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Research Article

Curcumin Enhance the Implantation Process in Endometriosis Induced Animal Model

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Abstract

This study mainly focused on down regulating or inhibiting endometrial lesion development by treating *Curcumin* in Diethylstilbestrol (DES) induced endometriosis rats. Female Sprague Dawley (SD) rats of 2 months old were subcutaneously injected with DES in three different concentrations (10, 15 and 20 µg/kg/bw/d) for 21 d. The development of endometrial lesion was confirmed by hormonal levels and estrous cycle. The endometriosis induced animals were treated with *curcumin* (48 mg/kg/bw) for 15 d. Endometriosis control group was maintained without any treatment for further process. After *curcumin* treatment, all female rats were cohabitated with male rats for a week. Cohabitated females left for gestation while copulatory vaginal plug and weight of the animals were also observed. At the end of the experimental period, it was noted to confirm whether *curcumin* treated groups could attain the implantation and produce the young ones or not. In addition, the implantation was also confirmed by histopathological study. The body weights of rats decreased from the initial period when compared with endometriosis induced rats. The *curcumin* (48 mg/kg/bw) treated groups significantly increased the bodyweight in 15 µg DES. Irregular estrous cycles were observed in endometriosis control group, except the *curcumin* treated groups. FSH and Estrogen levels increased significantly in DES induced group compared with normal and no significant changes in LH level. All DES induced with *curcumin* treated groups have attained the implantation and produced the litters, but not attained in endometriosis control groups. Further, histopathological observations showed abnormal and dilated endometrial lumen in endometriosis control groups. The present study provides that the *curcumin* as a potential therapeutic agent for endometriosis, through inhibition of endometrial lesion that retains the reproductive homeostasis for normal implantation process to produce young ones.

Keywords: Endometriosis, curcumin, diethylstilbestrol, implantation, reproductive homeostasis.

Introduction

Endometriosis is an estrogen dependent gynecological disorder and also defined as the presence of endometrial gland and stroma at outside the uterine cavity which affects the reproductive age women and causes infertility. Most of the endometriosis affected women's inability to produce the young ones due to the hormonal imbalance. All over the world, 176 million women suffer from endometriosis. In India, 26 million women affected by endometriosis and it can affect the menarche to menopause. In addition, 25-50% of infertile women has endometriosis reported by Missmer *et al.* (2004). The exact mechanism for the development of endometriosis remains unclear. Some of the research data suggest that a crucial role of estrogen in the establishment and maintenance of this disease. Estradiol can stimulate lesion development, which can initiate the pain induced by nerve fibers that contribute persistent inflammatory pain and inhibit neuronal apoptosis. An endometrial bleeding factor (EBAF) is responsible for bleeding, not rightly expressed and may contribute to uterine bleeding. The toxic effects of the inflammatory process on gametes and embryos due to the development of ectopic endometrium that is resistant to the action of progesterone regulation, and thus the endometrium is inhospitable to implanting embryo results infertility crisis (Giudice, 2010).

Diethylstilbestrol (DES) is a synthetic non-steroidal estrogen which is estimated to be five times more potent than the naturally occurring estrogen. DES can be metabolized in all species evaluated to produce either hormonally inactive compound that retain estrogenic activity. In this study DES is used to induce endometriosis in an animal model. The existing treatment for endometriosis involves pharmacological therapy and surgical removal of endometriotic lesions. The proliferation and long-term survival of ectopic endometrial tissue are estrogen dependent. The classical pharmacological therapies are primarily aimed at the suppression of endogenous estrogen production by the application of oral contraceptives, GnRH agonists, androgenic agents or aromatase inhibitors. However, this type of medication is associated with substantial side effects, which limits prolonged exposure. In addition, surgical removal can be technically challenging and bears the risk of urological or colorectal complications. Thus, there is an urgent need for the development of novel treatment strategies for endometriosis. For this purpose, a key process in the pathogenesis of endometriosis has been identified, which may serve as potential therapeutic agents.

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In order that, this study used a phytotherapeutic drug of *Curcumin*, which can act as pleiotropic interaction with numerous molecular targets including transcription factors, growth factors, protein kinases, inflammatory cytokines, and enzymes. It can lead to reduce endometrial lesion development can initiate the implantation.

Materials and methods

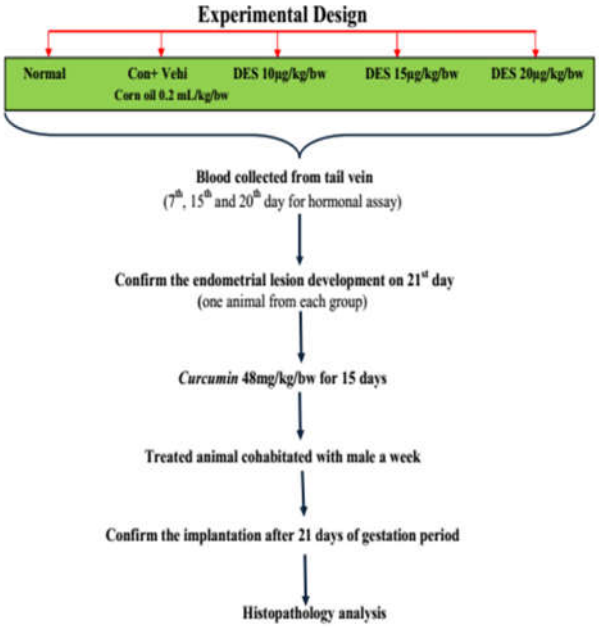
Materials: Diethylstilbestrol (DES) was purchased from Sigma Aldrich (purity ≥99% HPLC). The DES was suspended in corn oil and their concentrations are 10, 15, and 20 µg/kg bodyweight. *Curcumin* (99% purity) was obtained from SISCO Research Laboratories. FSH and LH level were quantified by adopting indirect DSI S.r.l EIA gonadotropin kit (Via Angelo Volonterio, Italy), while estrogen by competitive Pathozyme® oestradiol EIA kit from Omega Diagnostics Ltd., (Scotland, United Kingdom). All other chemicals were of analytical grade obtained from Medox Biotech (India) and Sigma Aldrich. The following experimental design explains shortly about present work (Fig. 1).

Animal experimentation: Two months old female Sparague dawley (SD) rats were purchased and all procedures were performed according to the Institutional Animal Ethical Committee for this study. Animals were housed under 12 h light/12 h dark cycle with controlled conditions. Animals were fed with standard pellet feed and water *ad libitum*. Bodyweight of the animals was measured control group, DES induction group and *curcumin* treatment group. The animals were divided into 5 groups, each containing 5 animals. In those 5 groups, the group 1 and 2 considers as normal control and control vehicle group. The other groups recorded as DES induced group in 3 different concentrations (10, 15 and 20 µg/kg bw). DES was dissolved in corn oil and given subcutaneously at alternative days up to 20 d. Meanwhile, body weight was taken at the digital balance; blood serum was collected through tail vein for hormonal analysis at 7th, 15th and 20th d. Then estrous cycle was analyzed by vaginal cytology. Anatomical observation of the three groups was noted in rats affected by DES and after *Curcumin* treatment whether those produce young ones or not.

Observation of body weight and estrous cycle: Bodyweight and the estrous cycle of all animals were monitored throughout the DES induction period. The animals weight were recorded at initial day and after the final day of DES induction and *curcumin* treatment. In addition, the final gain in bodyweight was calculated by subtracting the final weight from the initial weight of the animal. The detection of estrous cycle by evaluating vaginal cytology, vaginal cells are accepted as the most accurate method for identifying all stages of the estrous cycle (Long and Evans, 1922; Caligioni, 2009). Vaginal cells to be collected by vaginal smear on glass slide at early morning for 15 d and collected glass slide, air dried, stained, and viewed under light microscope. The frequency and number of estrous cycle observed and spread in the column to identifying whether it regular or irregular after DES induction.

Hormonal analysis: Blood was collected from control and DES induced group at 7th, 15th and 20th d of DES induction and allowed to stand for 30 min before centrifugation at 2,500 rpm for 10 min at 4°C for serum separation. FSH, LH and estrogen levels were quantified.

Fig. 1. Experimental design.



Anatomical observation: Animal from DES induced group was anaesthetically dislocating the abdomen and endometrial lesion development or abnormality in the reproductive organ. Then animal closed that skin layer surgically and it could be recovered to normal condition and taken to further treatment.

Inhibition of MMP activity: Matrix Metalloproteinase (MMP) activity was inhibited by treating *curcumin* (48 mg/kg/bw) were given intra peritoneal injection for 15 d in DES induced animal group except for the endometriosis control group.

Breeding of the animal groups: DES induced endometriosis control group and *curcumin* treated group were breed with same species of male rats leave for gestation.

Implanted animal groups: Implantation and producing F1 generation are an important process in reproductive cycle. During the gestation period of breeding animal to check vaginal plug and animal weight which is a preliminary indication for attaining implantation. Therefore, the breeding animals were observed to attain implantation to produce young ones or not.

Histopathology: It has to be done to find the architecture effect in disease control compare to normal under light microscopic examinations. Dissected fallopian and ovary tissues were fixed in Bouin's for 24 h, dehydrated in ethanol and embedded in paraffin wax. Sections were obtained at 5 µm thickness and stained with Harris haematoxylin and eosin. The sections were viewed and photographed in light microscope (Olympus BX51, Tokyo, Japan) with an attached camera (Olympus C-5050).

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Table 1. Bodyweight of the SD rats were observed from initial to after diethylstilbestrol induction and after *Curcumin* treatment.

Groups	Initial	Final	
Group-I Normal	190.0±11.54	198.0±11.54	
Group-II Cont+Vehi (0.2 mL/kgbw)	140.0±23.09	135.0±23.09	
		After DES	After *Curcumin* (48mg/kgbw)
Group-III (10 µg/kgbw)	140.0±23.09	140.0±23.09	140.0±23.09
Group-IV (15 µg/kgbw)	140.0±23.09	135.0±23.09	140.0±23.09 *
Group-V (20 µg/kgbw)	130.0±34.64	120.0±23.09	135.0±30.00 *

*Compared with initial Group-IV and Group-V P<0.05; Values are presented as the mean ± SD.

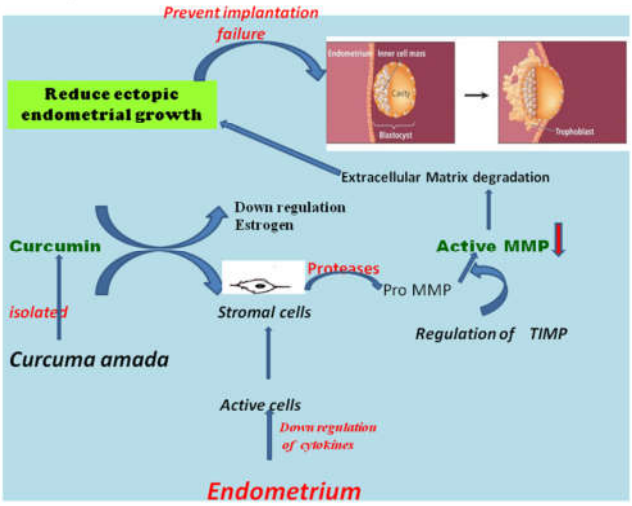
Statistical analysis: To analyze mean, standard deviation and their probability level by *t*-test and to find the significant levels were observed by SPSS version 16.

Results and discussion

Curcumin isolated from *Curcuma amada* can act on the endometrium of endometriotic tissue to down-regulate estrogen and the other hand, down-regulate cytokines to decrease production of stromal cells from active cells. The enzyme proteases convert proMMP to active MMP with help of tissue inhibiting matelloproteinase (TIMP) inhibit the active MMP production to stimulate the endometrial remodelling by extracellular matrix degradation to initiate the implantation process (Fig. 2). Diethylstilbestrol (DES) was administered subcutaneously to rats with the concentration of 10, 15 and 20 µg/kg bw showed significant difference appeared in the body weight of group IV (15 µg/kg bw) and V (20 µg/kg bw) from initial to final but not in the 10 µg DES induced group (Table 1) but compared to normal which did not showed much difference. The same DES induced group when treated with *curcumin* is significantly ($p<0.05$) related to normal, results were supported by Kizilay *et al.* (2017). DES exposure leads to a significant reduction in female bodyweight (Kyselovaa *et al.*, 2004), so the present results were consistent with the previous reports (Kang *et al.*, 2002).

Regulation of the reproductive cycle was determined by the hormonal balance in the reproductive organ, the first 1-6 d in DES induced animals have the LH level similar to control due to the inhibition progesterone level in estrous cycle, after 7 to 21 d, the FSH and estrogen levels were increased (Fig. 3a-c). This result is supported by alteration of hormonal level due to the exposure of DES induced group to increased estrogens and reduced progestins to cause the incidence of endometrial carcinoma of both human and animals (Marquardt *et al.*, 1999). However, the estrogen receptor may be the main signaling pathway for the hormonal agonist activity of DES induced mice (Korach and Couse, 1996). The regular estrous cycle was determined by vaginal cytology, in the present study, DES-exposed group was showed abnormal estrous cycle compared to the control. The duration of each stage was extended in DES induced group, particularly, met and diestrous stage than control (Fig. 4; Table 2). These results were correlated with the stable estrous cycle for a long time i.e. normal cycle formed by proestrous, estrous, metestrous and diestrous stages but in the case of DES exposed group, this cycle formation gets altered (Bouchard *et al.*, 1991).

Fig. 2. Proposed mechanism of *curcumin* in endometriosis.



Similarly, the dose dependent study of DES exposed can induce vaginal changes in the rat to indicate the abnormal status of anovulation and excess estrogen level (Yoshida *et al.*, 2011).

DES is an endocrine disruptor when it is exposed to organism specifically to target the reproductive organs. According to that, in this study, we observed some reproductive abnormalities in 10, 15 and 20 µg DES induced group on the 21st d. We observed fibroid in fallopian tube and lesion in 10 µg DES induced group, constricted fallopian tube shown in 15 µg DES, endometrial patches seen in 20 µg DES induced group (Fig. 5a-e). These results may confirm that the different concentrations of DES can alter the reproductive function and affect reproductive organs. Newbold and McLachlan (1982) reported similarly that DES exposed rodents lead to cause loss of uterotubal function, stratification of the uterine epithelium, disorganized uterine muscle layers, delayed and reduced uterine adenogenesis, and vaginal adenosis. While the critical window period of female reproductive tract development, DES potently inhibits the expression of some genetic molecules like HOXA10 and HOXA11 genes, which affect the female reproductive tract differentiation (Ma *et al.*, 1998; Miller *et al.*, 1998; Block *et al.*, 2000; Mericskay *et al.*, 2004). A similar reproductive functional change leads to reduced fertility (McLachlan, 1979).

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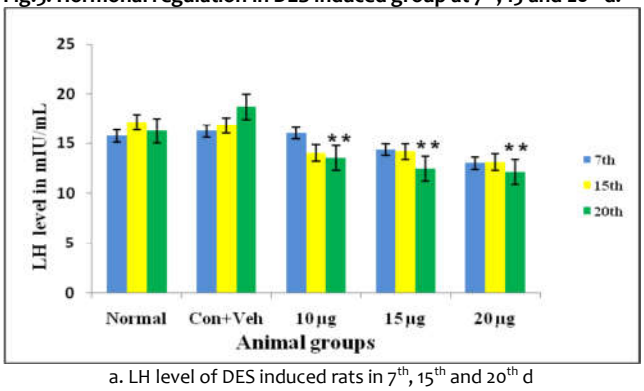
Table 2. Estrus cycle (15 d) in DES induced rat with curcumin treated group

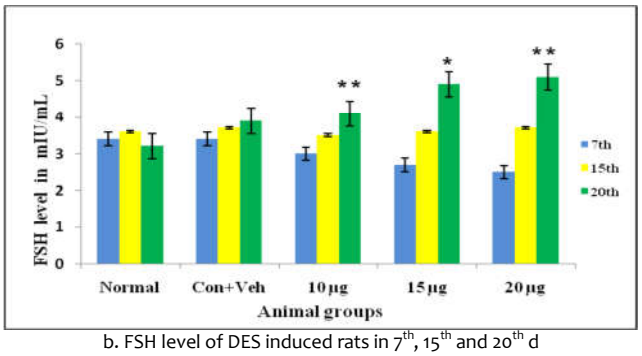
Groups		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
GI-Normal		M	D	P	E	E	M	D	P	E	M	D	P	E	M	D
GII-Con+Vehi		E	M	E	M	D	P	E	M	D	E	M	D	D	P	E
G III	10µg/kg bw	D	D	E	M	M	M	P	E	M	D	D	D	P	P	E
	Cur 48mg/kg bw	E	M	M	D	P	P	E	M	D	P	E	M	D	E	E
G IV	15 µg/kg bw	M	D	P	M	M	D	D	P	P	M	E	M	M	D	D
	Cur 48mg/kg bw	D	P	E	E	M	D	P	E	M	D	P	E	M	D	P
G V	20µg/kg bw	D	D	P	M	M	M	M	D	D	P	E	E	E	M	D
	Cur 48mg/kg bw	P	E	E	M	D	P	P	E	M	D	P	E	M	M	D

G-I: Regular Estrous cycle in Normal rats, G-II: Control vehicle 0.2 mL of corn oil; G-III: 10 µg DES treated by *curcumin* 48 mg/kg bw; G-IV: 15 µg DES treated by *curcumin* 48 mg/kg bw; G-V: 20 µg DES groups treated by *curcumin* 48 mg/kg bw.

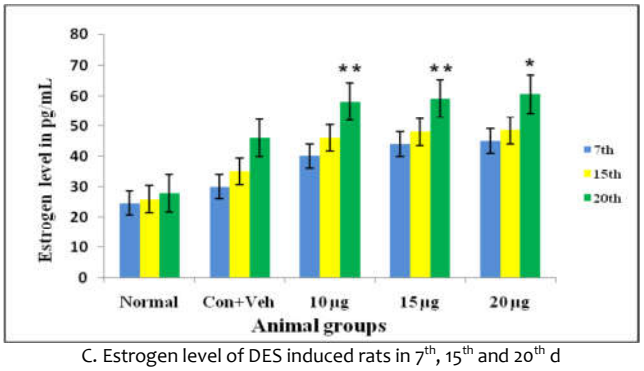
Fig.3. Hormonal regulation in DES induced group at 7th, 15 and 20th d.



a. LH level of DES induced rats in 7th, 15th and 20th d



b. FSH level of DES induced rats in 7th, 15th and 20th d



C. Estrogen level of DES induced rats in 7th, 15th and 20th d

Compared to control *P<0.01, ** P<0.05 significant

Fig. 4. Photomicrographs of unstained vaginal smear from female SD rats.

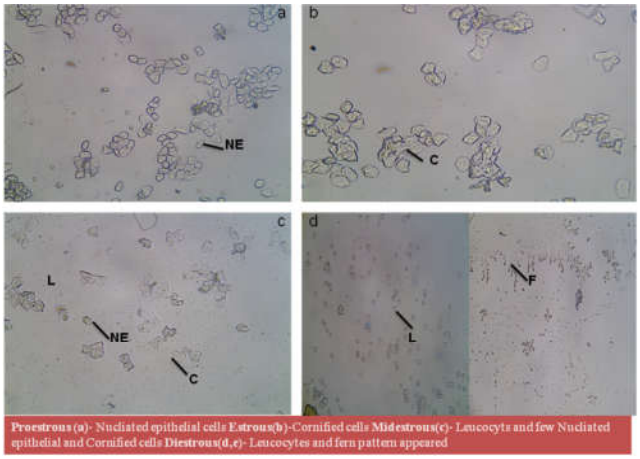
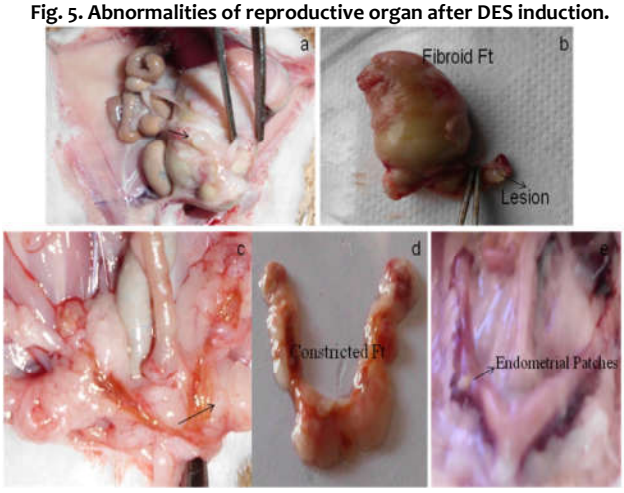


Fig. 5. Abnormalities of reproductive organ after DES induction.



Fibroid fallopian tube (FT) and lesion (a,b) shown in 10 µg DES, constricted fallopian tube (c,d) in 15 µg DES and endometrial patches on fallopian tube(e) in 20 µg DES.

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The impaired reproductive organs treated with *curcumin* may trigger the implanting suppressed genes (HOXA10 and HOXA11) can activate proteases to regulate the endometrial remodelling to reduce endometriosis development that it stimulates the implantation and produce the young ones. Similar strategies were observed in 15 µg DES treated with *curcumin* to produce young ones by reducing the reproductive abnormalities. The other group also produced young ones but did not attain regular implantation within stipulated period in 10 µg DES, and not stable and survival pubs in 20 µg DES (Fig. 6). Previous study suggests that follicular growth and oocyte quality were restored in early reproductive failure treated with *curcumin* (Tiwari-Pandey and Ram Sairam, 2009). It is also protected the ovary against experimentally-induced immune ovarian failure in mice (Voznesens'ka *et al.*, 2010). In addition, *curcumin* protected against toxin-induced damages in reproductive organ in rodents (El-Demerdash *et al.*, 2009).

Results showed that histo-architecture effect of fallopian tube and ovary in DES treated with *curcumin* compared to normal control and disease control groups. Endometrial lumen clearly observed in normal and *curcumin* treated group but not in the disease control group. The endometrial layer degenerated in DES induced without treatment group but not in the treated group that should be correlated with normal. Histopathological observation of ovary showed primary follicle, graffian follicle and vasculation are coinciding with normal and *curcumin* treated group. But in the case of DES induced group, appeared scratch of the follicle cells and reduction of the follicle cells (Fig. 7 and 8) were noted. Histopathological changes may occur due to endocrine disruptors (Wei *et al.*, 2007). Further, endocrine disruptors can alter reproductive hormones that lead to disturbance in folliculogenesis, oocyte maturation and endometrium (Billig *et al.*, 1993; 1996).

Conclusion

DES is effectively used to target reproductive organ to cause impaired abnormalities, it could be treated by phytotherapeutic compound of *curcumin*, which has an anti-inflammatary and anti-angiogenic property that could reduce the endometrial (tissues) patches, vascular constriction of uterine to initiate the implantation. In the present study, *curcumin* could be used to treat the endometrial related abnormalities in 15 µg DES/kg/bw and stimulate the implantation to produce young ones as effectively as normal group. The other *curcumin* treated group also showed to attain implantation but their survival and production was clearly observed in 15 µg DES/kg/bw group treated with *curcumin.*

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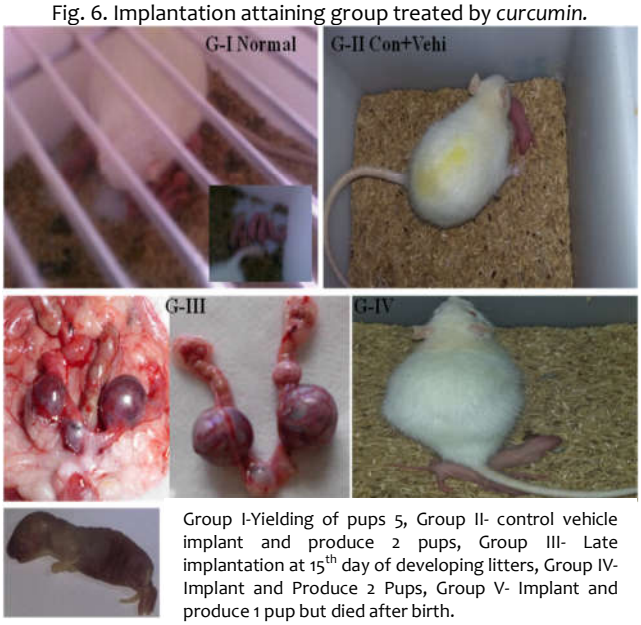
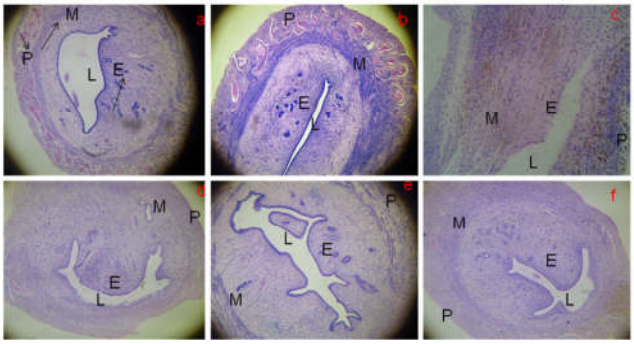
Fig. 6. Implantation attaining group treated by *curcumin.*



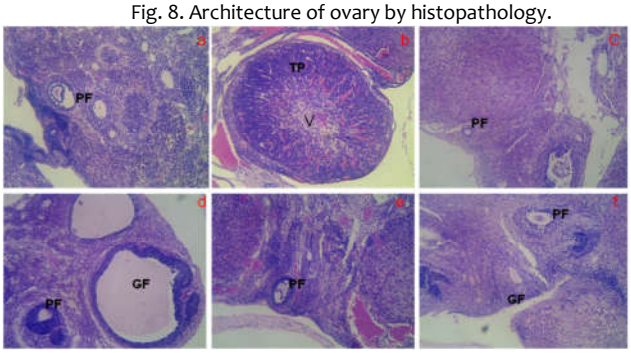
Group I-Yielding of pups 5, Group II- control vehicle implant and produce 2 pups, Group III- Late implantation at 15th day of developing litters, Group IV- Implant and Produce 2 Pups, Group V- Implant and produce 1 pup but died after birth.

Fig. 7. Architecture of uterine tube by histopathology.



L-Lumen E-Endometrium M-Myometrium P-Perimetrium, a) Normal; b) Control vehicle; c) Endometriosis control d)10 µg DES induced and treated with *Curcumin* 48mg/kgbw; e) 15 µg DES induced and treated with *Curcumin* 48mg/kgbw; f) 20 µg DES induced and treated with *Curcumin* 48mg/kgbw.

Fig. 8. Architecture of ovary by histopathology.



PF-Primary Follicle TP-Tissue Proliferation V-Vasculation GF-Graffian follicle, a) Normal; b) Control vehicle; c) Endometriosis control d)10 µg DES induced and treated with *Curcumin* 48mg/kgbw; e) 15 µg DES induced and treated with *Curcumin* 48mg/kgbw; f) 20 µg DES induced and treated with *Curcumin* 48mg/kgbw.

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