EFFECTS OF VITEX AGNUS CASTUS ON MICE FETUS DEVELOPMENT

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Abstract- *Vitex agnus castus* (chasteberry) is a popular treatment for the management of female reproductive disorders including corpus luteum insufficiency, premenstrual syndrome (PMS), menopausal symptoms, and insufficient milk production. According to developing situation of complementary medicine, and frequent use of this herb, it is important to examine its effects during pregnancy. In this research we studied its effects on mice development, and we focused on macroscopic parameters, such as CRL (Crown-Rump length) and the weight of embryos, and diameter and the weight of placenta, and microscopic parameters such as the diameters of eye and lens of embryos. We found that *Vitex* has special effects during different stages of mice development, for example it can improve the growth of embryos in 8th and 9th day of pregnancy (it causes significant increase in CRL and weight of embryos). Also, it may changes some microscopic parameters. These founding suggest that it should be used more cautiously during pregnancy.

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Key words: *Vitex agnus castus*, Mice fetus development

INTRODUCTION

V. agnus castus (chasteberry), has been traditionally used in treatment of women complaints (1). This plant is a shrub with finger-shaped leaved and slender violet flowers; it belongs to verbenacea, which is commonly native to Middle East and South Europe (2). Berries have a pepper-like taste; therefore, sometimes it has been named as monk's pepper. Dried fruits has been used for women health and recommended to treat complaints such as endometriosis, menopause, cyclical mastalgia and premenstrual syndrome (PMS).

Clinical studies demonstrated that extract of *Vitex* dried fruits may benefits the symptoms of PMS,abnormal menstrual cycling, amenorrhea, mastodynia and hyperprolac-tinemia witch are all due to increasing level of prolactin (3-9).

Studies show its significant effect on hypophysis, which is progesteronic-like and may decrease extra level of prolactin. It is demonstrated that *Vitex* extract can operate as dopaminergic agonists which decrease the expressing prolactin from hypophyseal cell cultures, *in vivo* (4, 10, 11). Studies show that chasteberry may stimulate LH and vise-versa can suppress the FSH hormone (12, 13).

Vitex agnus castus potentially contains following chemicals: iridoid glycosides: eurostoside, agnoside, acubin (14-16), flavonoids: Casticin, kampferol, quercetagetin, vitexin, iso rientin (12, 15, 17, 18), progestins: Progesterone, Hydroxyprogesterone (flowers and leavers), testosterone, epitestosterone (flowers), androstenedione (leaves) (17), alkaloids: viticin, volatile oil: 1, 8-cineol, limes, linalool,

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terpinyl acetate, pinenes and beta pinenes (19, 20), essential fatty acids: palmitic acid, oleic acid, linoleic acid (20), essential oil (21).

Clinical uses of *Vitex* are in (PMS, latent hyperprolactinemia, acne , complaint which worsen in the luteal phase including herpes simplex, mouth ulcers and post-traumatic epilepsy, amenorrhea, including post-pill amenorrhea , polymenorrhoea, eractic menstrual cycles including those associated with stress, eating disorders and drug abuse, cystic hyperplasia, infertility, poor lactation, post natal depression, menorrhagia: fibroids, symptoms accompanying the peri-menopausal including irregular menstruation, PMS and mood changes peri-menopausal migraines (7, 22, 23). The precise mechanism of *Vitex* actions and its active compounds are still unknown (24), however some constituents may have anti-inflammatory, sedative and analgestic properties. *Vitex* also has dopaminergic properties; although it remains unclear which active compound is responsible (25, 26). Also, there are few studies about its acute toxicity and the histomorphological changes which its toxic doses may produce in vital organs. (2, 27)

There is some evidence about its anti carcinogenic and anti-fungal effects (28). There are no clinical studies assessing the safety of *Vitex* in children and pregnant women. *Vitex* is generally not recommended in pregnancy due to its unknown effects on the pituitary. There is insufficient information on the safety of using *Vitex* during nursing. However, analysis for breast milk revealed no changes in composition (24, 29). Therefore, we decided to study the effects of *Vitex* extract on mice fetus development, and get new results about its effect on macroscopic parameters of growth (including the weight and CRL: Crown-Rump Length of fetuses and weight and diameter of placenta) and the microscopic parameters of growth (including liver megakaryocyte count and the diameter of eye and eye lens).

MATERIALS AND METHODS

Experimental animals

White mice were obtained from Pasture Institute (Iran, Tehran). Both non-pregnant and pregnant mice were utilized. To obtain timed pregnancies, females were caged overnight with fertile males. The vaginal plug was designated day 0 of pregnancy. The pregnant mice were divided to 7 groups: 1- Exp7 (Experimental group which injected with *Vitex* on the 7^{th} day of pregnancy) (n = 6), 2- Exp 8 (injected with *Vitex* on the 8^{th} day of pregnancy) (n = 7), 3- Exp 9 (injected with *Vitex* on the 9^{th} day of pregnancy) (n = 7), 4- Control (intact) (n = 8), 5- Sham7 (injected with ethanol in the 7^{th} day of pregnancy) (n = 6) , 6- Sham8 (injected with ethanol in the 8^{th} day of pregnancy) (n = 7) and 7- Sham 9 (injected with ethanol in the 9^{th} day of pregnancy) (n = 7). Since 1950's, the standard *Vitex* extract used for clinical research and treatment in Europe has been an alcohol-based tincture of the fruits of the plant known as "Agnolyt". 100 ml of the solution is standardized to contain 9 grams of the fruit. The recommended dosage is 20-40 drops daily (1). 0.045 mg/kg (clinical dose) of hydro-alcoholic extract of *Vitex* (Toliddaru Vitagnus production, Tehran, Iran) was injected peritoneally to the experimental groups (Exp 7, Exp 8, Exp 9) on certain times. The same dose of 50% ethanol was injected to experimental groups (Sham 7, Sham 8, Sham 9) and the control group didn't receive any injection. Embryos and placentas with their encapsulating decidual tissue were removed from the uterus in the 15^{th} day of pregnancy. Further dissections were performed with the aid of a stereo microscope. The placentas and embryo connections were removed by means of fine forceps and iridectomy scissors. Then embryos and placentas were transferred to the fixator for further analysis.

Weight and CR (Crown-Rump) length of embryos and, weight and diameter of the placentas were measured.

After removal of the Specimen, Sectioning (sagital, for embryo), and then Hematoxylin and eosin staining was performed. The microscopic analysis and measurement was done (The eye and lens diameter and the liver megakaryocyte.)

Data Analysis

Data were expressed as mean ± S.E.M analyzed by one-way ANOVA, using the SPSS software (version 11.5) in order to detect inter-group differences. $P < 0.05$ was considered to be statistically significant.

RESULTS

Fetus abnormalities

Malformations of the axial skeleton were observed, 50.25% in the Exp7 fetuses (experimental group injected with *Vitex*) (n = 74) to compare with 28.75% in the Control fetuses (n = 80) and 31.66% in the Sham7 fetuses (experimental group injected with 50 ethanol) (n = 60). Also, the percent of skeletal deformities in the Exp8 fetuses (injected with *Vitex*) (n = 82) was 75.60%, whereas it was 28.75% and 30% for the Control (n = 80) and Sham 8 (injected with 50 ethanol) (n = 50), respectively.

Skeletal abnormalities are shown in Fig.1.

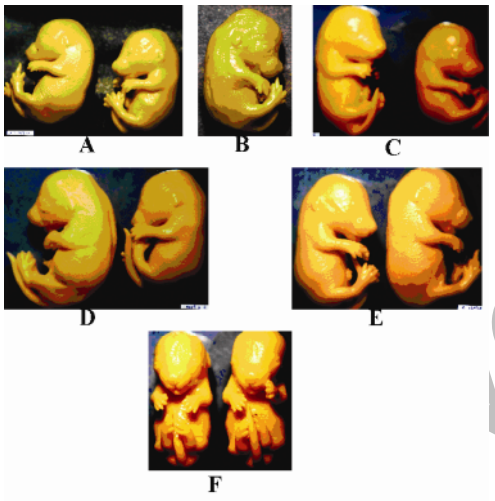

Fig. 1 Skeletal abnormalities in experimental groups (injected with *Vitex*). **A**, Control (intact) fetus (Right) and an abnormal Exp7 (*vitex* injected in the 7th day of pregnancy) (Left); **B**, skeletal abnormality (lordosis) in an Exp 7 (*vitex* injected in the 7th day of pregnancy) fetus; **C**, Control (intact) fetus (Right) and an abnormal (straight-form) Exp 8 (*vitex* injected in the 8th day of pregnancy) fetus (Left); **D**, Control (intact) fetus (Right) and an abnormal (lordosis) Exp 9 (*vitex* injected in the 9th day of pregnancy) fetus (Left); **E**, Control (intact) fetus (Left) and an abnormal (straight-shape) Exp 9 (*Vitex* injected in the 9th day of pregnancy) fetus (Right) [× 8].

Abnormalities percent in the Exp 9 fetuses (injected with *Vitex*) (n = 75) was 84%; to compare with 28.75% and 33.32% in the Control (n = 80) and Sham 9 (injected with ethanol) fetuses (n = 63), respectively. These. Other abnormalities such as malformation of brain (Fig. 2), malformations of digestive system (omphalocele, as shown in Fig. 3) and difference in eye and lens diameter were observed, too.

Macroscopic Results

The fetuses were investigated upon their body weight and CRL (Crown-Rump length). The diameter and the weight of placentas were measured, too.

Weight of Fetuses

The Exp7 group (injected with *Vitex*) didn't show any significant differences (0.3689 ± 0.0100 gr) upon their weight, to compare with control group (intact) (0.3525 ± 0.0084 gr) and Sham7 (injected with ethanol) (0.3509 ± 0.0593 gr).

Whereas Exp8 group (injected with *Vitex*) show significant increase ($P < 0.001$) of the weight (0.3904 ± 0.0714 gr) to compare with the control (intact) (0.3525 ± 0.0084 gr) and sham8 (injected with ethanol) (0.3451 ± 0.0071 gr) (Fig.4).

The Exp9 group (injected with *Vitex*) showed significant increase ($P < 0.001$) in their weight (0.4114 ± 0.0969 gr) to compare with the control group (intact) (0.3525 ± 0.0842 gr) and the Sham 9 group (injected with ethanol) (0.3350 ± 0.0066 gr).

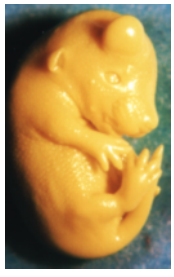

Fig. 2. Malformation of brain in Exp 7 (*Vitex* injected in the 7th day of pregnancy) fetus [× 8].

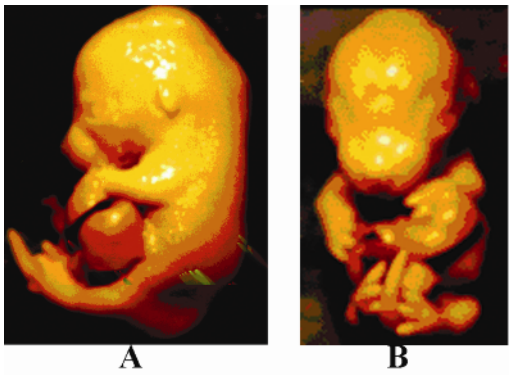

Fig. 3. A. Sagital view of malformations of digestive system (omphalocele) exp 9 (*Vitex* injected in the 9th day of pregnancy) fetus. **B.** Frontal view of the same fetus [× 8].

CRL (length of CR)

The Exp7 group (injected with *Vitex*) showed significant increase ($P < 0.05$) in their CRL (11.96 ± 0.11 mm), to compare with the control group (intact) and the sham7 group (injected with ethanol), which was, 11.91 ± 0.18 mm and 11.90 ± 0.12 mm, respectively (Fig.5).

The Exp 8 group (injected with *Vitex*) showed significant increase ($P < 0.001$) in their CRL (12.83 ± 0.10 mm), to compare with the control group (intact) and the sham 8 group (injected with ethanol), which was, 11.91 ± 0.18 mm and 12.00 ± 0.12 mm, respectively.

The Exp9 group (Injected with *Vitex*) showed significant increase ($P < 0.05$) in their CRL (12.48 ± 0.15 mm), to compare with the control group (intact) and the sham 9 group (injected with ethanol), which was, 11.91 ± 0.18 mm and 11.95 ± 0.18, respectively.

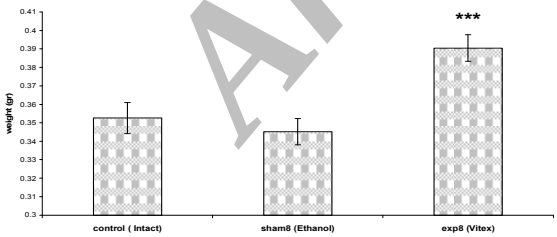

Fig. 4. Comparison of the weight of fetuses of control, sham 8 and exp 8 groups. Data are represented as Mean + S.E.M. ***. $P < 0.001$ as determined by ANOVA.

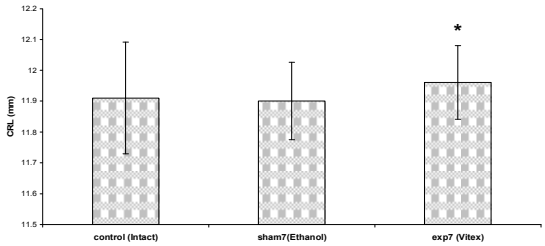

Fig. 5. Comparison of the length of CR of the fetuses between control, sham7 and exp7 groups. Data are represented as Mean + SEM*. $P < 0.05$ as determined by ANOVA.

Diameter of placenta

The Exp 7 group (injected with *Vitex*), didn't show any significant difference in their diameter of placenta (5.41 ± 0.10 mm), to compare with the control (intact) group (5.34 ± 0.08 mm) and the sham 7 group (injected with ethanol) (5.37 ± 0.09 mm).

The Exp 8 group (injected with *Vitex*), showed significant increase ($P < 0.001$) in their diameter ofplacenta (6.00 ± 0.13 mm), to compare with the control (intact) group (5.34 ± 0.08 mm) and the sham 8 group (injected with ethanol) (5.53 ± 0.06 mm) (Fig. 6).

The Exp 9 group (injected with *Vitex*), showed significant increase ($P < 0.05$) in their diameter of placenta (5.61 ± 0.69 mm), to compare with the control (intact) group (5.34 ± 0.08 mm) and the sham 9 group (injected with ethanol) (5.54 ± 0.10 mm) (Fig.7).

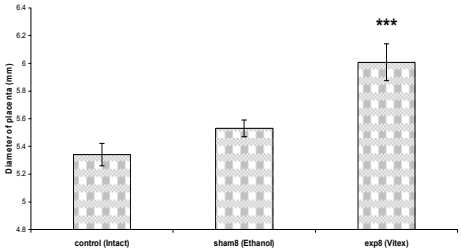

Fig. 6. Comparison of the diameter of placenta between control, sham 8 and exp 8 groups. Data are represented as Mean + S.E.M. ***. $P < 0.001$ as determined by ANOVA.

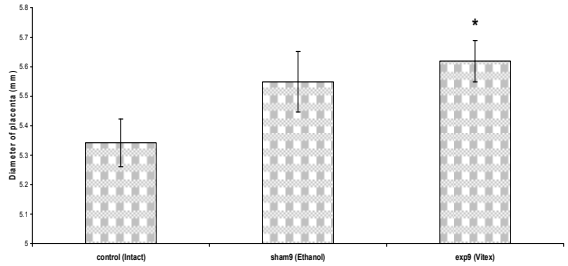

Fig. 7. Comparison of the diameter of placenta between control, sham9 and exp9 groups. Data are represented as Mean + S.E.M.*P<0.05 as determined by ANOVA.

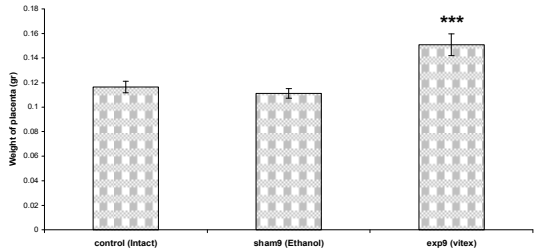

Fig. 9. Comparison of the weight of placenta between control, sham 9 and exp 9 groups. Data are represented as Mean + S.E.M. ***. $P < 0.001$ as determined by ANOVA.

Weight of placenta

The Exp7 group (injected with *Vitex*), showed significant increase ($P < 0.05$) in their weight of placenta (0.1246 ± 0.0055 gr), to compare with the Control (intact) group (0.1164 ± 0.0046 gr) and the Sham7 group (injected with ethanol) (0.1099 ± 0.0026 gr).

The Exp 8 group (injected with *Vitex*), showed significant increase ($P < 0.01$) in their weight of placenta (0.1259 ± 0.0041 gr), to compare with the Control (intact) group (0.1164 ± 0.0046 gr) and the Sham8 group (injected with ethanol) (0.1072 ± 0.0023 gr) (Fig. 8).

The Exp 9 group (injected with *Vitex*), showed significant increase ($P < 0.001$) in their weight of placenta (0.1510 ± 0.0088 gr), to compare with the Control (intact) group (0.1164 ± 0.0046 gr) and the Sham 9 group (injected with ethanol) (0.1111±0.0039 gr) (Fig. 9).

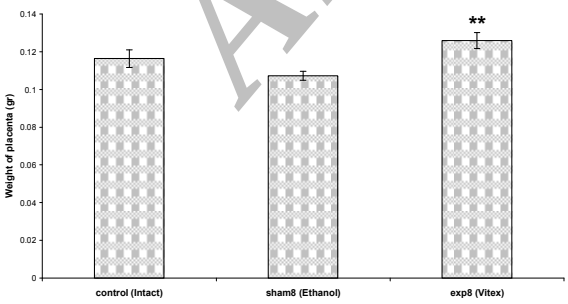

Fig. 8. Comparison of the weight of placenta between control, sham8 and exp8 groups. Data are represented as Mean + S.E.M.**P<0.01 as determined by ANOVA.

The eye and lens diameter

The eye and lens diameter of the Exp 7 group (injected with *Vitex*) didn't show any significant differences (423.33 ± 13.08 µm and 187.66 ± 15.64 µm, respectively) to compare with the Control (intact) group (436.66 ± 14.98 µm and 158 ± 12.33 µm) and Sham7 group (injected with ethanol) (433.33 ± 16.05 µm and 154.66 ± 15.51 µm).

The eye and lens diameter of the Exp8 group (injected with *Vitex*) didn't show any significant differences (390 ± 25.16 µm and 170 ± 14.37 µm, respectively) to compare with the Control (intact) group (436.66 ± 14.98 µm and 158 ± 12.33 µm) and Sham8 group (injected with ethanol) (413.33 ± 12.29 µm and 154 ± 7.91 µm).

The eye diameter in the Exp 9 group (injected with *Vitex*) showed significant decrease ($P < 0.001$) (360 ± 11.54 µm) to compare with the Control (intact) group (436.66 ± 14.98 µm) and Sham9 group (injected with ethanol) (441.33 ± 12.92 µm) (Fig. 10).

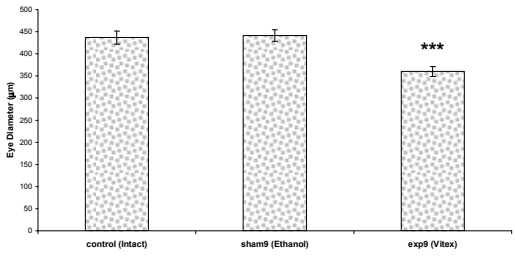

Fig. 10. Comparison of the eye diameter between control, sham 9 and exp 9 groups. Data are represented as mean + S.E.M. ***. $P < 0.001$ as determined by ANOVA.

But lens diameter in Exp 9 group (injected with *Vitex*) didn't show any significant differences (153.33 ± 5.02 µm) to compare with the Control (intact) group (158 ± 12.33 µm) and Sham 9 group (injected with ethanol) (160 ± 10.32 µm).

DISCUSSION

Human and animal studies have determined *Vitex* to be safe for most women of menstruating age, but they note that it should not be used during pregnancy, (1). The role for *Vitex* in various physiological and pathophysiological processes in pregnancy is still unknown.

Our data demonstrate a characteristic phenotype among experimental group (injected with *Vitex*), including increasing of fetal growth and developmental abnormalities of axial skeleton.

Our data indicate that the *Vitex* (or maybe its components) crosses the placenta and enters fetal tissues. The increase in the fetus growth is striking. According to Weiss et al. report, *Vitex* acts on the diencephalohypophyseal system *Vitex* increase LH production and mildly inhibits the release of FSH. The result is shift in the ratio of estrogen to progesterone, in favor of progesterone, (30)

The studies show that progesterone is critical to ensuring bone health. It offers neuroprotection, contributes to cardiovascular health, assists normal brain development, and provides protection from some types of cancers, (31). Therefore, *Vitex* which has progesteronic-like effect can have those benefits which are necessary for growth. It may submit the growth process, which is shown in results. Significant increase in CRL and the weight of fetuses). Also, *Vitex* contains flavonoids such as casticin, kampferol, quercetagetin, vitexin, (17).

Schroder *et al.* demonstrated that iodine-labled synthetic flavonoids administered to pregnant rats traverse the fetal blood-brain barrier and accumulate in higher levels in the fetal brain than in the mother herself (32). These results, suggest that the fetus may be exposed to high circulating levels of flavonoids, which may elicit toxic response that may otherwise be innocuous to the mother. The other species of the genus *Vitex* (*V.negundo*), causes histomorphological changes in rats (27).

Ohyama *et al.* demonstrated that cytotoxic activity of *Vitex* extract may be attributed to the growth activity of the respective cell, and showed that the possibility that the cytotoxicity is related to the cell cycle stage (2). Therefore *Vitex* because of its flavonoid components may affect the fetus. 8th day of pregnancy (8 PDC: post day coital) in mice (Theiler stage 12b) is the day that first somites unturned embryo with first appearance of somit pairs 5-7 somites. So the Exp 8, Exp 9 (injected with *Vitex*) showed increase in deformity of axial skeleton (such as lordosis, etc), whereas Sham8 and Sham9 groups (injected with ethanol) didn't show such abnormalities, so it maybe the result of *Vitex* extract. Also in the 9th day of pregnancy (9 dpc)(Theiler stage) the optic placodes are first evident and became indented to form the optic pits, so we can see significant decrease in eye diameter in Exp 9 (injected with *Vitex*). Sanderson *et al.* showed that low micromolar concentrations of naturally occurring flavonoid, quercetin, have inhibited cataractogenesis in a rat lens organ cultured model (33); however our results didn't show any significant changes in the lens diameter, in any groups. After all, our finding suggests that *Vitex* may have a dual effect on mice development, somehow it can improve growth parameter, but in the other hand its unknown toxic effects can inhibit normal growth of vital organs such as eye and liver development.

Conflict of interests

The authors declare that they have no competing interests.

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