOI, $n=4$), (SOII, $n=6$)]. Females in the Cu/LPDE/MVQ, Cu, and SOI groups were housed with fertile males for approximately three menstrual cycles. We assessed pregnancy by hysterectomy. Females in the Cu/LDPE/MVQ, Cu, and SOII groups underwent hysterectomy at about 4 months post-insertion for histologic assessment of morphologic changes of the endometrium, evaluation of materials using scanning electron microscopy (SEM), and evaluation of the inflammatory markers, including substance P receptor (SPR), associated with endometrial bleeding using enzyme linked immunosorbent assay, quantitative RT-PCR, and Western blot analyses.
Results: All of the SOI group females became pregnant (4/4, 100%). In contrast, no pregnancies occurred in either the Cu/LDPE/MVQ (0/5, 0%) or Cu (0/5, 0%) groups. We observed histologic features consistent with chronic endometrial inflammation in all females of the Cu group, but none of the SOII or Cu/LDPE/MVQ animals. Levels of inflammatory markers were significantly increased in the Cu group, compared with SOII or Cu/LDPE/MVQ groups ($p<.05$). SEM showed evidence of corrosion in the Cu wire not seen in the Cu/LDPE/MVQ group.
Conclusion(s): Cu/LDPE/MVQ material provided a contraceptive effect similar to Cu in macaques, with a lower impact on inflammation and inflammatory markers of the endometrium.
Implications: This study demonstrates the possibility of a Cu/LDPE/MVQ composite as an alternative to conventional copper device materials.

1. Introduction

Unwanted pregnancies and subsequently high rates of abortion remain a controversial issue worldwide [1]. Easy access to effective contraception is a significant factor in preventing unwanted pregnancies. Intrauterine devices (IUDs) are safe, cost-effective, reversible, and highly effective contraceptive devices, and they are the most commonly used long-acting reversible contraception worldwide [2–4]. It is well known that copper ions play an important role in the mechanism of Cu-IUDs. The cupric ions have a spermicidal effect and inhibit fertilization [5]. However, the copper ions result in local aseptic inflammatory reactions [6]. Although Cu-IUDs have undergone improvements such as the addition of hemostatic drugs and the alteration of the size and shape of the devices [7,8], adverse effects still inhibit the popularization of this contraceptive method. Large-scale epidemiological studies have shown that side effects such as pain, spotting, expulsion, and intermenstrual flow are the main reasons for the discontinuation of Cu-IUDs [9,10]. The adverse effects of Cu-IUDs have also been associated with the burst release phenomenon of the copper ions in the first few months after insertion [11]. Therefore, controlling the release rate of the copper ions should hopefully provide a valid way to alleviate the adverse effects and guarantee the antifertility effects of Cu-IUDs.

The burst release process is considered to result from the bulk copper coming into direct contact with uterine fluid. The micro-copper/low-density polyethylene/methyl vinyl silicone rubber (Cu/LDPE/MVQ) composite uses low-density polyethylene (LDPE) powder and methyl vinyl silicone rubber (MVQ) as a matrix to cover the copper micro-particles. As a result, the Cu^{2+} generated by the corrosion of the

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copper in the composite is diffused through the pore canals of the polymer matrix into the uterine cavity [12]. The LDPE powder and silicon rubber are commonly used high-polymer materials in the contraceptive field [13–15]. The LDPE powder possesses a high elasticity modulus, which makes it difficult to deform, and the silicon rubber is characterized by excellent flexibility, which contributes to its easy removal. In this study, we explored the potential of the Cu/LDPE/MVQ composite as a new contraceptive material. This potential IUD component material was prepared previously by the Department of Materials Science and Engineering of Huazhong University of Science and Technology, Wuhan, China. The experiments conducted in this study were performed to demonstrate the antifertility effectiveness of the Cu/LDPE/MVQ composite material and also its influence on the endometrium.

2. Materials and methods

2.1. General animal care

Twenty female rhesus macaques with regular menstrual cycles and proven fertility were provided by the nonhuman primate experiment center (Fujian Family Planning Institution, Fuzhou, China). The monkeys weighed 5–8.5 kg and were aged 6–13 years. All animal experiments in this study were performed according to the National Institutes of Health Guiding Principles in the Care and Use of Animals. All of the protocols were approved by the Ethical Committee of the Population Planning Research Institute, Fujian, China (NO.2015–01).

2.2. Treatment groups and surgery

The monkeys were divided into four groups: Cu/LDPE/MVQ composite (Cu/LDPE/MVQ, $n=5$), bare copper wire (Cu, $n=5$), and sham-operation [(SOI, $n=4$), (SOII, $n=6$)] groups. Three to five days after menstruation, the rhesus macaques in the Cu group, Cu/LDPE/MVQ group and the SO groups underwent anesthesia with 10% chloral hydrate (10 mg/kg intravenously), laparotomy and hysterotomy were performed, the corresponding materials were inserted into the uterine cavity through a small incision at the fundus of uterus, and then the incision was sewn up with a synthetic absorbable polyglycolic acid (PGA) suture, 3–0. The SO groups underwent the same operation, but no materials were inserted.

The rhesus macaques were monitored daily by vaginal swabbing to detect endometrial shedding denoting menstruation. When the menstrual flow recovered, females in SO I group and the Cu and Cu/LDPE/MVQ groups were mated with sexually mature, fertile male rhesus macaques to investigate the contraceptive effects. The menstrual cycles were monitored, and a rectal examination was performed when monkeys missed menses (15 days) to evaluate if the monkeys were pregnant. The breeding period lasted for approximately three menstrual cycles and all animals were evaluated the same way. The animals in SO II group did not undergo mating tests, and the females in SO II group together with the animals in the Cu and Cu/LDPE/MVQ groups were used to evaluate the influence of the contraceptive materials on the endometrium. After the mating experiment, the macaques in the four groups were anesthetized, laparotomy, hysterectomy and hysterotomy were performed for all of the animals. Pregnancy was confirmed by exploring the uteruses in SO I group and the Cu and Cu/LDPE/MVQ groups. The endometrial tissues and corresponding materials were collected from SO II group and the Cu and Cu/LDPE/MVQ groups. The uterine luminal fluid was gathered by uterine flushing. The endometrium was collected by scraping with a scalpel. All of the monkeys were sacrificed after sample collection.

2.3. Antifertility composite material

The Cu/LDPE/MVQ composite was provided by the Department of Materials Science and Engineering of Huazhong University of Science and Technology, Wuhan, China. The composite was made by combining the MVQ and LDPE powders with 15 wt% copper micro-particles (5 μm in diameter, with a purity of 99.9%). The composite was made by physicochemical methods (Supplementary Fig. 1). The LDPE/MVQ matrix was a controlled delivery carrier, with the copper micro-particles wrapped inside. The copper particles interacted with the simulated uterine solution through the gaps in the polymer matrix composite to control the copper ion release velocity (Supplementary Fig. 2). The composite material and bare copper we implanted were of cylindrical shape, 1 mm in diameter and 10.7 mm in length, with a surface area of 35 mm^2.

2.4. Fertility study

The animals were socially housed with one female and one male together, had ad libitum access to food and water. The breeding period lasted for approximately 3 menstrual cycles. After the mating experiment, the macaques were anesthetized with 10% chloral hydrate (10 mg/kg intravenously), laparotomy, hysterectomy and hysterotomy were performed for all of the females. Pregnancy was confirmed by exploring the uteruses of the SOI, Cu and Cu/LDPE/MVQ groups.

2.5. Evaluation of endometrium

2.5.1. Hematoxylin and eosin staining

The endometrium was cut into 3×3×3 mm^3 pieces, fixed with 10% paraformaldehyde solution, and embedded in paraffin blocks. Serial 4–5 μm thick sections were produced from the paraffin blocks. Next, the slides were stained with hematoxylin and eosin (HE) for observation under a light microscope.

2.5.2. ELISA detection

An enzyme linked immunosorbent assay (ELISA) was performed in line with the ELISA kit instructions (Cloud-clone Corp, Wuhan, China) to detect the platelet activating factor (PAF), tissue plasminogen activator (t-PA), platelet activating factor, prostaglandin E2 (PGE2) in the uterine luminal fluid. For each specimen, three-well parallel testing was implemented.

2.5.3. Real-time quantitative polymerase chain reaction (qRT-PCR)

The Trizol reagent was used for the total RNA extraction. The RNA concentration and OD260/280 ratio were determined by Nanodrop 2000 (Gene). The total RNA (1 μL) was used for the cDNA synthesis using the PrimeScript RT reagent kit (Takara). The specific primers of substance P receptor (SPR), and matrix metalloproteinase 9 (MMP9) and metalloproteinase inhibitor 1 (TIMP1) were synthesized previously (Supplementary Table 1). qPCR was performed using the New Light Cycler 96 Real-Time PCR Detection system (Roche) and SYBR Premix Ex Taq (Takara). The experiments were performed in triplicate for each sample. Water served as the negative control. Gene expression was obtained by normalizing the cDNA quantity to that of β-actin and calculated using the $2-\Delta\Delta Cq$ method.

2.5.4. Western blot determination

The Western blot protocol was performed as described previously [16]. The membrane was incubated with mouse-anti-human anti-SPR monoclonal antibody (Abcam, 1:1000 dilution), rabbit-anti-human anti-MMP9 monoclonal antibody (Abcam, 1:5000 dilution), and rabbit-anti-human anti-TIMP1 monoclonal antibody (Abcam, 1:1000 dilution). The mouse monoclonal anti-GAPDH antibody (Boster, Wuhan, China; 1:1000 dilution) was used as a loading control. All data were normalized to GAPDH.

2.6. Evaluation of materials

The contraceptive materials were prepared for scanning electron microscopy (SEM) observation (Tecnai G20 TWIN, USA). All of the

samples were gilded before observation. Three samples were used for each kind of material.

2.7. Statistical analysis

IBM SPSS 20.0 statistical software was used for the data analysis. All of the variable values are displayed as the mean±S.D. Comparison of inflammatory markers was made using one-way analysis of variance (ANOVA) and a Student-Newman-Keuls test. A chi-square test was used for statistical analyses in fertility study. A p value<.05 was considered to be statistically significant.

3. Results

3.1. Contraceptive effects of the materials

All of the SOI group females became pregnant (4/4, 100%). In contrast, no pregnancies occurred in either the Cu/LDPE/MVQ (0/5, 0%) or Cu (0/5, 0%) groups. The antifertility rates in the Cu and Cu/LDPE/MVQ groups were significantly higher than in the SO I group (100% vs.0, p<.05). There were no differences between the Cu and Cu/LDPE/MVQ groups.

3.2. Evaluation of endometrium

3.2.1. Histopathological analysis of the endometrium

The endometrial epithelial cells and stromal cells are arranged neatly in all of the SO II and Cu/LDPE/MVQ females. However, severe damage in the endometrium can be observed in females of the Cu group, including disordered arrangement and obvious exfoliation of the endometrial stromal cells, and mild cystic changes of the glands (Fig. 1).

3.2.2. ELISA evaluation of PAF, PGE2, t-PA in the uterine luminal fluid

The concentrations of PAF, PGE2, and t-PA in the Cu/LDPE/MVQ group (9.91±0.91, 11.88±4.07, 0.47±0.45 pg/ml, respectively) were significantly lower than in the Cu group (12.52±2.1, 25.91±8.57, 2.36±1.06 pg/ml, respectively), (p=.011, p=.006, p=.004). The concentrations were statistically different between the Cu group and SO II group (9.28±0.47, 7.14±6.08, 0.32±0.30 pg/ml, respectively), (p=.003, p=.001, p=.002). However, there was no difference between SO II group and the Cu/LDPE/MVQ group, (p=.474, p=.289, p=.767) (Fig. 2).

3.2.3. The mRNA expression of MMP9, SPR, TIMP1 in the endometrium

The MMP9 level in the Cu/LDPE/MVQ group was (1.75±0.34) times of the SO II group, (p=.009). The MMP9 level in the Cu group was (2.51±0.49) times of the SO II group, (p<.001). Moreover, the MMP9 level in the Cu group was higher than in Cu/LDPE/MVQ group, (p=.009). The TIMP1 level in the Cu/LDPE/MVQ group was (0.88±0.11) times of the SO II group, (p=.057). The TIMP1 level in the Cu group was (0.12±0.08) times of the SO II group, (p<.001). In addition, the expression level of TIMP1 in the Cu/LDPE/MVQ group was significantly higher than in the Cu group, (p<.001). The SPR level in the Cu/LDPE/MVQ group was (1.12±0.07) times of the SO II group, (p=.079). The SPR level in the Cu group was (1.93±0.12) times of the SO II group, (p<.001). Moreover, the SPR level in the Cu group was significantly higher than in the Cu/LDPE/MVQ group, (p<.001) (Fig. 3).

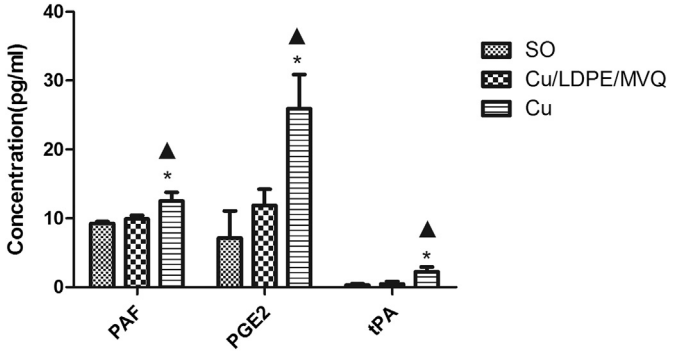

Fig. 2. Determination of the PAF, PGE2, and t-PA levels in the uterine luminal fluid of rhesus macaques by ELISA. SO, Cu/LDPE/MVQ, and Cu are the abbreviations for sham-operated group II, the Cu/LDPE/MVQ group, and the bare copper group, respectively. Compared with SO group II, *p<.05; Compared with the Cu/LDPE/MVQ group, ▲p<.05.

3.2.4. Protein expression levels of SPR, MMP9, and TIMP1 in the endometrium

The relative expressions of SPR and MMP9 in the Cu group (0.69±0.11, 0.92±0.17) were significantly higher than those in SO II group (0.23±0.05, 0.3±0.04), (p<.001, p<.001)and the Cu/LDPE/MVQ group (0.39±0.09, 0.69±0.06), (p<.001, p=.008). We observed elevated levels in the Cu/LDPE/MVQ group compared with SO II group, (p=.017, p<.001). A decrease of TIMP1 was also observed in the Cu group (0.29±0.06) compared with SO II group (0.94±0.19), (p<.001) and the Cu/LDPE/MVQ group (0.52±0.09), (p=.028). The TIMP1 level in the Cu/LDPE/MVQ group was lower than in SO II group, (p<.001) (Fig. 4).

3.3. Ultrastructural changes in the materials

The SEM images show significant ultrastructure alterations on the surface of the bare copper after usage, in contrast to the Cu/LDPE/MVQ. As shown in Supplementary Fig. 3B, serious corrosion can be seen in the bare copper, resulting in a rough surface, and sediment was absorbed on the surface. A tiny variance in the ultrastructure was observed after use in Cu/LDPE/MVQ. The surface was still smooth, with little sediment adhering to it (Supplementary Fig. 3).

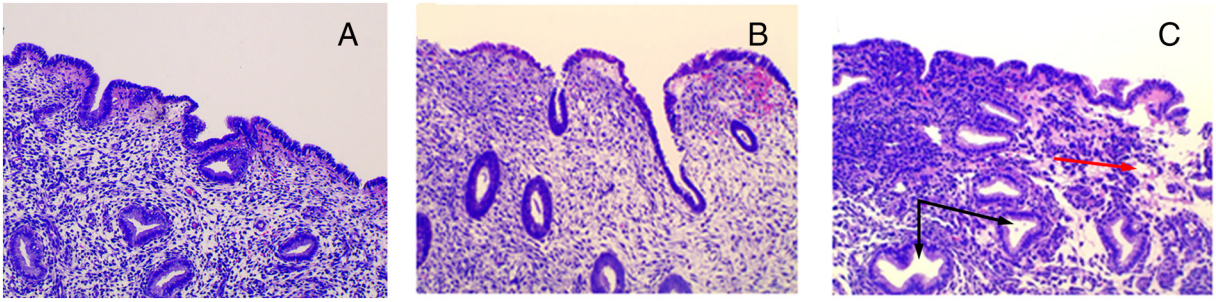

Fig. 1. Histopathological analysis of the endometrium in rhesus macaques by HE staining. (A) endometrium from sham-operated group II ×200; (B) endometrium from the Cu/LDPE/MVQ group ×200; (C) endometrium from the bare copper group ×200. Red arrowhead in C indicates disordered arrangement and exfoliation of the endometrial stromal cells, Black arrowhead in C indicates mild cystic changes of the glands.

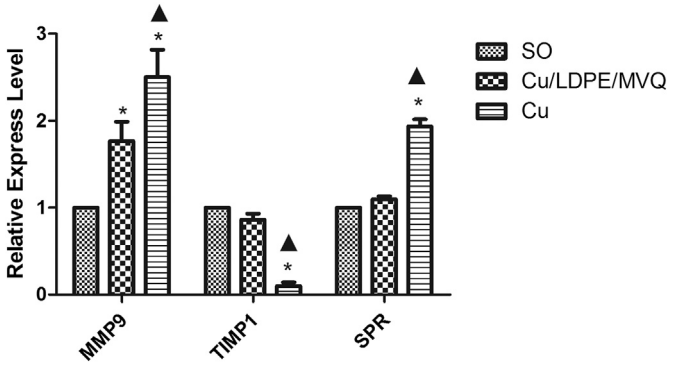

Fig. 3. The mRNA expression levels of SPR, MMP9, and TIMP1 in the endometrium of rhesus macaques by reverse transcription-quantitative polymerase chain reaction (qRT-PCR). SO, Cu/LDPE/MVQ, and Cu are abbreviations of sham-operated group II, the Cu/LDPE/MVQ group, and the bare copper group, respectively. Compared with SO group II, *p<.05. Compared with the Cu/LDPE/MVQ group, ▲p<.05.

4. Discussion

Since Zipper et al. found intrauterine copper had a suppression effect on fertility, Cu-IUDs have been widely used clinically for their satisfactory contraceptive effects [17]. However, conventional Cu-IUDs, consisting mainly of bare copper, are associated with several adverse effects such as pelvic pain, menorrhagia, and intermenstrual bleeding resulting from the burst release phenomenon [9,10]. To overcome the shortcomings of conventional Cu-IUDs, our team developed a novel Cu/LDPE/MVQ composite. The special effects of cupric ions and the controlled release technology contributed to the Cu/LDPE/MVQ composite preparation.

Rhesus macaques are non-human primates with one single uterus, and the uterus form of macaques is similar to that of humans. What's more, rhesus macaques give birth to one litter in each pregnancy. The physiological similarities between the rhesus macaques and human make the former an excellent model for exploring the effect of contraceptive materials. In this study we further demonstrated that the contraceptive effects of this novel composite was 100% in macaques, which was consistent with our previous experiments in rats and rabbits [16,18].

The immune response of the reproductive system is regulated by female sex hormones to balance immune protection [19]. Rhesus macaques are reported a median menstrual cycle length of approximately 28 days, which resemble that in humans [20,21]. Thus, the study of inflammatory factors generated from endometrium with response to contraceptive materials in rhesus macaques means the results provide further support for early phase clinical trials in women. The concentration of PAF, PGE2,t-PA in uterine luminal fluid, and the expression levels of SPR,MMP9 in the endometrium that are associated with genital tract inflammation [22–26], abnormal bleeding [27–29], pelvic pain [30,31] were significantly decreased in the Cu/LDPE/MVQ group, compared with the Cu group. Among the various factors that can damage the endometrium of animals, the surface condition of the contraceptive material might be important. The SEM images of the bare copper showed a rough surface and a lot of sediment, however, the surface of the Cu/LDPE/MVQ composite was smooth and clear. These findings suggest that Cu/LDPE/MVQ may relieve the side effects resulting from Cu-IUD insertion.

However, our current work had its limitations, considering the small sample size and short-duration. Thus , further researches with large sample size and longer duration are needed to test the effect of this novel composite material.

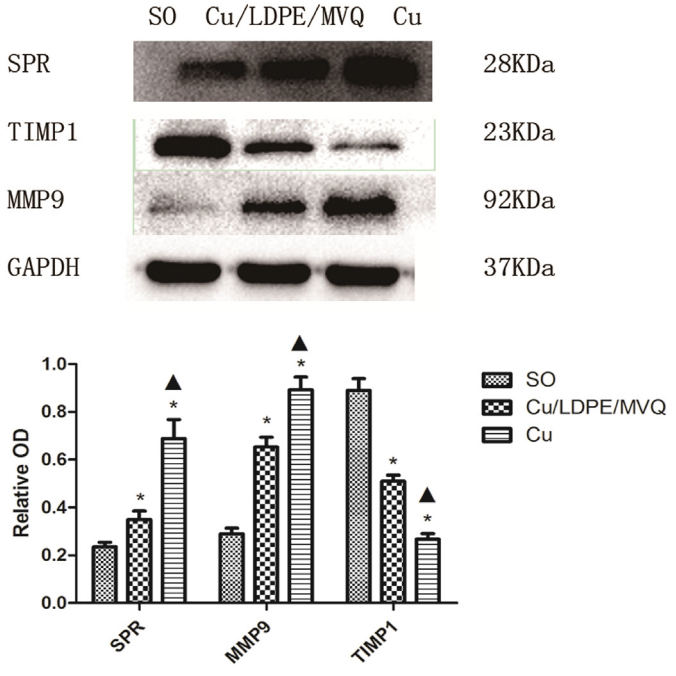

Fig. 4. Protein expression levels of SPR, MMP9, and TIMP1 in the endometrium of the rhesus macaques by Western blot. SO, Cu/LDPE/MVQ, and Cu are the abbreviations for sham-operated group II, the Cu/LDPE/MVQ group, and the bare copper group, respectively. The band above the bar diagram shows the protein bands from the Western blotting. The bar diagram exhibits the results of the analysis of the gray scale of the band. Compared with SO group II, *p<.05. Compared with the Cu/LDPE/MVQ group, ▲p<.05.

5. Conclusion

Cu/LDPE/MVQ exhibited satisfactory antifertility effectiveness with less influence on the environment of the endometrium and thus is a potential material for use in IUDs in the future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.contraception.2019.03.049.

Conflict of interest

None declared.

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