



Endometrial membrane response in *Mus musculus* during implantation by *Vitex negundo* Linn

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Abstract

The process of implantation of the blastocyst in the endometrium is considered analogous to a “proinflammatory” response, and a possibility exists that any compound showing good anti-inflammatory activity may also possess a significant anti-implantation potential. Leaf extract from the shrub *Vitex negundo* Linn has previously been studied for its analgesic and anti-inflammatory activities. Hence, anti-implantation activity of the methanolic extract of leaves of *Vitex negundo* Linn was investigated. Pregnant female mice were dosed with the extract (500 mg/kg body weight) from Days 1 to 6 of pregnancy. No implantation sites were observed in treated animals when they were surgically opened on Day 15 of pregnancy. Biophysical alterations were observed in the endometrium in treated animals, especially on Day 5 (4:40 a.m.), the day of implantation. A sharp increase in superoxide anion radicals that was seen in the endometrium from control animals was altered in treated animals. Thus, the physiological alterations induced by extract of *Vitex negundo* Linn during the process of implantation may serve as a good lead for further research on natural contraceptive targets.

Keywords: Blastocyst implantation, superoxide dismutase, superoxide radical, lipid peroxidation, *Vitex negundo* Linn.

Introduction

Implantation is a crucial event in reproductive physiology. Several biochemical, biophysical, and hormonal changes take place prior to this event (Laloraya, 1990). Studies have shown that endometrial membrane conditions are important for blastocyst implantation (Pal *et al.*, 1985). Progesterone, estrogen, superoxide anion radical, and superoxide dismutase regulate implantation (Aitken, 1979; Laloraya, 1990).

The process of blastocyst implantation is considered analogous to a “proinflammatory” response. Thus, participation of various inflammatory mediators is speculated in the process of implantation. For example, prostaglandins (PGs) are implicated as important mediators of increased endometrial vascular permeability during

implantation. Also, COX-2 deficiency has been reported to result in defective ovulation, fertilization, implantation, and decidualization (Chakraborty *et al.*, 1996).

Vitex negundo Linn (Fam. Verbenaceae) is a large shrub available throughout India (Gupta *et al.*, 2005). Leaves of this plant have been studied thoroughly for their varied therapeutic activities, such as CNS depressant activity (Gupta *et al.*, 1999) inhibition of rat peritoneal cavity mast cell degranulation (Nair *et al.*, 1995), and prevention of genotoxicity (Balboa and Lim-Sylianico, 1993). Mosquito repellent effects (Hebbalkar *et al.*, 1992), antiulcerogenic (Sahni *et al.*, 2001), antiparasitic (Parveen, 1991), antimicrobial (Rusia and Srivastava, 1998), and hepatoprotective (De *et al.*, 1993) potential of the leaves have also been studied.

Vitex negundo Linn has been investigated extensively for its anti-inflammatory (Ravishankar *et al.*, 1985; Jana *et al.*, 1999; Telang *et al.*, 1999; Valamathi *et al.*, 2000) and analgesic (Ravishankar *et al.*, 1985; Telang *et al.*, 1999) activities, but it was Telang *et al.* (1999) who noticed the inhibitory activity of the extract on PG biosynthesis and confirmed NSAID-like activity with selective COX-2 inhibition activity (Ravishankar *et al.*, 1985; Telang *et al.*, 1999). Thus, it was hypothesized that a plant possessing a significant anti-inflammatory activity may also demonstrate a potential anti-implantation activity.

It has been noted that the dynamic oxyradical-antioxidant balance serves as a good marker for biophysical and biochemical changes occurring in any biological system (Tiwari, 2001). Hence, superoxide anion radical and superoxide dismutase have been used previously to study any physiological changes occurring in the uterine milieu (Nivsarkar *et al.*, 2001, 2002, 2005, 2006).

The objective of the present study was to investigate the anti-implantation potential of the methanolic extract of the leaves of *Vitex negundo* Linn with respect to its effect on the oxyradical-antioxidant balance in the uterus.

Materials and Methods

Animals

Mature, inbred female mice (Balb/c, 2-3 months

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Received: November 1, 2006
Accepted: August 27, 2007



old) housed in temperature-controlled ($27 \pm 1^\circ\text{C}$) rooms at light: dark regimen of 14:10 hours were used for the study. The experimental protocol was approved by the Institutional Animal House Ethics Committee (IAEC), constituted by the Ministry of Social Justice and Empowerment, Government of India.

Only those females that showed a regular 4-5 day estrous cycle were used. Vaginal smears were examined daily according to Stockard and Papanicolaou (1917), and only those females that showed a proestrus smear (Day 0) were mated with a male of proven fertility in the same evening. The presence of a vaginal plug the following morning confirmed mating and was designated as Day 1 of pregnancy. The pregnant females were sacrificed on Days 1, 2, 3, 4, 5 (4:40 a.m.), 5 (10:00 am), and 6 of pregnancy. The uterus was excised from each animal, cleaned from adhering fat, washed with saline, and then used for estimation of lipid peroxidation and assay of superoxide dismutase activity.

Preparation of methanolic extract of Vitex negundo Linn

Fresh green leaves of *Vitex negundo* Linn were collected from the botanical garden of our Institute and cleaned, shade dried, and extracted in methanol by cold extraction process (3 cycles) followed by evaporation in a water bath at $60\text{--}70^\circ\text{C}$. The extract was stored at -20°C until use and suspended in 0.2% agar prior to dosing.

Treatment of animals

All animals were dosed at 10:00 a.m. each day with the water extract of *Vitex negundo* Linn (500 mg/Kg body weight) from Day 1 to Day 6 of pregnancy. A few selected animals that were not dosed after Day 6 of pregnancy, were sacrificed on Day 15 of pregnancy, and the uterus was exposed for observing the sites of implantation. Six animals were utilized per group.

Estimation of lipid peroxidation

The uterine tissue was taken in 5 ml of Hank's balanced salt solution (HBSS, pH 7.4) and homogenized at 5000 rpm by using a Polytron homogenizer (3 cycles of 30 sec each; Kinematica, Switzerland). The homogenate was then centrifuged at 3500 rpm for 10 min. The pellet was resuspended in 0.1 ml of HBSS that was then used for estimation of lipid peroxidation.

Lipid peroxidation was measured in terms of malonaldehyde (MDA):thiobarbituric acid (TBA) reaction as reported by Okhawa *et al.* (1979). The reaction mixture contained 0.1 ml of tissue homogenate (as described above), 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with 1 M NaOH), and 1.5 ml of 0.8% aqueous solution of TBA. The reaction mixture was made to a volume of 4 ml with the addition of 0.7 ml of double distilled

water and heated at 95°C for 1 h in a water bath. After cooling, 1 ml of double distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) was added, and the mixture was shaken vigorously on a vortex mixer for 5 min. This mixture was centrifuged at 3000 rpm for 7 min, the upper organic layer was separated and the amount of MDA formed in this layer (extinction coefficient of MDA is $1.45 \times 10^5/\text{min}/\text{cm}$) was measured at 532 nm using an ultra violet/Visible spectrophotometer (Systronics, India). Appropriate controls were used at different steps during this estimation.

Assay of superoxide dismutase activity

The uterine tissue was taken in 4 ml of chilled Tris buffer 50 mM (pH 8.2) and was homogenized at 13000 rpm (3 cycles of 30 sec each) using a Polytron homogenizer. The homogenate was treated with 1 ml of 0.1% Triton X 100 (v/v) for 20 min at 4°C . Homogenate was then centrifuged at 15000 rpm at 4°C for 30 min using a Sorval high-speed centrifuge (Sorval, USA) with a fixed angle rotor (SS34). The supernatant was used for the assay of superoxide dismutase (SOD) activity by the method of Marklund and Marklund (1974). All calculations were made as per gram fresh weight.

Statistical analysis

All data have been represented as mean \pm SEM. Data were analyzed using paired *t*-tests within groups and $P < 0.05$ was considered significant. Linear correlation was established between the LPO and SOD values on Day 5, 4:40 am and the correlation coefficient was calculated.

Results

Treated animals, which were dosed with the water extract of *Vitex negundo* Linn for the first 6 days of pregnancy, showed 100% inhibition in blastocyst implantation (0/6) as compared to the control (53/6) animals. These animals were sacrificed on Day 15 of pregnancy, the uteri were removed, and the implantation sites were counted.

Figure 1 shows SOD and superoxide anion radical levels (measured as MDA levels) in control animals from Days 1-6 of pregnancy. A sharp decrease in SOD levels and a sharp increase in superoxide anion radicals (MDA levels) were observed at the time of implantation (Day 5, 4:40 a.m.; $P < 0.05$) when compared to Days 4 and 5 (10:00 a.m.). Thus, the levels of superoxide anion radicals and SOD in the uterus were negatively correlated ($r = -0.9$) at the time of implantation. However, the treated animals did not show the characteristic peak in superoxide anion radicals at the peri-implantation time. Moreover, a sharp



decrease ($P < 0.05$) in SOD activity was noted when compared to Days 4 and 5 (10:00 a.m.) as observed in the control group (Fig. 2). In this case, the uterine SOD

and superoxide anion radicals did not demonstrate a significant negative correlation at the time of implantation as seen in control animals (Fig. 1).

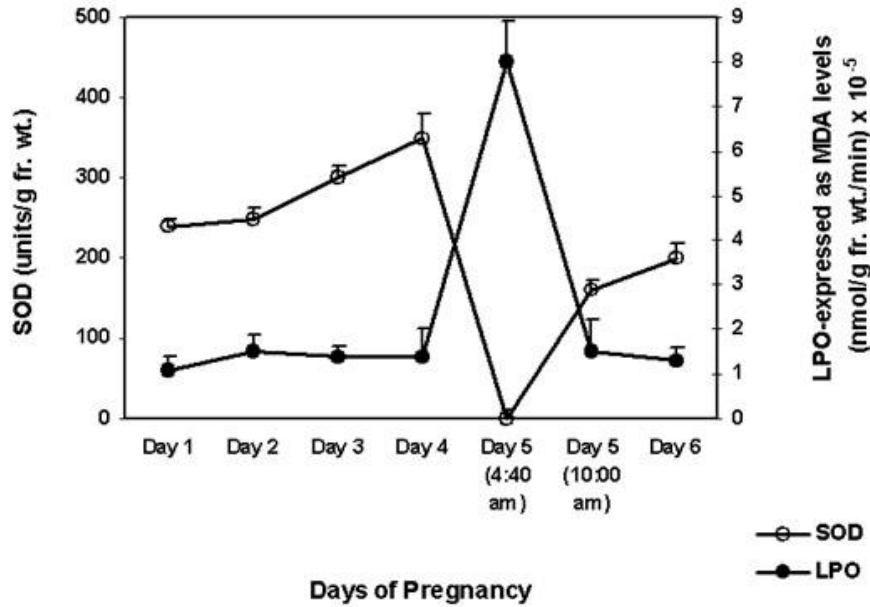


Figure 1. Superoxide dismutase (SOD) activity and lipid peroxidation (LPO; MDA levels) on different days of pregnancy (Days 1-6) in uterus of control animals (n = 6).

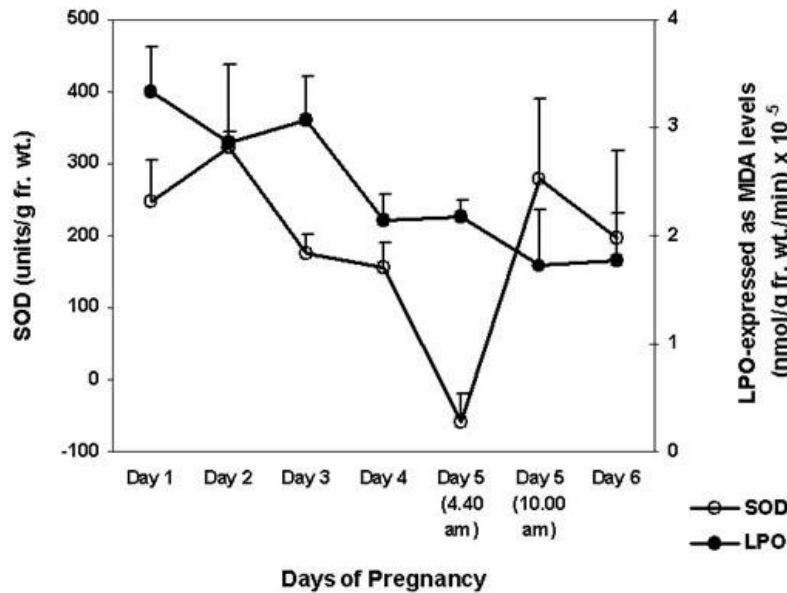


Figure 2. Superoxide dismutase (SOD) activity and lipid peroxidation (LPO; MDA levels) on different days of pregnancy (Days 1-6) in uterus of *Vitex negundo* Linn leaf extract treated animals (n = 6).

Discussion

The results show that failure of implantation cannot be due to interference with tubal transport of the

zygote but a consequence of inadequate environment of the endometrium. The pre-implantation and peri-implantation periods of the embryo and the endometrium include a number of biochemical and



biophysical events. The successful completion of these events involves a complex series of synchronized changes in the blastocyst and the endometrium. The fertilized ovum must follow the correct pathway or perish. It is critical that the fertilized ovum reach the uterus at an appropriate progesterational stage of the ovarian cycle and when the endometrium has reached a precise stage of maturity (Nivsarkar et al., 2005). In mouse, the entry of ovum into the uterus is timed to coincide with the beginning of the luteal phase, which occurs 3 days after fertilization (Hafez, 1973; Kabir et al., 1984). The arrival of the zygote in the endometrium is not sufficient to ensure implantation; hormone-dependent changes and the increase in membrane fluidity, also called "receptive endometrium", are also necessary. The superoxide anion radical surge at the time of implantation has been implicated for induction of endometrial membrane fluidity ("receptive endometrium"). An estrogen surge at the time of implantation has been shown to be responsible for a decrease in SOD levels and an increase in superoxide anion radical levels (Laloraya et al., 1996).

The characteristic decrease in SOD activity at the time of implantation was observed in both control and treated groups and might suggest that the *Vitex negundo* Linn extract does not have a significant anti-estrogenic activity. Furthermore, a reduction in the free radical peak was noted at the same time, implicating the involvement of some other entity in controlling this typical peak. Membrane fluidity, a prerequisite for implantation, is achieved by high free radicals levels, and therefore a "non-receptive endometrium" resulted in the treated animals. Thus, the anti-inflammatory potential of the extract may interfere with the biophysical events during implantation by altering the physiology of the endometrium, changing the oxyradical status of the endometrium, and thus making it "non-receptive", which may lead to failure of blastocyst implantation.

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