Prevention of intrauterine fetal growth restriction by administrating C1q/TNF-related protein 6, a specific inhibitor of the alternative complement pathway

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Abstract

Purpose The latest treatments do not sufficiently prevent miscarriage and fetal growth restriction (FGR) in pregnant women. Here, we assessed the effects of a human protein, CTRP6, that specifically inhibits the activation of the alternative complement pathway on miscarriage, fetal and placental development.

Methods Pregnant CBA/J mice mated with DBA/2 male mice as a model of spontaneous abortion and FGR were randomly divided into the control and CTRP6 groups. In the CTRP6 group, the mice were intravenously administered CTRP6 on days 4.5 and 6.5 post-conception (dpc). The abortion rate, fetal and placental weights on 14.5 dpc were examined. Remodeling of the spiral artery was also assessed.

Results The abortion rate in the CTRP6 group (13%) was reduced compared to the control group (21%), but there was no statistical difference. The placental and fetal weights in the CTRP6 group were also heavier than those in the control ($P < 0.05$). Moreover, the thickness of the blood vessel wall in the CTRP6 group was significantly thinner than that in the control ($P < 0.05$) and comparable to that in the non-abortion model (CBA/J x BALB). The ratio of the inner-per-the-outer diameter of the spiral artery increased more in the CTRP6 group than that in the control ($P < 0.05$). As well, the Th1/Th2 cytokine ratio was significantly reduced by CTRP6 treatment.

Conclusions Taken together, the supplementation with a protein that regulates the alternative complement pathway in vivo improves FGR and promotes spiral artery remodeling in a mouse model of miscarriage and FGR.

Key words: Abortion, Complement Alternative pathway, Recurrent pregnant failure, Spiral artery

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Authors' roles

M.K., A.T. and S.H. designed the experiment, interpreted the results, and wrote the manuscript with help from all authors. M.K., Y.M., and D.T. supervised the project.

Conflict of interest

The authors declare that no conflict of interest exists.

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Introduction

Miscarriage causes physical and mental distress for pregnant women. The causes of miscarriage include chromosomal abnormalities, uterine morphology, endocrine disorders, and infections [1]. On the other hand, unexplained miscarriages are often observed. Despite the introduction of technology into clinical practice to identify abnormalities in the chromosome number of fertilized ova prior to embryo transfer, which has been shown to reduce the miscarriage rate, miscarriages in early pregnancy have not been fully overcome [2, 3]. Since the fetus is semi-allogeneic, the maternal immune system shows an immune tolerance to the fetus [4]. Because the fetus is able to escape from the immune attack by the mother and differentiate and grow in an environment of immune tolerance, it is thought that a unique immunosuppressive mechanism is at work in the placenta [5–8].

The feto-maternal site of the placenta has its own unique immune system, such as uterine natural killer (uNK) cells [9], macrophages, T cells, dendritic cells, B cells, and NKT cells. The balance of Th1/Th2 cytokines varies according to the stage of pregnancy, but is characterized by a predominance of Th2-type immunity after implantation due to feto-maternal immune tolerance [10, 11]. It is reported that the administration of excess amounts of Th1-type cytokines induces abortion in mice [12].

The complement system, particularly the alternative pathway, has been shown to be involved in the development of this unexplained spontaneous abortion, fetal growth restriction (FGR) and

preeclampsia [13]. The complement system is a basic immune system in the body but, when it becomes strongly activated, it can damage its own cells through anaphylactic shock reactions and membrane injury of the membrane attack complex (MAC) [14]. It has also been shown that the deposition of C3, the most abundant complement component in the body, in the placenta can cause miscarriage [15]. The activation of the complement system is accomplished by three pathways: the classical pathway, the alternative pathway, and the mannose-binding lectin (MBL) pathways. The complement factor MBL2 has been shown to be associated with adverse pregnancy outcomes and to play a role in preeclampsia and abnormal placentation [16–20]. However, the classical and lectin pathways are characterized by a specific immune response by antigen-antibody complexes, while the alternative pathway is a non-specific innate immune response. In humans, the alternative pathway has been shown to be activated in women with recurrent miscarriage and preeclampsia [21]. Therefore, specific inhibition of the activation of the alternative complement pathway is expected to prevent these complications to pregnancy. Indeed, the suppression of the alternative pathway by antibody injection improves the abortion rate in mice [13]. However, the antibodies used in mouse experiments do not exist in vivo, and it is unclear how they would affect humans, especially pregnant women. Therefore, their clinical application is considered to be difficult.

C1q/TNF-related protein 6 (CTRP6; gene symbol C1qtnf6) specifically suppresses the alternative pathway of the complement system and is known to be highly expressed in the placenta [22]. CTRP6

has been shown to be an endogenous complement regulator and could be used for the treatment of complement-mediated diseases [23]. Furthermore, it has been shown that C1qtnf6 gene-deficient mice would be semi-lethal, suggesting that the gene-deficiency could potentially affect fetal development [23]. In humans, it has been suggested that CTRP6 might be involved with the pathogenesis of polycystic ovary syndrome which is one of the most common endocrine disorders affecting females of reproductive age [24]. Patients with preeclampsia have been shown to have a significantly higher frequency of SNPs in C1qtnf6 gene compared to normal pregnant women, but the mechanism is unclear [25]. However, the role of CTRP6 in pregnancy maintenance and fetal growth has not yet been fully elucidated. In addition, CTRP6 specifically regulates only the alternative pathway among the three complement activation pathways, and it is expected to reduce side effects such as increased susceptibility to bacterial and fungal infections. Because CTRP6 inhibits the first reaction of the alternative pathway, it is expected to be more efficient than therapies targeting other complement components.

Here, we investigated the role of CTRP6 in the prevention of miscarriage and FGR using CBA/J female mice mated with DBA/2 male mice as a model of spontaneous abortion, which is known to exhibit similar pathology as recurrent miscarriage in humans [26, 27] and FGR [28].

Materials and Method:

Mice

Female CBA/J JAX® mice (Charles River, Yokohama, Japan), male BALB/cAJcl (Japan CLEA, Tokyo, Japan) mice and male DBA/2JJc1 mice (Japan CLEA) were reared under controlled temperature (22 ± 2 °C) and a 12-hour light: 12-hour dark schedule with water and food ad libitum. Eight- to 12-week-old virgin female mice were mated with 8- to 12-week-old male mice. The presence of a vaginal plug was designated as 0.5 days post-conception (dpc). Pregnant females on 14.5 dpc were sacrificed under anesthesia, and blood, fetus and placenta samples were collected. Implantation sites were divided into normal and resorption sites, which were visually identified by the presence of a normally developed or dead fetus. Fetal and placental weights were measured after the fetuses and placentas were separated. The placentas were either frozen and stored at −80°C in 500 µl RNAlater™ Stabilization Solution (AM7020, Thermo Fisher Scientific, Inc., Waltham, MA, USA) (for RNA and protein) or immediately fixed in 4% (w/v) paraformaldehyde overnight at 4°C and stored in 70% (v/v) ethanol at 4°C until being embedded in paraffin by standard procedures for morphology. This study was approved by the Institutional Animal Care and Use Committee of Osaka City University (Permission number: 18041). In addition, all experiments involving live animals were performed in accordance with relevant guidelines and regulations and were reported as described by the recommendations in the ARRIVE guidelines.

Administration protocol

Pregnant CBA/J mice mated with DBA/2 male mice were randomly divided into two groups: control (N: 9) and CTRP6 (N: 11, Fig.1). In the CTRP6 group, the mice were intravenously administered 3 μg of recombinant human CTRP6 (RD172156100 Lot#AP-16-033 Z, Biovendor Laboratory Medicine, Czech Republic) on 4.5 and 6.5 dpc. In the control group, the mice were injected with phosphate-buffered saline (PBS) on 4.5 and 6.5 dpc. The dose concentration of the CTRP6 was determined taking into consideration that the CTRP6 protein concentration in the blood of mice was originally 1 μg/ml [23]. The injection day was determined with reference to a complement inhibition experiment in CBA/J × DBA/2 mice [13] and a complement activation experiment in CBA/J x BALB/c mice [29].

Immunostaining and Histology

For immunostaining, the rehydrated paraffin-embedded placenta sections (4 μm) were treated with 0.01 M citrate buffer (pH 6.0) and 3% H_2O_2 in methanol and then incubated with rabbit polyclonal anti-CTRP6 antibody (ab36900, Abcam, Cambridge, UK). Specific binding of the primary antibody was detected with Histofine® Simple Stain MAX PO(R) (Nichirei Bioscience, Tokyo, Japan). Peroxidase activity of the secondary antibody was detected by diaminobenzidine, and the sections were counterstained with hematoxylin.

To evaluate the remodeling of the spiral artery, sections were stained with the periodic acid Schiff's (PAS) procedure. Images were captured using phase-contrast light microscopy (BZ-X800, Keyence, Osaka, Japan). Serial sections were cut sagittal to the midline. The outer and inner diameters of a short section of the blood vessel were measured and half of the outer diameter value minus the inner diameter value was calculated as the thickness of the blood vessel wall using 18 blood vessels in 12 placentae of 5 mice in the control group, 24 blood vessels in 12 placentae of 5 mice in the CTRP6 group, and 8 blood vessels in 4 placentae of 3 mice in a non-abortion model (CBA/J female mice mated with BALB/c male mice, Fig. 1). Although BALB/c mice have the same MHC class antigens as DBA/2 mice, mating CBA/J female mice with BALB/c male mice results in a low abortion rate, so this mouse pair has been used as a non-abortion model when analyzing CBA/J female and DBA/2 male mice [13, 29]. RNA Extraction, cDNA Synthesis, and quantitative PCR (qPCR)

Total RNA were extracted from the uteri or placentas and purified with ISOGEN (331-02501, NIPPON GENE CO., LTD., Toyama, Japan) according to the protocol. A fixed amount of total RNA was reverse-transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manual. The expression of target genes was evaluated by qPCR (Rotor-Gene Q, Qiagen) with the following reaction system: 0.6 µl of cDNA was incubated with 25 pmol of both reverse and forward primers from the gene of interest and 12.5 µl of SYBR Green master mix (QuantiFast SYBR Green PCR Kit, Qiagen) and was made up to a final volume of 25 µl with RNase free water. Analysis of the genes

was performed by the delta-delta CT method, and relative expression levels were calculated in comparison to GAPDH as a control. We measured the gene expression levels of CTRP6 and vascular endothelial growth factor (VEGF)-A, placental growth factor (PlGF), transforming growth factor-beta (TGF-β), and insulin-like growth factor 1 (IGF-1) as vascular growth factors in the placenta. In addition, tumor necrosis factor-alpha (TNF-α) were measured as Th1-related cytokines, and interleukin (IL)-6 and IL-10 were measured as Th2-related cytokines (see supplementary table for primer sequences). We examined the CTRP6/GAPDH expression from 3-4 placentae at each stage (non-pregnant, 4.5, 6.5, 8.5, 10.5, 12.4 and 14.5 dpc). Because sufficient amounts of RNA could not be obtained, we compared data from 7 placentae for the control and 10 placentae for the CTRP6 group for VEGF-A, 8 placentae for the control and 9 placentae for the CTRP6 group for PlGF, 8 placentae for the control and 10 placentae for the CTRP6 group for TGF-β, and 8 placentae for the control and 10 placentae for the CTRP6 for IGF-1. Seven placentae for the control and 8 placentae for the CTRP6 group for TNF-α/IL-6, and 5 placentae for the control and 8 placentae for the CTRP6 group were used for TNF-α/IL-6. One placenta was obtained from each mouse.

ELISA

Serum was collected from 8 mice in the control group and 10 mice in the CTRP6 group on 14.5 dpc to measure the concentration of the secreted soluble fms-like tyrosine kinase-1 (sFLT-1). Serum levels of sFLT1 were measured by the Quantikine® ELISA kit (MVR100, R&D systems, Minneapolis, MN, USA).

The optical density was measured at 450 nm and 540 nm by a multimode microreader (Varioskan LU, Thermo Scientific, Tokyo, Japan). The average of the duplicate readings for standard, control, and individual samples was used for the analyses.

Statistical analysis

Differences between the two groups were analyzed using the unpaired Student's t-test. When more than two groups were compared, analysis of variance (ANOVA), followed by the Tukey–Kramer test, was used. Normality and homogeneity of variances were confirmed by the Shapiro-Wilk and the Levene tests before parametric analyses (t-test and ANOVA) were run. A p-value less than 0.05 was considered to be significant. Statistical analysis was performed using Excel Statistics or R programming.

Results

Expression of CTRP6 in the placenta

Prior to the start of the CTRP6 administration study, the expression pattern of CTRP6 in the placenta during pregnancy was investigated. An increase of CTRP6 mRNA expression in the placenta was observed as the pregnancy progressed (Fig. 2a). In particular, the gene expression significantly increased on 14.5 dpc, compared with the expression in the non-pregnant mice ($P < 0.01$). The results of immunostaining experiments showed that the CTRP6 protein was localized in the endometrium of the pregnant mice (Fig. 2b-d).

Abortion rate, fetal weights and placental weights

We tested whether CTRP6 administration affects the miscarriage rate and fetal growth of CBA/J female mice mated with DBA/2 male mice as a model of spontaneous abortion. Although there was no significant difference in the abortion rate per mouse 14.5 dpc (Fig. 3a), the rate in the CTRP6-injected group (CTRP6, 13%) declined compared that in the control (21%) group. There was also no difference in the number of implantation sites per dam (Fig. 3b). The placental and fetal weights in the CTRP6 group (placenta: 104 ± 16 mg; fetus: 163 ± 25 mg) was heavier (placenta: $P < 0.05$; fetus: $P<0.01$) than those in the control (placenta: 97 ± 13 mg; fetus: 148 ± 21 mg) group (Fig. 3cd). Moreover, CTRP6 supplementation improved the FGR in the FGR mouse model.

Blood vessel thickness of spiral arteries in the decidua basalis

Remodeling of the spiral artery is known to be important for continuing pregnancy [30, 31]. Remodeling of spiral arteries consists of the process of increasing the vessel lumen diameter and thinning of the vessel wall [32]. To assess the effect of CTRP6 on the remodeling of the spiral artery, we measured the thickness of the blood vessel wall in decidua basalis on 14.5 dpc (Fig. 4a). The thickness of the blood vessel wall in the CTRP6 group (12.7 ± 3 μm, Fig. 4b) and the non-abortion group (11.8 ± 4 μm) was significantly thinner than that in the control group ($P < 0.05$, 18 ± 4 μm). The ratio of the inner-per-the-outer diameter of the spiral artery increased more in the CTRP6 group than in the control group ($P < 0.05$, Fig. 4c). The thickness of the blood vessel wall and the ratio of the inner-per-the-outer diameter of the spiral artery in the CTRP6 group were similar to those in the non-abortion group, respectively. These data indicated that CRTP6 administration supported the remodeling of the spiral artery. The abortion rate of CBA/J x BALB/c mice was 15.1 ± 1.4 % (n=3).

Gene expression of cytokines and angiogenic factor in placenta

We assessed the effect of CTRP6 on cytokine gene expression in placentas on 14.5 dpc.

CTRP6 administration increased the expression levels of VEGF-A ($P < 0.05$, Fig. 5a), compared with the control group. However, levels of TGF-β, PlGF and IGF-1 were no different between the two groups. The ratio of the Th1/Th2 balance, TNF-α per IL-6 and TNF-α per IL-10 in the CTRP6

group were all significantly lower ($P < 0.05$, Fig. 5b) than those in the control group.

Amount of sFlt1 in blood samples

During preeclampsia, sFLT1 acts as a decoy receptor for VEGF receptors by reducing free circulating levels of the proangiogenic factors of the VEGF family, including PlGF. Thus, sFLT1 is thought to be a key player in preeclampsia pathology and a main cause of maternal hypertension and proteinuria [33, 34]. To explore why the blood vessel walls of the spiral artery were thinning, we measured sFlit1 levels. However, CTRP6 administration didn't affect these levels (Fig. 6).

Discussion

The data in the present study showed that treatment to a mouse model of abortion and FGR with CTRP6, a protein that inhibits the activation of the alternative complement pathway in vivo, results in a thinning of the vessel wall thickness of the spiral arteries and an improvement in fetal growth.

Although CTRP6 gene expression is known to be higher in the placenta than in other tissues [22], the localization of the protein and whether it changes during gestation are unknown. We performed CTRP6 immunostaining of the uterus and placenta and found that CTRP6 is expressed in the endometrium during implantation and is localized in the placenta at the maternal-fetal interface, especially on the fetal side (Fig. 2). The expression pattern of CTRP6 gene in the placenta during pregnancy was also examined and compared with that in the non-pregnant uterus; CTRP6 expression in the placenta increased with increasing gestational age and was significantly higher at 14.5 days of gestation. This suggests that CTRP6 may play a role in the maintenance of pregnancy.

In human recurrent spontaneous abortion, serum C3 and C4 are elevated, and complement is activated [35], thus indicating that complement activation affects miscarriage and FGR in humans as it does in mice [36]. Furthermore, an inhibition of complement activation at the fetoplacental compartment [37, 38] is essential for the maintenance of a normal pregnancy [13]. Therapeutic agents that inhibit complement activity include C1 inhibitors for the treatment of hereditary angioedema and C5 inhibitors for the treatment of paroxysmal nocturnal hemoglobinuria and

atypical hemolytic uremic syndrome, both of which have been reported for use in pregnant women [39, 40]. However, concerns that C1 inhibitor products cannot completely inactivate parvoviruses during the manufacturing process, and that C5 inhibitors increase the risk of meningococcal infections [41], have led to the need to develop safer treatments.

In this study, CTRP6, an in vivo protein that regulates the activation of the complement alternative pathway [23], was administered to CBA/J x DBA/2 mice and improved the FGR (Fig. 3). In addition, CTRP6 administration showed a decreasing trend in the miscarriage rate. Compared to the improvement of miscarriages obtained by suppressing the alternative pathway by daily administrating an antibody against factor B from 4.5 to 10.5 dpc [13], the improvement of miscarriages by supplementation with CTRP6 was a little low. The improvement of IUGR by supplementation of CTRP6, a protein that regulates upstream the activation of the alternative complement pathway localized in the endometrium, is expected to offer great hope to patients suffering from unexplained miscarriages.

We investigated which mechanism had a positive effect on fetal weight. Remodeling of the spiral arteries at the decidua, the maternal-fetal interface, is said to be important in maintaining a normal pregnancy. Therefore, we measured the size of spiral arteries and investigated the expression of the VEGF-A gene and the protein level of sFLT1, both of which are related to angiogenesis. Treatment with CTRP6 resulted in a thinning of the spiral artery wall and an increase in the internal/external

diameter ratio (Fig. 4). The remodeling of spiral arteries is completed by the replacement of trophoblasts with smooth muscle cells and vascular endothelial cells [42]. Residual vasoconstriction due to impaired remodeling reduces the blood supply to the intervillous spaces, thus leading to placental abruption, delayed fetal growth, and an increased risk of miscarriage. Our results showed that FGR in CBA/J x DBA/2 mice due to inadequate vascular remodeling were improved by supplementation with CTRP6, which normalized the remodeling of the spiral arteries via the inhibition of complement activation. Consequently, CTRP6 treatment improved FGR and lead to a downward trend in the miscarriage rate.

Although the balance of Th1/Th2 cytokines changes according to the stage of pregnancy [10, 11], Th2 dominance in the balance is important for pregnancy maintenance [12, 43, 44]. Therefore, we investigated the gene expression of Th1 and Th2 related cytokines in the placenta. The Th1/Th2 cytokine balance ratio was significantly reduced by CTRP6 treatment (Fig. 5). This result agreed with a report that an elevated Th1/Th2 cytokine balance ratio reduces placental function and causes miscarriage [29], and those results suggested that the increase in VEGF-A has a pro-angiogenic effect and that the predominance of Th2 cytokines over Th1 cytokines is immunologically responsible for the inhibition of FGR.

There was a trend towards a reduction in miscarriage rates with CTRP6 in this study. We believe that the lack of statistical difference was due to an insufficient sample size. The amount of drug used in

this experiment was calculated to be the in vivo concentration of the mouse upon administration [23]. Therefore, if the administered mice were not deficient in the CTRP6 protein, the amount would be double the amount they originally had. As a result, the risk of infection caused by alternative pathway inhibition needs to be considered in the future. Further studies on the various conditions regarding the dose and date of administration of CTRP6 are required. Results of the present work show that supplementation with a protein that regulates the alternative complement pathway in vivo improves fetal growth and promotes spiral artery remodeling in a mouse model of miscarriage and FGR.

Figure legends

Fig. 1. Experimental design. The presence of a vaginal plug was designated as 0.5 dpc. Pregnant CBA mice mated with DBA male mice were randomly divided into two groups: CTRP6 and control. In the CTRP6 group, the mice were administered CTRP6 on 4.5 and 6.5 dpc. In the control group, the mice were injected with PBS on 4.5 and 6.5 dpc. Pregnant females were sacrificed on 14.5 dpc, and blood, fetus and placenta samples were collected. CBA female mice mated with BALB male mice were sampled similarly to assess the remodeling of the spiral artery as a non-abortion model.

Fig. 2. Gene expression levels and localization of CTRP6 in the uterus and placenta. CTRP6 gene expression levels in the uterus and placenta of pregnant mice increased as the pregnancy progressed. Parentheses show the number of examined mice. *P < 0.05 by Tukey Kramer test following ANOVA. (b-d) CTRP6 protein was localized at the endometrium and maternal-fetus interface, as shown by brown-staining with anti-CTRP6 antibody immunostaining. Uterus of non-pregnant mouse (b), 4.5 dpc (c), and 10.5 dpc (d). Scale bars are as shown in the figures b-d.

Fig.3. Effect of CTRP6 on abortion and fetus weight. (a) The abortion rate per dam on 14.5 dpc showed a declining trend by the CTRP6 injection (13%, n=11 mice) compared with the control (21%, n=9 mice). (b) There was no difference in the number of implantation sites per dam. control: n=9 mice, CTRP6: n=11 mice. (c) The average placental weights were 102 mg in the CTRP6 and 99 mg in the control . Control: n=50 placentae, CTRP6: n=66 placentae. In the CTRP6 group, the placental weight was heavier than that in the control group (P < 0.05). (d) The fetal weight in the CTRP6 group (163 mg) was heavier (P <0.01) than in the control (148 mg) group. Control: n=50 fetuses, CTRP6: n=66 fetuses. Data are presented as a box plot with median, interquartile range ± upper/lower extreme. X: Mean value. *P < 0.05, **P < 0.01 by the student's t-test.

Fig.4. Effect of CRTP6 on the remodeling of the spiral artery. (a) An image of the spiral artery. Broken circle shows the blood vessel wall. Red and black dimension lines show the inner diameter and outer diameter of the spiral artery, respectively. Scale bar shows 100 μm.
Arrowhead shows a vascular endothelial cell. (b) Vascular wall thickness was thinner in CTRP6 and non-abortion groups than that in control. Control: n=18 blood vessels in 12 placentae of 5 mice, CTRP6: n=24 blood vessels in 12 placentae of 5 mice, non-abortion: n=8 blood vessels in 4 placentae of 3 mice. (c) The inner per outer diameter ratio increased in CTRP6 group compared with that in control group. control: n=18, CTRP6: n=24, BALB: n=8. Data are presented as a box plot with median, interquartile range ± upper/lower extreme. X: Mean value. **P < 0.01 by Tukey Kramer test following ANOVA.

Fig.5. Effect of CTRP6 on the gene expression for cytokines and angiogenic factors in the placenta on 14.5 dpc. (a) CTRP6-administration in the CTRP6 group increased the expression levels of VEGF compared with that in the control group. However, levels of TGF-β, PlGF and IGF-1 were no different between the two groups. For VEGF-A, control n: 7 placentae, CTRP6 n: 10 placentae. For TGF-β, control n: 8 placentae, CTRP6 n: 10 placentae. For PlGF, control n: 8 placentae, CTRP6 n: 9 placentae. For IGF-1, control n: 8 placentae, CTRP6 n: 10 placentae. (b) The ratios of TNF-α per IL-6 and TNF-α per IL-10 in the CTRP6 group were significantly lower than those ratios in the control group. For TNF-α/IL-6, control n: 7 placentae, CTRP6 n: 8 placentae. For TNF-α/IL-6, control n: 5 placentae, CTRP6 n: 8 placentae. One placenta was obtained from each mouse. Data are presented as a box plot with median, interquartile range ± upper/lower extreme. X: Mean value. *P < 0.05 by student's t-test.

Fig.6. Effect of CTRP6 on the serum concentration of the soluble FLT-1 in CBA/J mated with DBA/2 mice on 14.5 dpc. There was no difference between the two groups. control: n=7 mice, CTRP6: n=5 mice. Data are presented as a box plot with median, interquartile range ± upper/lower extreme.

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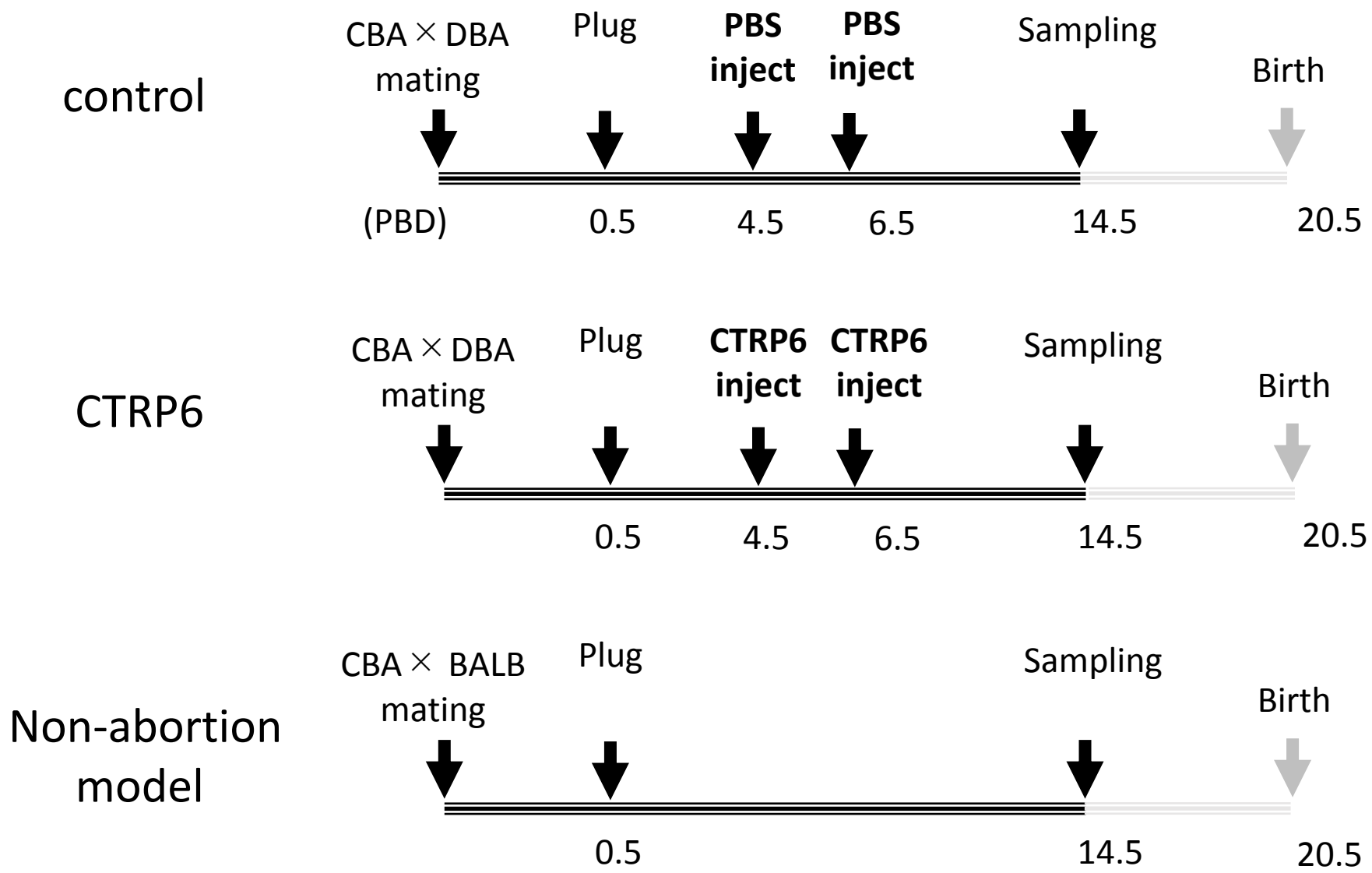

Fig. 1. Experimental design. The presence of a vaginal plug was designated as 0.5 dpc. Pregnant CBA mice mated with DBA male mice were randomly divided into two groups: CTRP6 and control. In the CTRP6 group, the mice were administered CTRP6 on 4.5 and 6.5 dpc. In the control group, the mice were injected with PBS on 4.5 and 6.5 dpc. Pregnant females were sacrificed on 14.5 dpc, and blood, fetus and placenta samples were collected. CBA female mice mated with BALB male mice were sampled similarly to assess the remodeling of the spiral artery as a non-abortion model.

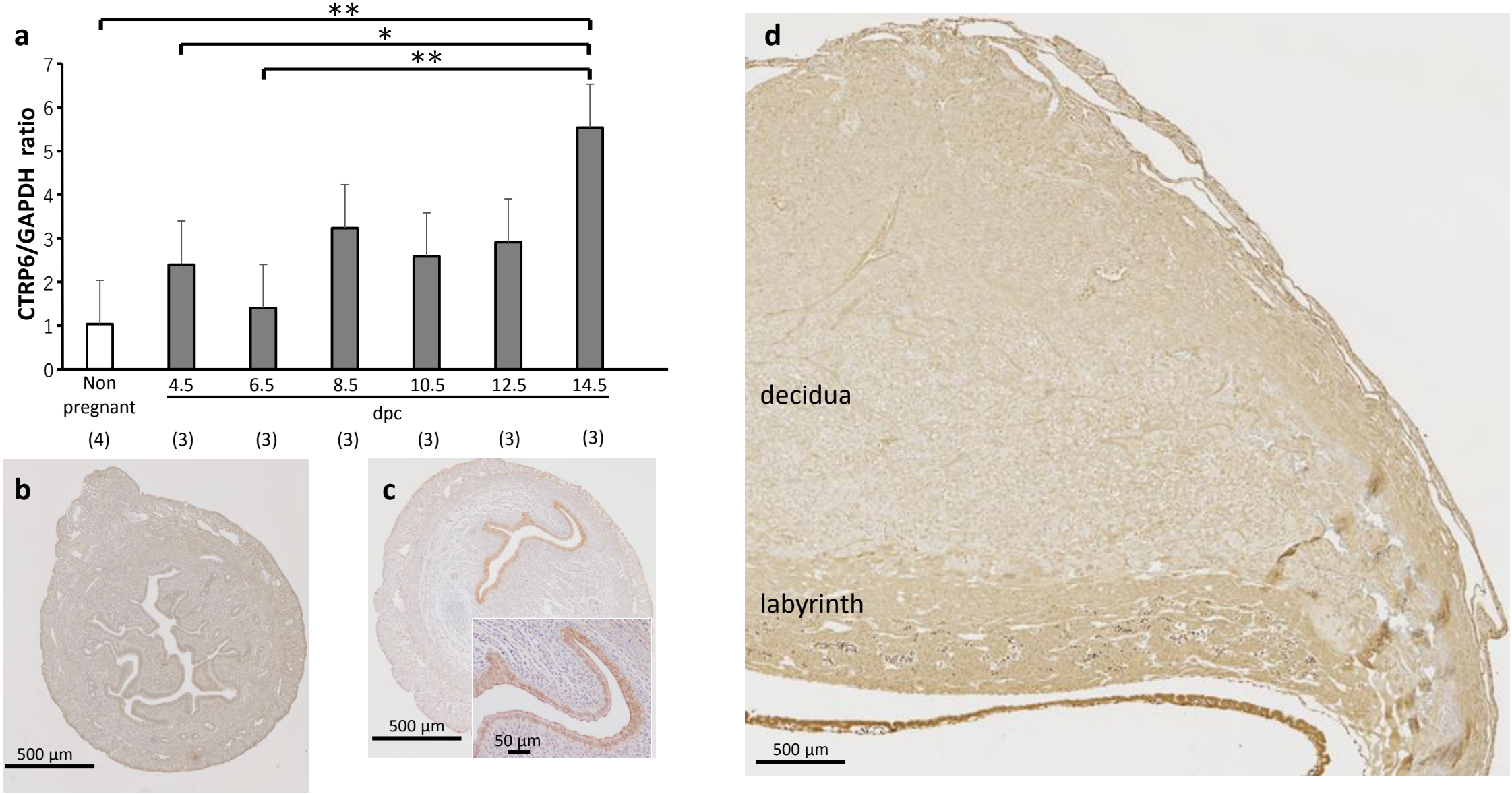

Fig. 2. Gene expression levels and localization of CTRP6 in the uterus and placenta. CTRP6 gene expression levels in the uterus and placenta of pregnant mice increased as the pregnancy progressed. Parentheses show the number of examined mice. *P < 0.05 by Tukey Kramer test following ANOVA. (b-d) CTRP6 protein was localized at the endometrium and maternal-fetus interface, as shown by brown-staining with anti-CTRP6 antibody immunostaining. Uterus of non-pregnant mouse (b), 4.5 dpc (c), and 10.5 dpc (d). Scale bars are as shown in the figures b-d.

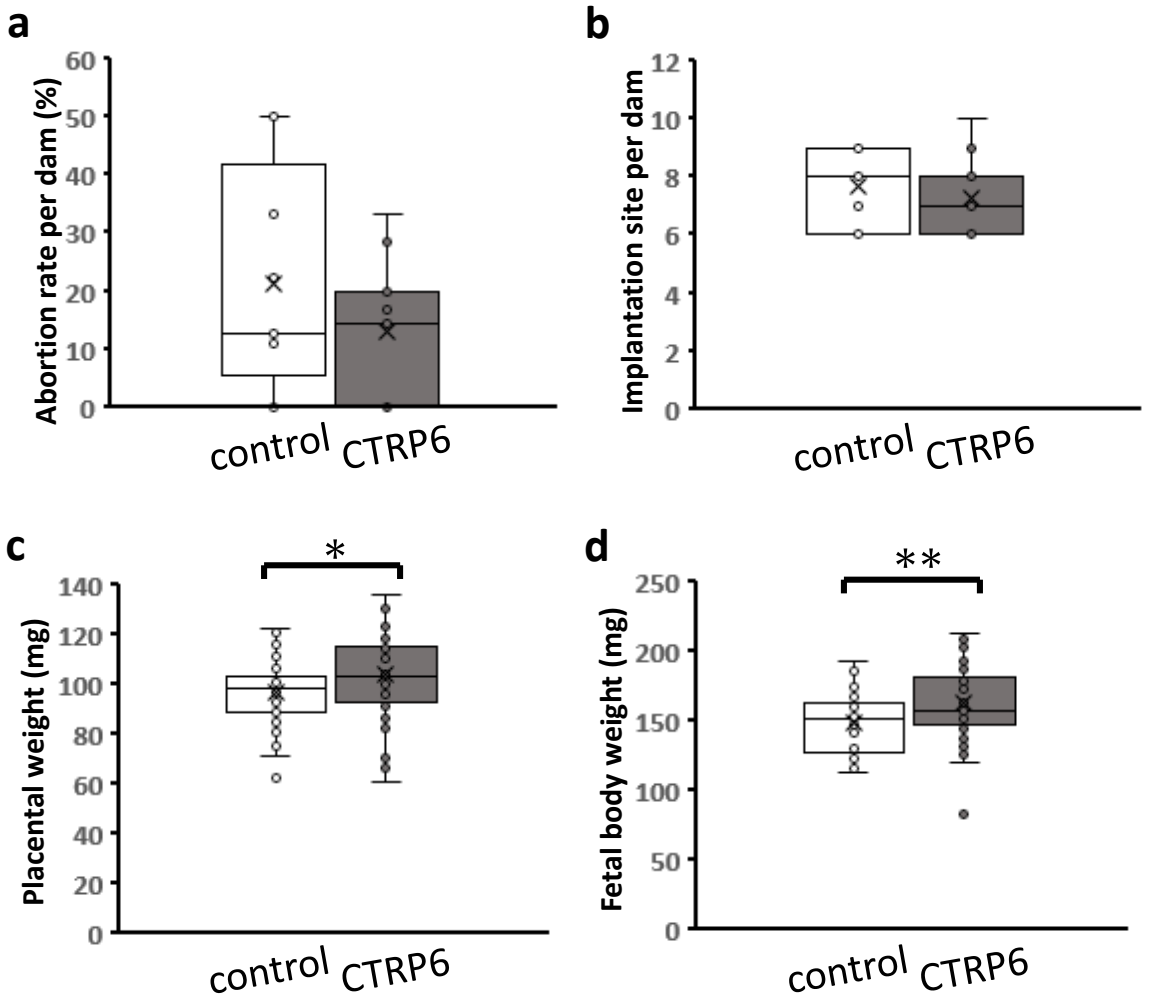

Fig.3. Effect of CTRP6 on abortion and fetus weight. (a) The abortion rate per dam on 14.5 dpc showed a declining trend by the CTRP6 injection (13%, n=11 mice) compared with the control (21%, n=9 mice). (b) There was no difference in the number of implantation sites per dam. control: n=9 mice, CTRP6: n=11 mice. (c) The average placental weights were 102 mg in the CTRP6 and 99 mg in the control . Control: n=50 placentae, CTRP6: n=66 placentae. In the CTRP6 group, the placental weight was heavier than that in the control group ($P < 0.05$). (d) The fetal weight in the CTRP6 group (163 mg) was heavier ($P < 0.01$) than in the control (148 mg) group. Control: n=50 fetuses, CTRP6: n=66 fetuses. Data are presented as a box plot with median, interquartile range ± upper/lower extreme. X: Mean value. *$P < 0.05$, **$P < 0.01$ by the student's t-test.

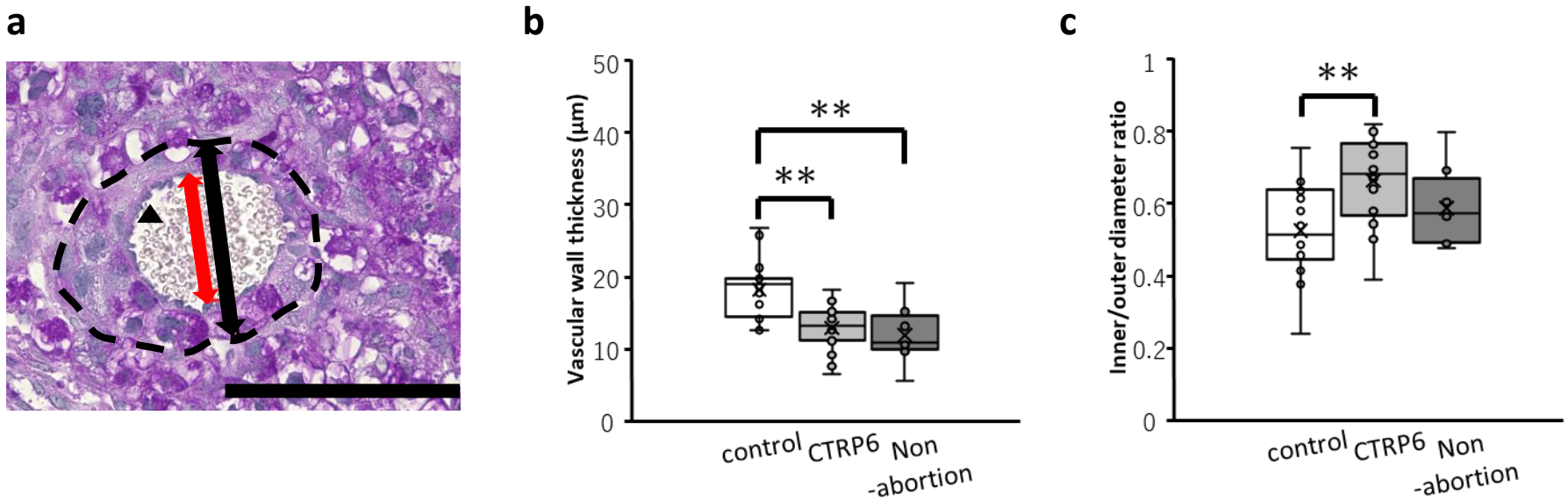

Fig.4. Effect of CRTP6 on the remodeling of the spiral artery. (a) An image of the spiral artery. Broken circle shows the blood vessel wall. Red and black dimension lines show the inner diameter and outer diameter of the spiral artery, respectively. Scale bar shows 100 μm. Arrowhead shows a vascular endothelial cell. (b) Vascular wall thickness was thinner in CTRP6 and non-abortion groups than that in control. Control: n=18 blood vessels in 12 placentae of 5 mice, CTRP6: n=24 blood vessels in 12 placentae of 5 mice, non-abortion: n=8 blood vessels in 4 placentae of 3 mice. (c) The inner per outer diameter ratio increased in CTRP6 group compared with that in control group. control: n=18, CTRP6: n=24, BALB: n=8. Data are presented as a box plot with median, interquartile range ± upper/lower extreme. X: Mean value. **P < 0.01 by Tukey Kramer test following ANOVA.

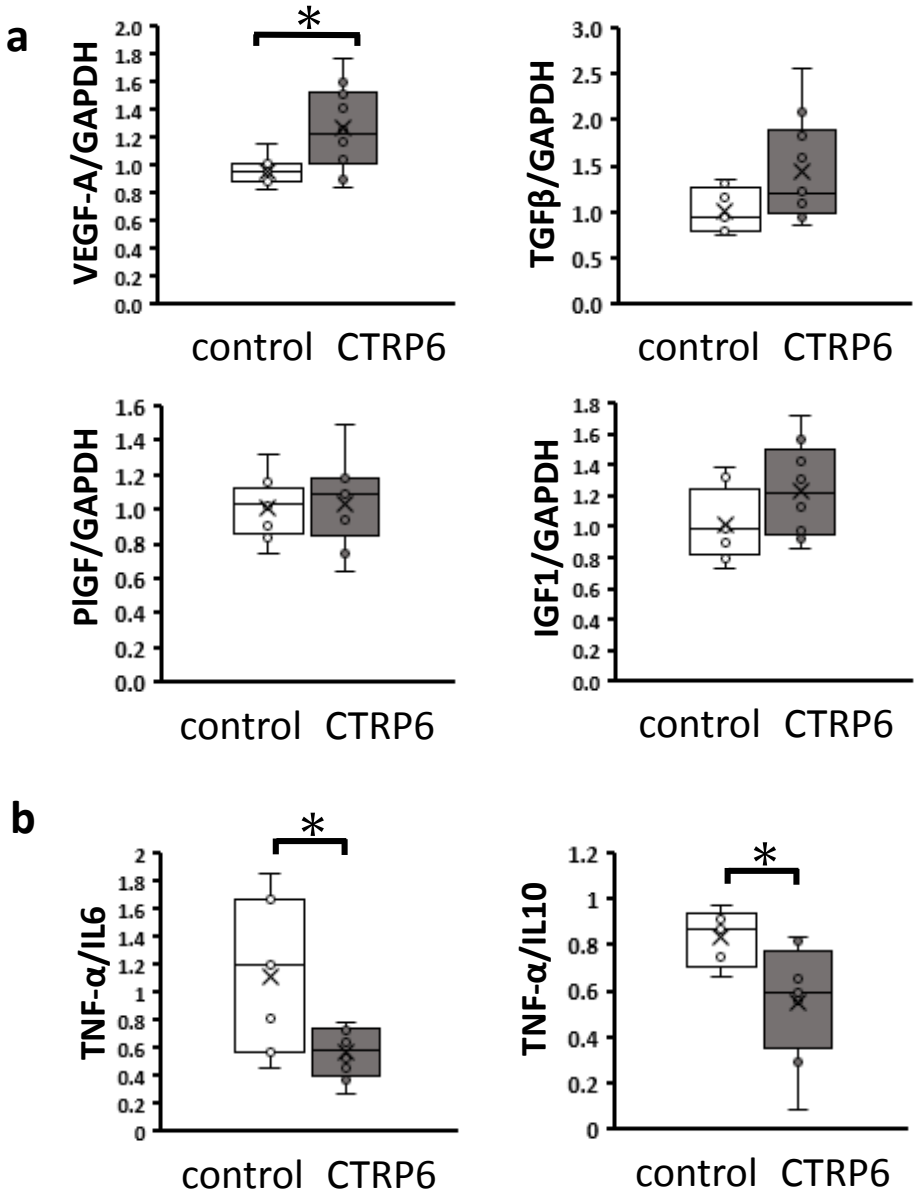

Fig.5. Effect of CTRP6 on the gene expression for cytokines and angiogenic factors in the placenta on 14.5 dpc. (a) CTRP6-administration in the CTRP6 group increased the expression levels of VEGF compared with that in the control group. However, levels of TGF-β, PlGF and IGF-1 were no different between the two groups. For VEGF-A, control n: 7 placentae, CTRP6 n: 10 placentae. For TGF-β, control n: 8 placentae, CTRP6 n: 10 placentae. For PlGF, control n: 8 placentae, CTRP6 n: 9 placentae. For IGF-1, control n: 8 placentae, CTRP6 n: 10 placentae. (b) The ratios of TNF-α per IL-6 and TNF-α per IL-10 in the CTRP6 group were significantly lower than those ratios in the control group. For TNF-α/IL-6, control n: 7 placentae, CTRP6 n: 8 placentae. For TNF-α/IL-6, control n: 5 placentae, CTRP6 n: 8 placentae. One placenta was obtained from each mouse. Data are presented as a box plot with median, interquartile range ± upper/lower extreme. X: Mean value. *P < 0.05 by student's t-test.

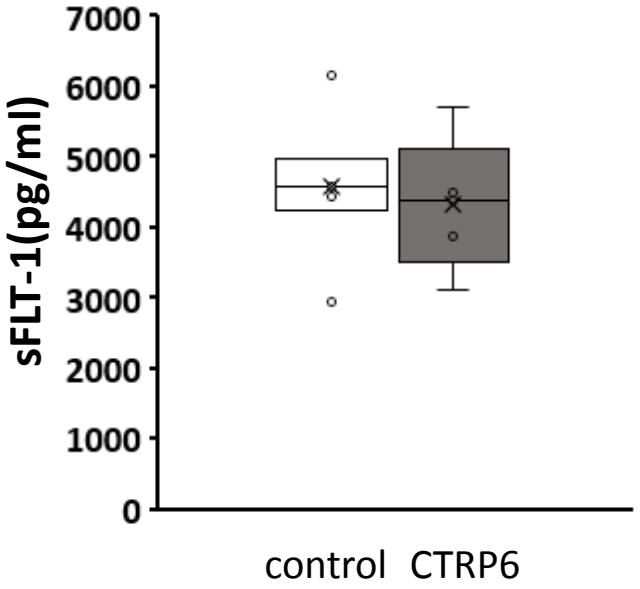

Fig.6. Effect of CTRP6 on the serum concentration of the soluble FLT-1 in CBA/J mated with DBA/2 mice on 14.5 dpc. There was no difference between the two groups. control: n=7 mice, CTRP6: n=5 mice. Data are presented as a box plot with median, interquartile range ± upper/lower extreme. X: Mean value.