Influence of antiangiogenic fraction from *Diogenes avarus* (Heller) on fertility and implantation in mice

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The methanol extract isolated from hermit crab, *D. avarus* degenerated ovarian and uterine tissues in cyclic and pregnant mice, treated before and after the implantation. Immunohistochemical staining using CD31 and Factor VIII specific to endothelial cells showed reduction in microvessel density. The hormonal assay showed decrease in the progesterone secretion in all experimental mice.

**Keyword**: Antiangiogenic fraction, CAM, *Diogenes avarus*, Implantation, Mouse

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Angiogenesis or neovascularisation is formation of new blood vessels from capillary sprouts. It is a tightly regulated by angiogenic and antiangiogenic factors and is switched on and off over a time schedule. Under normal conditions in adults it occurs infrequently and briefly. Physiological angiogenesis is also associated with repair of wounds, peptic ulcers and myocardial infarction. Physiological angiogenesis is unique to the female reproductive system. Angiogenesis plays an important role in the growth of the ovarian follicle, corpus luteum, the endometrium and placentation. Intense, local angiogenesis occurs during the growth of the ovarian follicle and corpus luteum. High vascularisation is necessary to provide the luteal cells with large amounts of cholesterol needed for progesterone synthesis and for the delivery of the progesterone to the circulation. The growth of the endometrial vasculature differs during the sequence of events that occur during estrous/menstrual cycle. It starts during the proliferative phase and continues throughout most of the secretory phase. After implantation the spiral arteries grow in presence of progesterone and growing conceptus. Increased vascular blood flow to the placental tissues satisfies the steadily increasing demand of the embryonic development and foetal growth.

The angiogenic process is therefore crucial in functioning of the female reproductive system and determining the fertility. The present work describes the effect of an antiangiogenic extract isolated from a hermit crab *Diogenes avarus* (Heller) on the reproductive function of female mice.

**Materials and Methods**

The extract was prepared as described previously. Briefly, hermit crab *D. avarus* was collected from the shores of Versova, Mumbai, removed from shell and extracted by cold percolation in methanol. The concentrated methanol extract was then washed with solvent ether. The ether washings were pooled, dried over sodium sulphate and solvent was removed completely under reduced pressure. The ether portion (HCM1) was dissolved in dimethyl sulphoxide (DMSO) and administered to mice.

**Mice**—Swiss albino mice (6 weeks old) weighing 20-24 g were acquired from Haffkine Institute, Parel, Mumbai (India) and were acclimatized for 1 week. The animals were housed in 12:12hr light:dark schedule with free access to readymade chow and water. Their stages of estrous cycle were checked by visual inspection of vagina according to the criteria of Champlin and also with vaginal smear. Animals exhibiting a normal 4-5 day estrous cycle only were included in the study. The mice were divided into two groups of six animals. The first group consisted of cycling mice and the second group consisted of inseminated mice of proven fertility. Experiments

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were carried out in triplicate and according to the rules laid by Animal Ethical Committee, Government of India.

**Treatment of cycling mice**—The first experiment was set up to determine the effect of antiangiogenic extract on non-pregnant cycling mice. Female mice (6 per cage) were randomized into three groups. Two experimental groups received a predetermined dose (40 mg/kg) of *Diogenes* extract subcutaneously every alternate day for 16 days (about 4 cycles). The control group received saline.

On day 16, mice from control group and one of the experimental group were sacrificed by one of cervical dislocation. The ovaries, and uterus were resected en bloc, weighed, fixed in Carnoy’s fixative/neutral formalin and embedded in paraffin for histology and for immunohistochemical localization of endothelial cells. The tissues were fixed and processed for electron microscopy separately. Blood was collected from the mice by heart puncture for serum progesterone content. The females in the second experimental group were kept for recovery and studied for mating and subsequently for the litter size or any other abnormalities.

**Treatment of inseminated mice**—Mice of proven fertility were collected and mated (3 females: 1 male) in the evening and the vaginal plugs checked the next morning. The day of plugging was termed as day 1 of pregnancy.

Two sets of experiments were carried out on the pregnant females. In the first, the plugged females were injected, sc, with 0.1 ml HCM1 (40mg/kg body weight) on day 1. The females were sacrificed on day 7 of insemination and the tissues were preserved for histopathology as well as electron microscopy and immunohistochemical localization of endothelial cells. Simultaneously, blood was collected and analysed for serum progesterone.

In the second set of experiment, 6 female mice were injected, sc, with 0.1 ml HCM1 (40mg/kg body weight) on day 7 of insemination and sacrificed on day 9. The numbers of implantation sites were counted. Simultaneously, a control group of six pregnant female mice were injected saline.

**Immunohistochemical localization of endothelial cells**—To determine the microvessel density, the Cornoy's fixed sections were processed according to manufacturers', manual (Dako Labsystems). Endothelial cells were stained using mouse monoclonal antibody and factor VIII antibody.

Briefly, sections were deparaffinized, rehydrated through graded ethanol, rinsed in distilled water and immersed in 0.3% H2O2 for 10 min to inhibit endogenous peroxidase activity. Sections were rinsed in PBS, pH 7.2 and incubated for 30 min. Sections were then incubated for 30 min with peroxidase-labelled antimouse IgG (Dako) washed in PBS. The tissues were counterstained with haematoxylin and mounted in DPX. Each count was expressed as the number of microvessels identified within a selected field. Ten such fields were counted per section.

**Electron microscopy**—The tissues for electron microscopy were processed at Jaslok Hospital and Research Center, Mumbai. The tissues were cut into small pieces and fixed in glutaraldehyde (3%) for 1-2 hr, washed in sodium cacodylate buffer and post-fixed in osmium oxide (1.5%) for 2 hr. The tissues were dehydrated in alcohol, passed through propylene oxide and were embedded in Araldite in BEEM capsules. The blocks were cut 1-2µm in size, stained with toluidine blue and area marked for ultra-thin sections. Tissues were spread on copper grid mesh. The tissues were subjected to alcoholic uranyl acetate (10%) followed by lead citrate for 30 min to enhance the contrast. The grids were observed on JEOL-100S electron microscope at 80kV accelerating voltage.

**Serum progesterone levels**—The blood from control and experimental mice was collected and centrifuged (1000 g) for 10 min at 4°C and the serum was stored at -20°C until use. Serum progesterone was determined by a progesterone immunoassay. The method used was fully automated VIDAS system by Biomerieux, France. The results of all experimental groups were compared with control.

**Results**

Study on the effect of a non-toxic antiangiogenic extract on female reproductive tissues has shown it to have an extensive effect on all the experimental groups.

The treatment showed morphological changes in the ovary and uterus in all experimental groups. Blood island formation on the uterus was not proper and the tissues appeared to be pale. The weight of the ovary and uterus reduced. There was about 23.3% reduction in the weight of the ovary and 22.3% reduction in the weight of the uterus (unpublished data).

**Histological findings**—The histological examination of the serial sections of the ovary of treated cycling mice (Fig. 1a, b) showed that all the
growing follicles were in various stages of atresia and the corpus luteum was absent. Larger follicles showed distinct antral atresia. These follicles showed condensed nuclei in the granulosa cells closest to the antrum or in the antrum.

The luteal cells of the day one treated inseminated mice showed degenerative changes. The nucleus became condensed and the endothelial lining of the blood vessels broken. The corded appearance of the luteal cells was also lost. Inseminated mice treated on day 7 also showed similar changes (Fig. 2 a, b).

The endometrium of the cycling mice showed a reduced lumen of the endometrial glands. In inseminated mice treated before placentation, placentation did not occur and the embryo was malformed. No distinct embryonic layers were seen. Nucleated red blood cells and labyrinth structure were absent in the placenta. In the inseminated mice treated on day 7 i.e. after implantation, the embryo was aborted and few remnants of the endometrium were visible (Fig. 3 a, b, c).

**Determination of microvessel density (MVD)**—To detect and evaluate the effect of the extract on the vascular tree, a highly specific staining technique was used to stain the endothelial cells. The endothelial cells were localized using antibodies to factor VIII.

![Fig. 1](#) — Section of ovarian follicles stained with Haematoxylin-Eosin of normal cycling mice and (b) atretic follicle observed in treated cycling mice (4x). A= antrum and DC= degenerating cell.

![Fig. 2](#) — Section of corpus luteum of 9 day inseminated mice stained with Haematoxylin-Eosin. (a) control and (b) treated (20x). L= luteal cell and E= endothelial lining.
and CD31 (Fig. 4 a, b). All the experimental groups and both tissues, uterus and ovary showed a marked reduction in the microvessel density by about 40-60% (Fig. 5 a, b).

Electron microscopic observations—Electron microscopic studies revealed generalized cellular deterioration in all the treated animals. The cells undergoing death showed a delineation of nuclear and cell membrane resulting in spaces formed around the nucleus and cells losing contact with each other. The chromatin material condensed and the fragmentation of
the nucleus occurred. There was a corresponding loss in the cytoplasmic organelles also.

The granulosa cells of the treated cycling mice showed nuclear fragmentation, loss of cell-cell contact and blebbing. The luteal cells showed a total collapse of the secretory machinery. Rough endoplasmic reticulum and smooth endoplasmic reticulum were absent and mitochondria ruptured. However, large secretory vesicles were seen in the cells (Fig. 6 a, b).

The cells of the endometrium of the treated cycling mice and the inseminated mice showed disintegration and fragmentation of rough endoplasmic reticulum and smooth endoplasmic reticulum (SER). Secretory granules were accumulated and can be seen as electron dense bodies (Fig. 7 a, b).

The electron micrograph of the endothelial cell lining the blood vessel of the corpus luteum showed nuclear fragmentation indicating early apoptotic changes in the endothelial cell (Fig. 8 a, b).

Progestrone levels—Evaluation of serum progestrone levels showed decrease (Fig. 9), as a result normal estrus cycle was lost. The animals remained in diestrus for a very long time and mating did not occur. The progestrone levels of both the treated, pregnant mice groups were also lower than the control groups.

Discussion

A non-toxic antiangiogenic extract of a marine crustacean has been reported. HCM-1, an ether soluble fraction at a concentration of 400 g/kg body weight is non-toxic to mice. It was found to be an inhibitor of angiogenesis in chick chorioallantoic membrane assay. The female reproductive organs the ovary and uterus are few of the adult tissues, which exhibit periodic growth and regression associated with equally rapid changes in the blood flow.

Follicular development and formation of corpus luteum require the growing follicle to recruit blood supply from the surrounding stroma. Absence of corpus luteum and follicular atresia in the treated cycling ovary indicates inhibition of maturation of the ovarian follicle, ovulation and development of corpus luteum. That the effect is through hampered blood vessel development in the ovary is substantiated by reduction in the microvessel density of the treated ovaries. The absence of corpus luteum results in the reduced progesterone levels in these mice. These changes however are temporary and complete recovery of the treated cycling mice takes place, as indicated by normal and healthy litter. The primordial and primary follicles do not require blood supply of their own. They may not be affected by the treatment since the activity of extract is restricted to the endothelial cells.

The inhibition of angiogenesis in the corpus luteum in the inseminated treated mice results in failure of development of the microvascular tree, which affects the luteal cells functioning restricting its ability to secrete progestrone. The toxic changes observed in the histological and electron microscopic examination of luteal and endometrial cells may be as a result of absence of angiogenesis leading to reduced nutrition of the tissues. The accumulation of the secretory material in the cells may be due their non-secretion into the blood vessels.
Fig. 6—Electron micrograph of (a) granulosa cell of control ovary and (b) granulosa cell of treated ovary (x5000). N= nucleus, FN= nuclear fragmentation and S= secretory vesicle.

Fig. 7—Electron micrograph of endometrial cell of 9 day inseminated mice (a) control and (b) treated (x 12K). N= nucleus, M= mitochondria, ER= endoplasmic reticulum and V= vacuole.

Fig. 8—Electron micrograph of endothelial cell (a) control and (b) treated animal (x5000). E= endothelial cell, N= nucleus and L= lumen of blood vessel.
The exact mechanism by which the angiogenesis is inhibited in these tissues needs to be investigated. Primarily, the effect seems to be through inhibition of endothelial cells directly. All other changes are subsequent to the failure of the development of the extensive vasculature necessary for proper functioning of the female reproductive system.

A number of angiogenic inhibitors such as angiostatin, endostatin, and cytokines have been extensively described as potent inhibitors of pathological angiogenesis, especially tumor angiogenesis. However, the use of these inhibitors of regulation of angiogenesis of the reproductive system has not been investigated. Of the large number of antiangiogenic compounds entering the clinical trials, only one, TNP470, has been shown to affect the reproductive system of female mice. Antiangiogenesis is being extensively studied as a strategy for intimidating malignant tumours. A number of drugs are being studied for their antiangiogenic activity and use in diseases such as cancer, rheumatoid arthritis, psoriasis. Keeping this information in view, it would be interesting to study Diergeni extract for antifertility activity.

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