Comparative Immunological Effect of Anticoagulant and Antioxidant Therapy in the Prevention of Abortion in Mice

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Problem
Considering the potential adverse effects of anticoagulation in abortion treatment, we investigate whether antioxidants might exert the same immunoprotection. Although the fertility properties of Vitamin E have been associated with its antioxidant capacity, its effect on cytokine balance during pregnancy is still unknown.

Method of study
Pregnant females from CBA/J × DBA/2 abortion model were orally supplemented with Vitamin E or inoculated intraperitoneally with enoxaparin. Foeto-placental units were scored at 14.5 days of pregnancy, and abortion rate was calculated. Cytokine placental levels were determined by enzyme-linked immunosorbent assay.

Results
Vitamin E (15 mg/day) has been able to decrease abortion rate and to increase IL-6 placental levels, while both treatments increased vascular endothelial growth factor (VEGF) placental levels.

Conclusion
Vitamin E and enoxaparin are able not only to prevent foetal wastage but also to balance IL-6 and VEGF placental levels, presenting a new potential therapeutic alternative for patients with recurrent abortion not associated with thrombophilias.

Introduction

Because thrombophilia is highly associated with recurrent pregnancy loss,[1,2] anticoagulant treatment is the most common therapy adapted to fertility programmes. Over the last years, the immunomodulatory effect of heparin associated with its protective effect on pregnancy. Low-molecular weight heparin (LMWH) decreases TGF-β, which is implicated in controlling trophoblast invasion.[3] Heparin induces STAT3 activation via JAK/STAT pathway,[4,5] whereas STAT3-deficient mice show embryo lethality because of placental defects.[6,7]

Furthermore, LMWH stimulates IL-6 production by peripheral blood mononuclear cells in a dose-dependent manner,[8] whereas serum IL-6 deficiency has been associated with recurrent miscarriages.[9] Moreover, we have previously demonstrated that LMWH enoxaparin has identical effects to that of recombinant IL-6 reducing embryonic absorption in the CBA/J × DBA/2 murine model. In this study, we also demonstrated that foeto-placental units from high-resorption rate CBA/J × DBA/2 secrete lower IL-6 levels than normal CBA/J × BALB/c mating.[10]

On account of these non-anticoagulant effects exerted by heparin, together with the lack of

therapeutic alternatives for those patients without abnormal thrombophilic markers, anticoagulant therapy is often prescribed in the absence of sufficient evidence. Therefore, it is essential to find other agents that might exert the same immunoprotection with less adverse effects.

Although Vitamin E has been considered a fertility factor for more than 80 years, knowledge about how it prevents abortion is still scarce.[11] Vitamin E is implicated in placental and embryonic development, whereas its deficiency affects embryo survival.[12–14] Alternative effects of Vitamin E have been recently reported as unrelated to its antioxidant capacity.[15] It induces the expression of vascular endothelial growth factor (VEGF) promoter,[16] as well as reduces the production of inflammatory cytokine.[17]

It is known that recurrent abortion might be associated with the impaired expression of VEGF[18] and that IL-6 is capable of inducing VEGF synthesis.[19] Therefore, we decided to investigate the effect of Vitamin E supplementation on abortion rate and placental levels of IL-6 and VEGF in the CBA/J × DBA/2 model. Furthermore, this effect was compared to the enoxaparin treatment.

Materials and methods

Mice

The present investigation followed the 'Guidelines for care and use of laboratory animals'.[20] Four-month-old CBA/J female mice were acquired from the Animal Center of Atomic Energy National Argentine Commission. The DBA/2 male mice were obtained from the Animal Center of the Faculty of Pharmacy and Biochemistry of the University of Buenos Aires. CBA/J female mice were mated with DBA/2 males. The temperature of the experimental animal room was 20–25°C. Lighting was artificial, being the sequence 14 hr light and 10 hr dark. Day 0.5 of pregnancy was determined by the presence of vaginal plug.

Foetal Resorption

To determine foetal wastage, CBA/J pregnant mice were euthanized at day 14.5 of pregnancy. Lost and normal foeto-placental units were scored, and the percentage of resorption (%R) was evaluated as: %R = 100 × Re/Re + F (Re: number of resorted embryos and F: number of viable embryos).

Placental Culture Supernatants

Total placentas from non-resorption units were separated from uterine implantation sites, decidua was peeled-off and each placenta was weighted. Explants were minced in small fragments as it was described by Chaouat et al., 1995.[21] Cultures from individual placentas were performed in one well of the 24-well tissue culture plates using Roswell Park Memorial Institute medium (RPMI 1640; GIBCO, Carlsbad, California, USA) supplemented with 10% of foetal bovine serum (SFB; GIBCO) and 1% of penicillin/streptomycin (GIBCO). Incubation was set up at 5% CO_2 humid atmosphere at 37°C, during 48 hr. Supernatants were collected, centrifuged at 2000 and 8000 × *g* and kept at −70°C for cytokine analysis.

Cytokine Quantification by ELISA

Levels of placental cytokines IL-6, IL-10, TNF-α and VEGF were determined with a double antibody-sandwich ELISA method[22] in explants culture supernatants by using a commercially available kit (R&D, Minneápolis, MN, USA). Briefly, 96-well plates were coated with anti-cytokine monoclonal antibody overnight at room temperature (RT). After successive washes in phosphate buffer solution (PBS) (0.01 M PO_4HNa_2/PO_4HNa y 0.15 M ClNa) pH 7.4, free sites were blocked with 1% bovine seroalbumin (BSA; SIGMA, Saint Louis, MO, USA) in PBS and incubated at RT for 1 hr. Washes were repeated with PBS added Tween-20 0.05% (Tween; Anedra, San Fernando, Argentina). Samples and recombinant cytokine as standard were cultured for 2 hr RT. After successive washes, the biotinylated detection antibody was cultured at RT for 1 hr. A new wash cycle was followed by the addition of horseradish peroxidase (HRP) with streptavidine for 20 min RT. Colour reaction was developed by 3,3′, 5,5′- tetramethylbenzidine (TMB; MP Biomedicals, OH, USA) and stopped with 1 M solution H_2SO_4. Optical density was measured at 450 nm using an automatic plate reader (Metertech, Taipei, Taiwan). To normalize cytokine levels to placental size, each placenta was weighted for analysis.

Antioxidant Treatment with Vitamin E

Mice – CBA/J pregnant females – were divided into four groups (n = 10 per group). Two groups received

different doses (3, 15 mg/day) of D-α-tocopheryl acetate (Vitamin E) with specific activity of 1342 UI/g (MP Biomedicals) reconstituted with soy oil as a vehicle (12 UI/100 g of D-α-tocopherol acetate), administered daily from day 0.5 to day 12.5 of pregnancy,[23] by intragastric catheter (Thomas Scientific, Swedesboro, New Jersey). Two groups served as control. One treated with placebo (soy oil) and the other one was monitored but not treated.

Anticoagulant Treatment with Enoxaparin

For the anticoagulant treatment, we used enoxaparin (Clexane, Aventis Pharma, France) and PBS as a vehicle.

Mice – CBA/J × DBA/2 females – were divided into two groups ($n = 10$ per group). The first group was supplemented intraperitoneally with a daily dose of 0.7 mg/Kg of enoxaparin on day 6.5; 8.5 and 10.5 of pregnancy.[10] This route, with confirmed systemic absorption,[24] has been used in previous studies which demonstrated pregnancy-protective effects.[10,25] The second group served as control, being treated with sterile PBS.

Statistical Analysis

Using one-way ANOVA, groups were compared with Tukey test as post-test or unpaired Student's *t*-test depending on the number of compared variables. GraphPad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for analysis. Value of $P < 0.05$ was considered statistically significant.

Results

Vitamin E was used to prevent abortion in animal models.[23,26] Doses of 3 and 15 mg/day were assayed in CBA/J × DBA/2 murine model based on previous data reported in rats.[23]

The outcome demonstrated that 3 mg/day of D-α-tocopherol acetate was not able to modify resorption rate (data not shown). However, 15 mg/day has shown to reduce abortion by 93% (Fig. 1a).

Considering that Vitamin E modulates *in vitro* cytokine expression,[16,17] we decided to analyse the influence of Vitamin E supplementation of CBA/J × DBA/2 model on cytokine placental levels by ELISA. Gestational treatment with 15 mg/day of Vitamin E increased placental IL-6 production of CBA/J × DBA/2 mating in a considerable manner, in comparison with placebo treated group (23.6 ± 41.3 and 10.9 ± 6.2 pg/mL, respectively, Fig. 1b).

Vitamin E supplementation was not able to affect IL-10 and TNF-α placental levels (data not shown); 15 mg/day of Vitamin E increased placental VEGF levels of the abortion combination, in comparison with abortion females treated with placebo (23.55 ± 33.7 and 12.58 ± 20.7 pg/mL, respectively, Fig. 1c).

Because we have previously demonstrated that inoculation of low-molecular weight heparin prevents abortion and regulates IL-6 placental levels,[10] we decided to compare the effect of Vitamin E with enoxaparin treatment on IL-10, TNF-α and VEGF placental levels. Enoxaparin was not able to modify IL-10 and TNF-α placental levels (data not shown). However, 0.7 mg/kg of enoxaparin increased placental VEGF levels with regards to control group (41.7 ± 30.8 and 19.09 ± 23.5 pg/mL, respectively, Fig. 1d).

Effect of the Antioxidant and Anticoagulant Treatment on Placental Growth

We compared placental weights of treated and control animals. Results showed that there was no significant difference in the weight of day 14.5 placentas of abortion mice treated with Vitamin E ($P = 0.21$) or enoxaparin ($P = 0.09$) when compared with the placebo (119 ± 25 versus 124 ± 26 mg and 103 ± 20 versus 109 ± 17 mg, respectively, data not shown).

Discussion

Experimental evidence suggests that antioxidant supplementation protects endothelium from injury induced by oxidative stress and pro-inflammatory factors.[27] Taking these facts into account, we decided to investigate the effect of D-α-tocopheryl acetate (natural Vitamin E) for the prevention of abortion and cytokine production of CBA/J × DBA/2 murine model. Our results demonstrate a dose-dependent protective effect for Vitamin E on pregnancy, accompanied by the modulation of IL-6 and VEGF placental levels. However, this vitamin was not able to modulate expression of IL-10 or TNF-α. Further studies should be conducted to investigate the necessary treatment conditions (dose, duration, etc.) to obtain the same protective effect of Vitamin E in women.

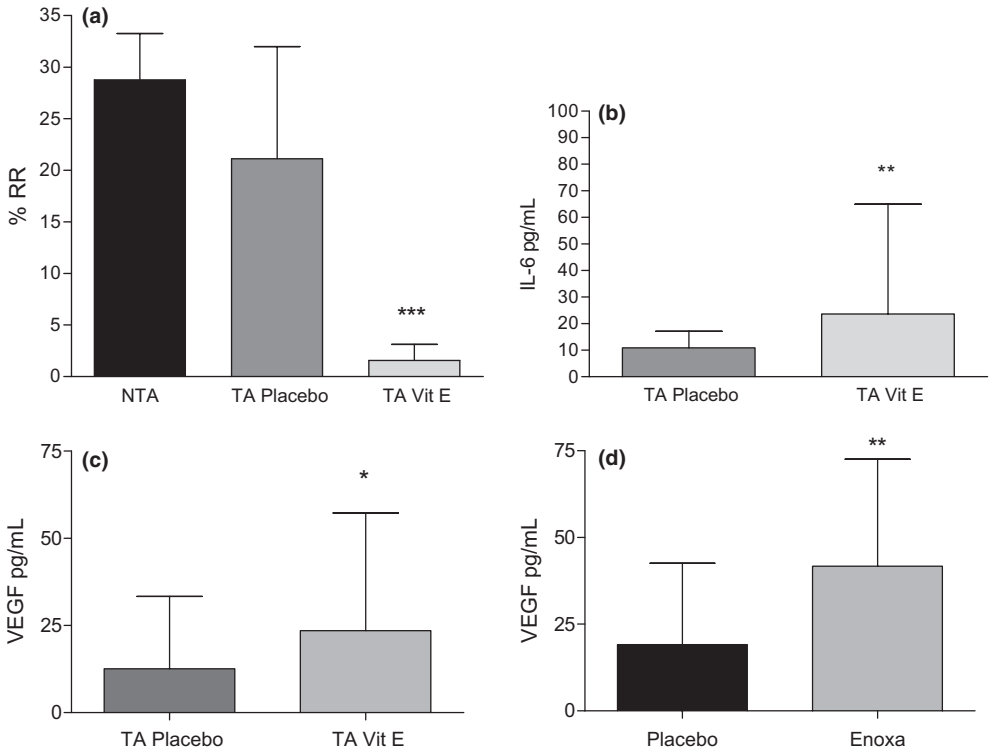

Fig. 1 (a) Percentage of Resorption rate (RR) was measured in CBA/J × DBA/2 abortion murine model in a gestational treatment with 15 mg/day of acetate α –tocopherol (Vit E). Significant differences using one way ANOVA test and Tukey (***$P < 0.0001$) were found in RR with regard to placebo (soy oil containing 15 μg/day of Vitamin E) and No Treated Aborted (NTA) females. Ten mice were used in each group. (b) Placental levels of IL-6 in culture supernatants of CBA/J × DBA/2 females treated with Vitamin E (15 mg/day) or placebo. Cytokine concentration was determined by ELISA as indicated in Material and Methods. Results are expressed as pg/mL of IL-6. Significant differences using Student́s *t* test (**$P < 0.001$) were found between means of Vitamin E and placebo group (23.6 ± 41.3 and 10.9 ± 6.2 pg/mL, respectively). Ten mice were used in each group. (c) Placental levels of vascular endothelial growth factor (VEGF) in culture supernatants of CBA/J × DBA/2 females treated with Vitamin E (15 mg/day) or placebo. Cytokine concentration was determined by ELISA as indicated in Material and Methods. Results are expressed as pg/mL of VEGF. Significant differences using Student́s *t* test ($P < 0.05$) were found between means of Vitamin E and placebo group (23.55 ± 33.7 and 12.58 ± 20.7 pg/mL respectively). Ten mice were used in each group. (d) Placental levels of VEGF in culture supernatants of CBA/J × DBA/2 females treated with Enoxaparin (0.7 mg/kg) or placebo. Cytokine concentration was determined by ELISA as indicated in Material and Methods. Results are expressed as pg/mL of VEGF. Significant differences using Student́s *t* test ($P < 0.005$) were found between means of Enoxaparin and placebo group (41.7 ± 30.8 and 19.09 ± 23.5 pg/mL respectively). Ten mice were used in each group.

Because we had demonstrated that anticoagulant therapy with enoxaparin exerts the same protective effect on CBA/J × DBA/2 abortion rate and IL-6 placental levels,[10] we decided to investigate the effect on placental IL-10, TNF-α and VEGF. Results showed that enoxaparin increased VEGF levels, without modifying IL-10 and TNF-α production (Fig. 1d). This immunomodulatory effect is the same to the one produced by Vitamin E.

Moreover, considering that neither Vitamin E nor enoxaparin affected placental weights, differences in cytokine values for each treatment could be associated with an effect on cytokine synthesis more than with an increase in placental blood flow.

IL-6 has controversial roles during pregnancy. Increased values of this cytokine were found in CBA/J × DBA/2 serum,[28] while its expression is reduced in the endometrium of recurrent abortion patients.[29] We postulate the role of IL-6 in the regulation of the end of an inflammatory predominance during implantatory window towards placental angiogenesis. Deficient placental IL-6 levels may induce a deregulation leading to an exacerbated inflammation, a poor angiogenesis and foetal death by isquemia. However, more research should be conducted to confirm this hypothesis.

These results allow us to postulate a preventive effect of Vitamin E treatment on recurrent abortion

associated to an immunomodulatory action, besides its antioxidant properties. This opens new lines of research towards new therapeutic alternatives in reproductive medicine.

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