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DIETHYLSTILBESTROL (DES) INDUCED ENDOMETRIOSIS IN BALB/C MICE TREATED WITH MMP-2 AND 9 INHIBITORS-I

Mohan Rajeswari and Balamuthu Kadalmani *

Department of Animal Science, Bharathidasan University, Tiruchirappalli - 620024, Tamil Nadu, India.

Keywords:

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Correspondence to Author:

Dr. B. Kadalmani

Assistant Professor,
Department of Animal Science,
Bharathidasan University,
Tiruchirappalli - 620024,
Tamil Nadu, India.


E-mail: kadalmanibdu@rediffmail.com

ABSTRACT: Diethylstilbestrol (DES) is an endocrine disturbing compound it can mimic and perform like a natural hormone, which mimics estradiol by binding to the estrogen receptor. This compound mainly reproductive target, so in this study used to induce endometriosis. Endometriosis is an estrone dependent constant gynaecological disorder. Here it, intends to induce endometriosis and treated by the MMP inhibitor to check whether it could attain the implantation or not. Hence the present study induces endometriosis by three different concentrations such as 10, 15 and 20 µg/kg body weight for 20 days at alternatively while to check the estrous cycle, hormonal regulation at 7th, 15th and 20th day also to record the body weight and reproductive organ weight from initial to final day of DES induction. Endometrial lesion developments were confirmed by anatomical observation. The above result reveals 20 µg DES induced group leads to endometrial cancer, so other 10 & 15 µg DES induced group carry out to further analysis. The endometrial lesions were treated by 10 µM concentrations of MMP 2 & 9 inhibitor I by interaperitonealy at 7 days. The determination of protein band and proteolytic activity of matrix metallo protein by SDS-Page and gelatin zymogram, to promote the histogram which is an intensity of protein band by using excel. Based on the results we consolidated 15 µg des induce group can induced the endometrial lesion and it treated by MMP 2 & 9 inhibitor compound to attain implantation to produce young ones. Further these reports were confirmed by vaginal protein, implantation attained animals and histopathological result.

INTRODUCTION: The production and growing up of human generation is a part of women reproductive system. Typically Menarche, the inception of menses and fertility can occur in young women between the ages of 9 - 14. During this time there are some changes that occur in a woman's reproductive organs.

The ovaries produce the steroid hormones, progesterone, and estrogen, which along with neural activity can regulate these changes.

Further, that occurred during the menstrual cycle in response to hormonal regulation, if any abnormal changes in the reproductive tract, functional and mechanistic differences can create some women disorders. Endometriosis is one of the women reproductive disorders in women in which some of the tissue has spread elsewhere such as the ovaries or other than the abdominal cavity also served a benign gynecological disorder characterized by the presence of endometrial-like glands and stromal occurring outside the uterine cavity.

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It affects 10 - 15% women of reproductive age and is often associated with chronic pelvic pain which can be extremely devastating leads to 40 - 50% of women infertility ¹. The development of endometrial lesion due to the abnormal regulation of hormone especially upregulation of estrogen to stimulate angiogenic factors and cytokines causes endometriosis.

Risk factors for endometriosis include mullerian anomalies and exposure to diethylstilbestrol (DES), prolonged exposure to endogenous estrogen cause early menarche, late menopause, or obesity, short menstrual cycles, low birth weight, and also exposure to endocrine-disturbing chemicals ^{2, 3, 4, 5}. Laparoscopic is the gold standard tool to diagnostic endometriosis.

Diethylstilbestrol is a synthetic ethinyl estrogen, it's an endocrine disturbtors ⁶. In the normal endometrium, estradiol is associated with cellular proliferation as well as growth-related expression of matrix metalloproteinases (MMPs) enzymes, which activate extracellular matrix degradation and turnover including an endometrial breakdown at menstruation. In addition, MMP is important in proper orchestrating physiological function of the endometrium, hence the alteration of MMPs appear to be improperly expressed in the endometrium is crucial factor for the development of endometriosis in association with a reduced sensitivity to progesterone and increased sensitivity to estrogen ⁷. Aberrant expression of MMPs is associated with invasive and destructive endometrial like disorders.

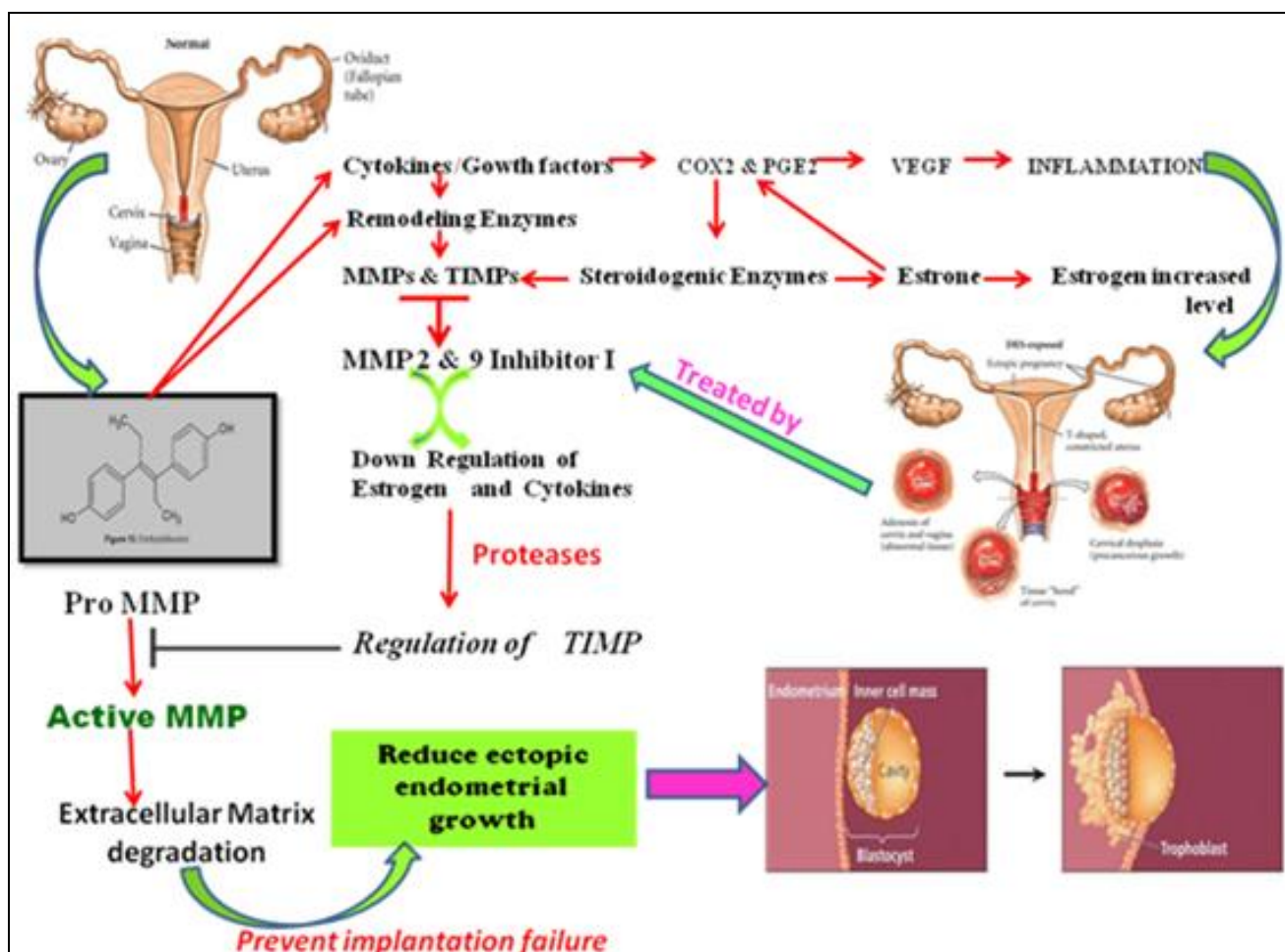


FIG. 1: THE NORMAL UTERUS EXPOSED TO DIETHYLSTILBESTROL (DES) TO STIMULATE THE CYTOKINES AND GROWTH FACTORS TO ENHANCE THE INFLAMMATORY AND ANGIOGENIC FACTORS LIKE COX-2 AND VEGF AT THE SAME AUGMENT OF THE STEROIDOGENIC ENZYME TO INHIBIT THE EXTRACELLULAR MATRIX DEGRADATION TO CAUSE REPRODUCTIVE DISORDERS LEADS TO INFERTILITY. WHEN IT TREATED BY MMP 2 & 9 INHIBITOR-I, COULD BE DOWN REGULATE THE CYTOKINES AND ESTROGEN LEVEL TO REGULATE THE TIMP WITH HELP OF PROTEASES TO INHIBIT PROMMP TO ACTIVE MMP PROMOTE EXTRACELLULAR MATRIX DEGRADATION TO REDUCE ECTOPIC ENDOMETRIAL GROWTH MAY PREVENT IMPLANTATION FAILURE

The expression of MMP genes were regulate along with their natural inhibitors (tissue inhibitors of metalloproteinases, TIMPs) largely reflects the presence or absence of reaction elements within the promoter of any given gene; The relationship of TIMP expression to the expression of MMPs is the main role of balancing extracellular matrix (ECM) degradation and congregation during tissue remodelling. If TIMP levels fluctuate less during the menstrual cycle than those of the MMPs, were appears to provide an additional element of control over the cell-specific expression of these enzymes⁸. The main symptoms of endometriosis are pain and infertility, but exact pathological mechanism still unclear^{9, 10, 11}. Endometriosis has considered to include different stages, whether it early, minimal, mild and moderate. Due to that decay endometriosis women reproductive organ show, have some anatomical obstruction and difficulties to conceive and maintaining pregnancy¹². Herein, hypothesized that DES is an endocrine disruptor to induce endometriosis and it inhibited by using MMP 2 & 9 Inhibitor-I too find to attain implantation or not, which is analyzed by body weight, vaginal cytology, hormonal analysis, anatomical observation, gelatine zymogram, histopathology and number of implantation attaining animal groups. The following flow chart explained the perception of this study **Fig. 1**.

MATERIALS AND METHODS:

Materials: Diethylstilbestrol (DES) was purchased from Sigma Aldrich (purity $\geq 99\%$ (HPLC)). The DES was suspended in corn oil and their concentrations are 10, 15, and 20 $\mu\text{g}/\text{kg}$ body weight. MMP 2/MMP 9 Inhibitor I (Purity $>99\%$) were purchased from Abcam. FSH and LH level was quantified by adopting indirect DSI S.r.l EIA gonadotropin kit (*via* Angelo Volonterio, Italy), while estrogen by competitive Pathozyne® oestradiol EIA kit from Omega Diagnostics Ltd., (Scotland, United Kingdom). All other chemicals were of analytical grade and were obtained from Medox Biotech (India) and Sigma Aldrich.

Animal Experimentation: 7 - 8 weeks old female BALB/c mice were purchased and all procedures performed by the Institutional Animal Ethical Committee (Ref. No. (BDU/IAEC/2015/NE/32/Dt. 17.03.2015) for this study. Animals were housed under 12 h light / 12 h dark cycle with controlled

conditions. Animals were fed standard food and allowed water *ad libitum*. Body weight and organ weight of the animals was measured at initial and final induction period. The mice were randomly 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 that are 10, 15 and 20 $\mu\text{g}/\text{kg}$ body weight which is dissolved in corn oil were given subcutaneously at alternative days up to 20 days. Meanwhile, body weight and organ weight were taken at the digital balance; blood serum was collected through retro orbital puncture for hormonal analysis at 7th, 15th and 20th day. Then estrous cycle was analyzed by vaginal cytology. Anatomical observation of the four groups, three were used as treatment groups and one as the endometriosis control group.

Observation of the Body Weight and Estrous Cycle:

Body weight and the estrous cycle of all animals were monitored throughout the DES induction period. The animal weight was recorded at initial and after the final day of DES induction. In addition, the final gain in body weight 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^{13, 14}. Vaginal cells to be collected by vaginal smear on glass slide at early morning for 15 days, 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 day of DES induction then it allowed standing for 30 min before centrifugation at 2,500 rpm for 10 min at 4 °C for serum separation. FSH, LH and estrogen level were quantified by adopting indirect DSI S.r.l EIA gonadotropin kit (*via* Angelo Volonterio, Italy), while estrogen by competitive Pathozyne® oestradiol EIA kit from Omega Diagnostics Ltd., (Scotland, United Kingdom).

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

Gelatin Zymogram: Gelatin zymography is a method, which is to detect the activity of gelatinase enzymes that are matrix metalloproteinases (MMPs) of MMP-2 and MMP-9. Fallopian tissue was collected from DES induced animal groups and MMP 2 and 9 inhibitor-I treated group then it centrifuged at 10,000 g for 15min at 4 °C, followed by the supernatant was taken to quantify the protein by Bradford assay.

Aliquots 30 µg proteins from each sample were mixed with non-reducing Laemmli's sample buffer (62.2mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 0.025% bromophenol blue) and it loaded 10% SDS-PAGE gels, it was polymerized in the presence of 1% gelatine.

After electrophoresis, SDS was eliminated from the gels by extensive washing solution containing 2.5% Triton X-100. Then the MMP activity was developed by incubating gels at 37 °C for 18 h in a buffer containing 50 mM Tris HCl (pH 7.5) 200 mM NaCl, 4 mM CaCl₂ and 0.2% Brij@35. The position of the proteolysis band by staining (0.05% coomassie brilliant blue in 25% methanol and 10% acetic acid) and destain the gels up to gelatinolytic of the clear band appear in the dark background.

Inhibition of MMP Activity: Matrix metallo-proteinase activity was inhibited by treating MMP 2 and 9 inhibitor-I (10 µM) were given intra peritoneal injection at 7 days. This treatment applied in DES induced animal group except for the endometriosis control group.

Vaginal Protein by SDS-PAGE: Estimation and separation of vaginal protein observed in two stage such as estrous and diestrous stage. In this method 50 µl of PBS (pH 7.2) taken in a 500 µl eppendorf tube. Apply and pipette out 50 µl of PBS in a vaginal orifice at superficially, then flexed and refill at the same eppendorf tube. Before protein extraction could identify the estrous cycle stage by light microscope.

Breeding of the Animal Groups: DES induced group and MMP 2 and 9 inhibitors-I treated group was breed with same species of male animal leave for gestation.

Implanted Animal Groups: Implantation and producing new generation is an important in reproductive life cycle. During the gestation period of breeding animal to check vaginal plug and animal weight which is a preliminary indication for attaining implantation group. Therefore, to observe whether that breeding animal groups was attain implantation to produce young ones or not.

Histopathology: Histopathology For histological light microscopic examinations, the fallopian tissues were dissected and the tissue samples were fixed in Bouin's fixative solution for 14 - 18 h, processed in a series of graded ethanol solutions, and embedded in paraffin. Paraffin sections were cut with a microtome at 5 mm thickness and stained with haematoxylin and eosin. The sections were viewed and photographed on a light microscope (Olympus BX51, Tokyo, Japan) with an attached camera (Olympus C-5050; Olympus Optical Co Ltd., Japan).

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

RESULTS AND DISCUSSION:

Determination of Body Weight and Organ Weight: Diethylstilbestrol was administered subcutaneously to Balb/c mice with the concentration of 10, 15 and 20 µg/kg bw shown that results are not a significant difference in the body weight from initial to final but slightly reduced in DES induced group compared to the normal.

The organ weight of uterine weight significantly ($p < 0.01$, $p < 0.05$) reduced in 10 and 15 µg induced group compared to the control but not in the 20 µg DES induced group **Table 1** and there is no significant change in the ovary. Similar results¹⁵ were recorded but EE₂ or DES exposed to fetuses body weight was less than control also organ weight of uterus reduced but not in the ovary. DES exposure leads to a significant reduction in female body weight¹⁶, so the present results were consistent with the previous report¹⁷.

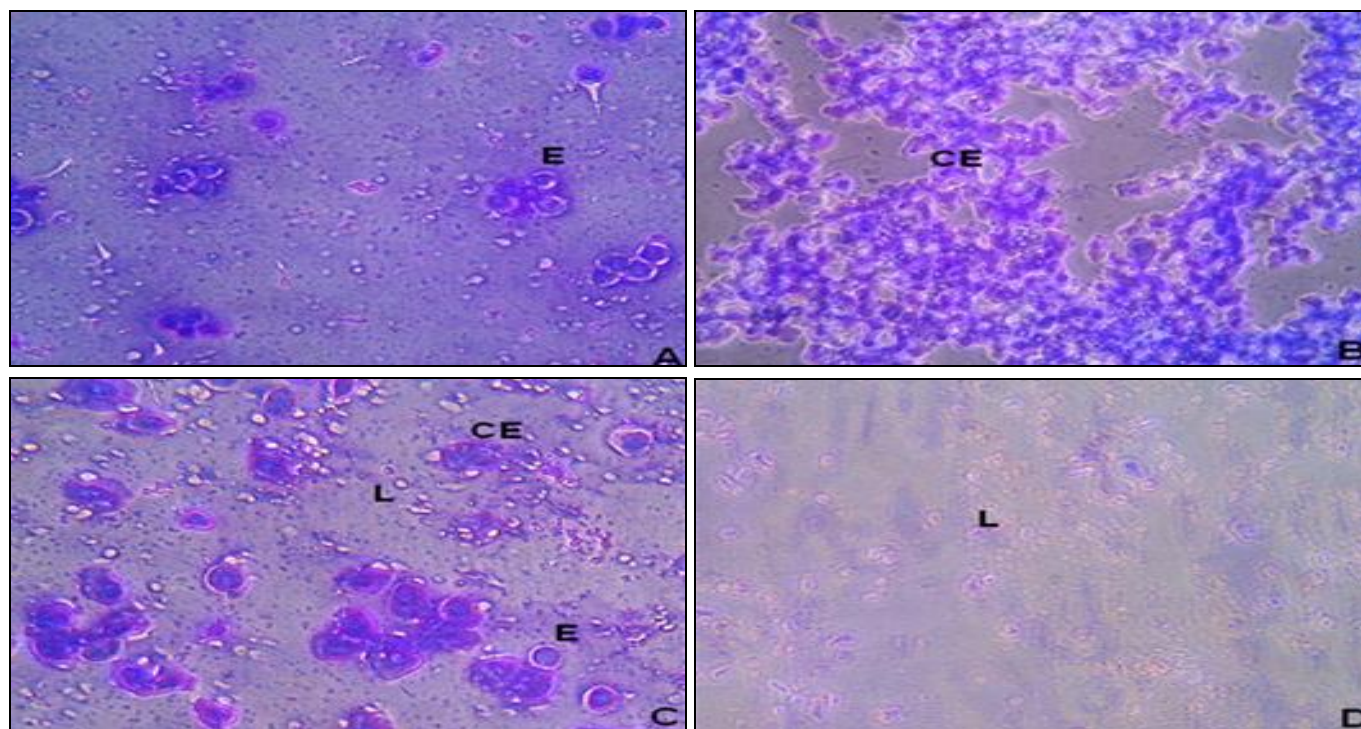
TABLE 1: DETERMINATION OF BODY WEIGHT AND ORGAN WEIGHT IN DES INDUCED MICE

Groups	Body weight in gram		Reproductive organs weight in gram at 21 st day	
	Initial	Final	Uterine Horn	Ovary
Control	30.0 ± 0.8	32.0 ± 2.6	0.28 ± 0.01	0.034 ± 0.03
Con + Vehi	31.3 ± 1.5	31.0 ± 2.0	0.28 ± 0.02	0.033 ± 0.02
Group I 10µg/kg BW	32.3 ± 3.5	31.3 ± 2.0	0.17 ± 0.01**	0.032 ± 0.01
Group II 15µg/kg BW	35.0 ± 4.0	32.6 ± 1.5	0.30 ± 0.02 *	0.033 ± 0.02
Group III 20µg/kg BW	36.6 ± 5.01	38.0 ± 6.0	0.44 ± 0.01	0.034 ± 0.03

Abbreviations: Control-Normal, Con + Vehicle- Corn oil as vehicle Control, SD- Standard Deviation; Bw- Body Weight
Values are represented as Mean ± SD of 5 Balb/C mice in each group ** significant At P<0.01, * significant at P < 0.05

Evaluation of Vaginal Cytology: The regular estrous cycle were determined by vaginal cytology, regarding at present results evaluate the DES-exposed group were showed abnormal estrous cycle compared to the control. The duration of the each stage was extended in DES induced group that is the frequency of met and diestrous stage higher in DES group compare to control **Fig. 2, Table 2.** These results were correlated with estrous cycle

stable at long time in one of the stages that is normally cycle formed by following way pro-estrous, estrous, metestrous and diestrous but in the case of DES expose group this cycle formation altered¹⁸. Similarly the dose dependent study of DES exposed can induce vaginal changes in rat to indicate the abnormal status with anovulation and excess estrogen levels¹⁹.

**FIG. 2: PHOTOCOPY OF STAINED VAGINAL CYTOLOGY IN BALB/C MICE**

Vaginal smear observed under stained resulting in A-presents of epithelial cells (E) denotes the proestrous stage B- cornified epithelial cells (CE) considered as a estrous stage C- leukocytes (l) along with CE and E denotes as metestrous D- only leukocytes presents as diestrous

TABLE 2: EVALUATION OF ESTROUS CYCLE IN CONTROL AND DES INDUCED GROUP

Groups	Shown the estrous cycle for 15 days of DES induction														
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Control	M	D	P	E	E	M	D	P	E	M	D	P	E	M	D
Con+Vehi	E	M	E	M	D	P	E	M	D	E	M	D	D	P	E
G-I 10µg	D	D	E	M	M	M	P	E	M	D	D	D	P	P	E
G-II 15µg	M	D	P	M	M	D	D	P	P	M	E	M	M	D	D
G-III 20µg	D	D	P	M	M	M	M	D	D	P	E	E	E	M	D

Vaginal cytology to determine the estrous cycle in DES induced group at 20 days regular estrous cycle in control and control vehicle group, abnormal regulation were observed in DES induced group as group I, II AND III. P-Proestrous, E-Estrous, M-Metestrous, D-Diestrous

Hormonal Analysis from Serum: Regulation of the reproductive cycle determined by the hormonal balance in the reproductive organ, the first 1 - 6 days in DES induced animals have the LH level similar to control due to the inhibition progesterone level in estrous cycle after 6 days up to 21st day the FSH level increased at the same estrogens level

increased **Fig. 3A - C**. This present results were supported by alteration of hormonal level in neonatal mice induced by diethylstilbestrol at 21st day of DES exposure²⁰. However, the estrogen receptor may be the main signalling pathway for the hormonal agonist activity of DES induced mice²¹.

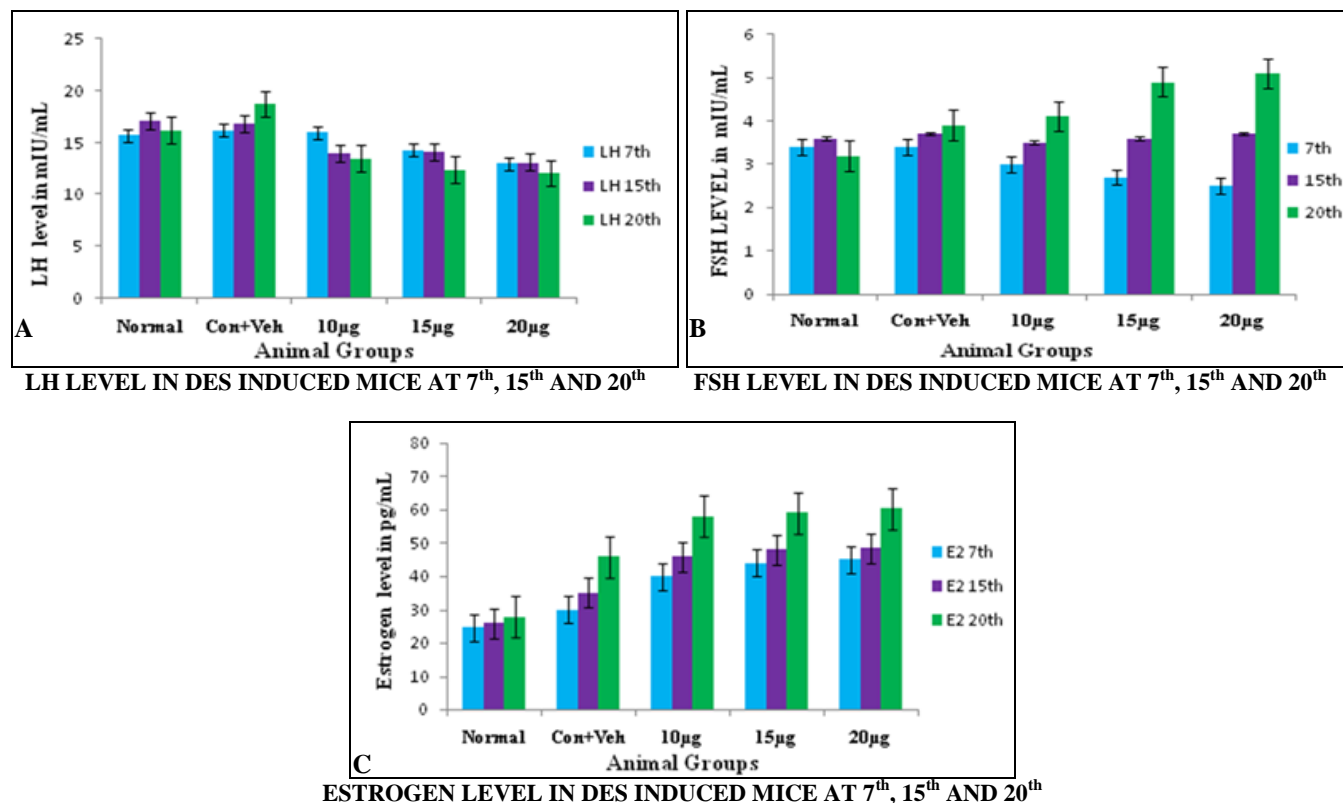


FIG. 3: HORMONAL REGULATION IN DES INDUCED GROUP AT 7th, 15th AND 20th

Estimation of hormone regulation in DES induced group at 7th, 15th and 20th day A- down regulation of leuteal hormone (LH) B- gradual increase of follicle stimulating hormone (FSH) C- estrogen (e) level was increased in des induced group

Selection of Dose Based on the Anatomical Observation: Reproductive abnormalities were observed in 10, 15 and 20 µg DES induced group on 21st day. Regarding that report shown some complex abnormalities such as constructive fallopian tube, infected fallopian tube with lesion development and tumour infected fallopian tube were observed in 10, 15 and 20 µg/kg BW of DES induction **Fig. 4A - E**. Therefore, 20 µg DES-induced group were excluded for further studies due to the tumour infection. The above results were comparable to the effect of female genital tract associated with uterus hyperplasia due to prenatal exposure of DES²² then reproductive functional changes leads to reduced fertility²³ and tubal malformation of reproductive tract shown in Diethylstilbestrol exposure group²⁴.

Analysis of Matrix Matello Protein by Gelatine Zymogram: Matrix Matello proteins extensively participate in endometrial tissue remodeling. The present result MMP-2 and 9 inhibitors-I treated group was significantly reduced the MMP level in 15 µg treated group at the same time MMP level highly expressed in endometriosis control group compared to that of Normal **Fig. 5A, B**. The above results identify the molecular weight of 72KDA protein of MMP-2 in 15 µg DES treated with MMP 2 and 9 inhibitor-I can be reduced the MMP activity than 10 µg DES treated group. The above results were concluded 15 µg DES treated with MMP is selected dose for further study and 10 µg DES treated group was excluded. The intensity of protein band was determined by histogram **Fig. 5C**.

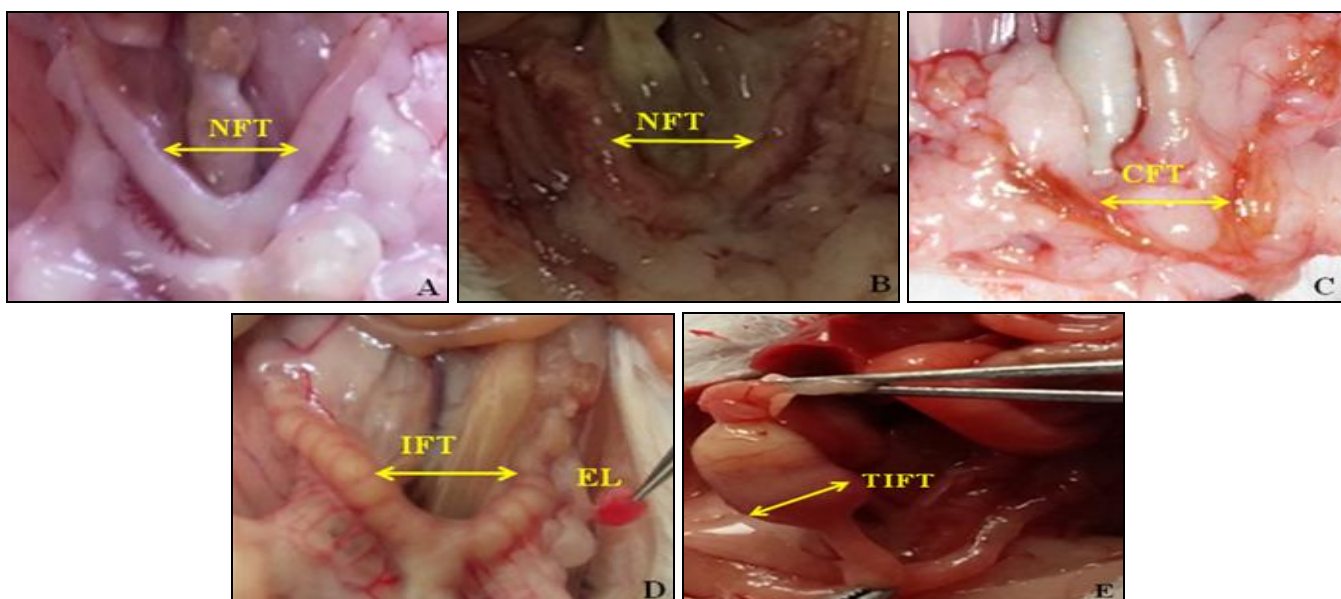
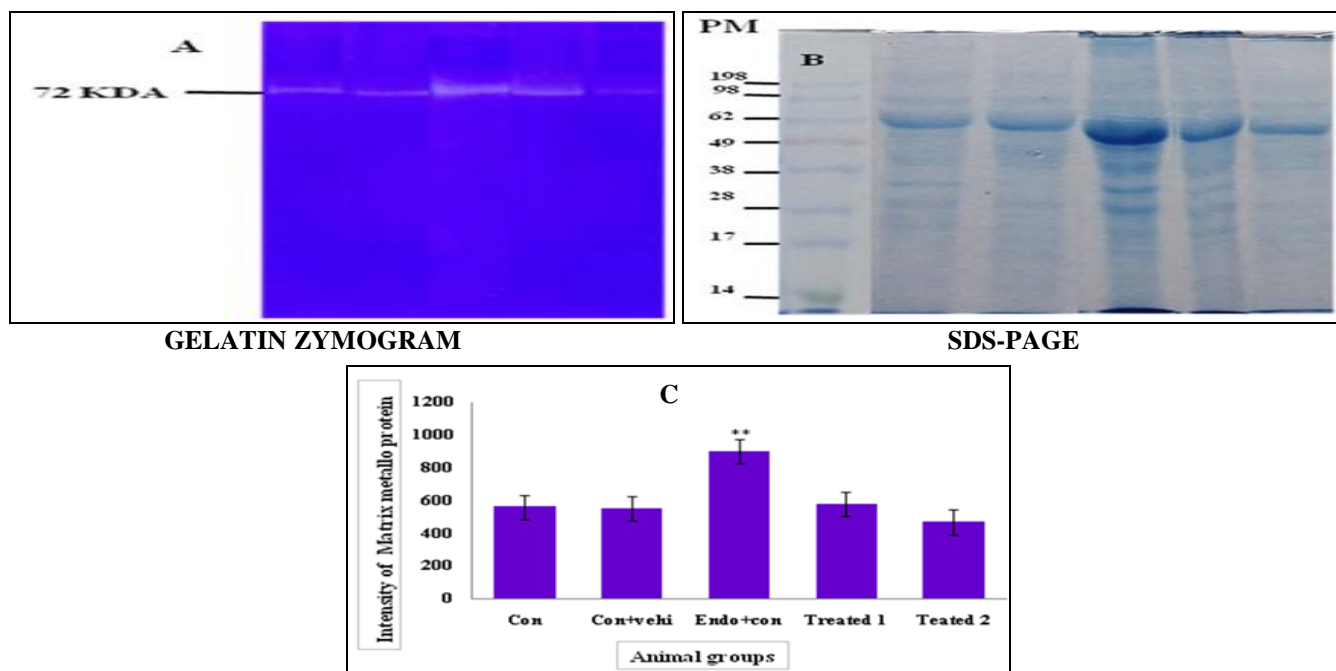


FIG. 4: ANATOMICAL OBSERVATION OF ENDOMETRIAL LESION DEVELOPMENT IN DES INDUCED GROUP
 Anatomical observation of fallopian tube A- normal fallopian tube, B- fallopian tube of control vehicle C- constricted fallopian tube (CFT) in 10µg DES D- endometrial lesion (EL) and infected fallopian tube in 15 µg DES E- tumour infected fallopian tube in 20 µg DES induced group

This present result was supported by the expression of MMP-2 significantly increased, owing to a complex level of regulation that may trigger E2 production of signal compounds which is enhance MMP-2 production²⁵. But in these results differ from those by Kaitu'u and colleagues reported that batimastat reduced the MMP activity but did not

alter endometrial breakdown in a mouse decidualization model²⁶. An estrogenic resemblance of DES can induced may reflect a difference in matrix processing induce of abnormal tissue growth on the uterus as compared to tissue regression due to progesterone withdrawal in a decidualization model²⁵.



THE INTENSITY OF MATRIX MATELLO PROTEIN BAND BY HISTOGRAM

FIG. 5: MATRIX MATELLO PROTEIN ANALYSIS BY GELATIN ZYMOGRAM

Presence of protein observed by SDS page (B) specific protein of matrix matello protein identified by zymogram (C) intensity of MMP protein band analysed by histogram. ** increased matrix matello protein in endometriosis control group.

Further gelatinase result, expressed MMP 2 but not MMP 9, this was strongly hold up following report of gelatinase A (MMP 2) acts on ECM components and may be involved in the degradation that occurs during decidualization²⁷ gelatinase B (MMP 9) also acts on similar substrates, but not expressed during decidualization *in vivo*²⁸.

Vaginal protein by SDS-PAGE Analysis: Estrous stage identified and their proteins were observed by SDS-page analysis. Among the 4 stage of estrous cycle, this study focused and observed the protein in an estrous and diestrous stage, which is coming under long phase and short phase considered as proestrous and medestrous, based on the duration of time spent in the stage of estrous cycle, it can deviate above mentioned 2 phase²⁹.

The vaginal protein of 118, 98, 62 and 49 KDA protein expressed in estrous stage and the reduction of 118, 98, 62 KDA protein was shown in diestrous stage **Fig. 6**. The contrast of above results was protein band in an estrous comparatively decreased band in diestrous stage³⁰.

Uterine Lumen by Histopathology: Histopathological analysis reveals that uterine lumen in DES induced group of endometriosis control group were destructed degenerative endometrium and reduced stromal cells were shown but in DES-induced endometriosis treated with MMP inhibitor clear uterine lumen improved endometrium related to that of normal **Fig. 7**. Results were compared to that of hyperplasia infected mice treated with inhibitors before the onset of puberty, the endometrium was entirely normal on histology³¹.

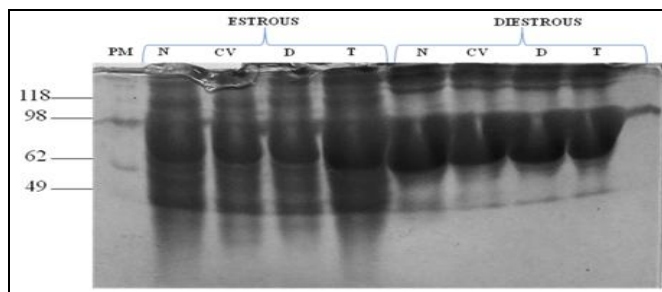


FIG. 6: SEPARATION OF VAGINAL PROTEIN BY SDS-PAGE ANALYSIS

Vaginal protein loaded in each well shown the protein level in estrous and diestrous stage of animal groups N-Normal, CV-control vehicle, D-Diseased, T-Treated

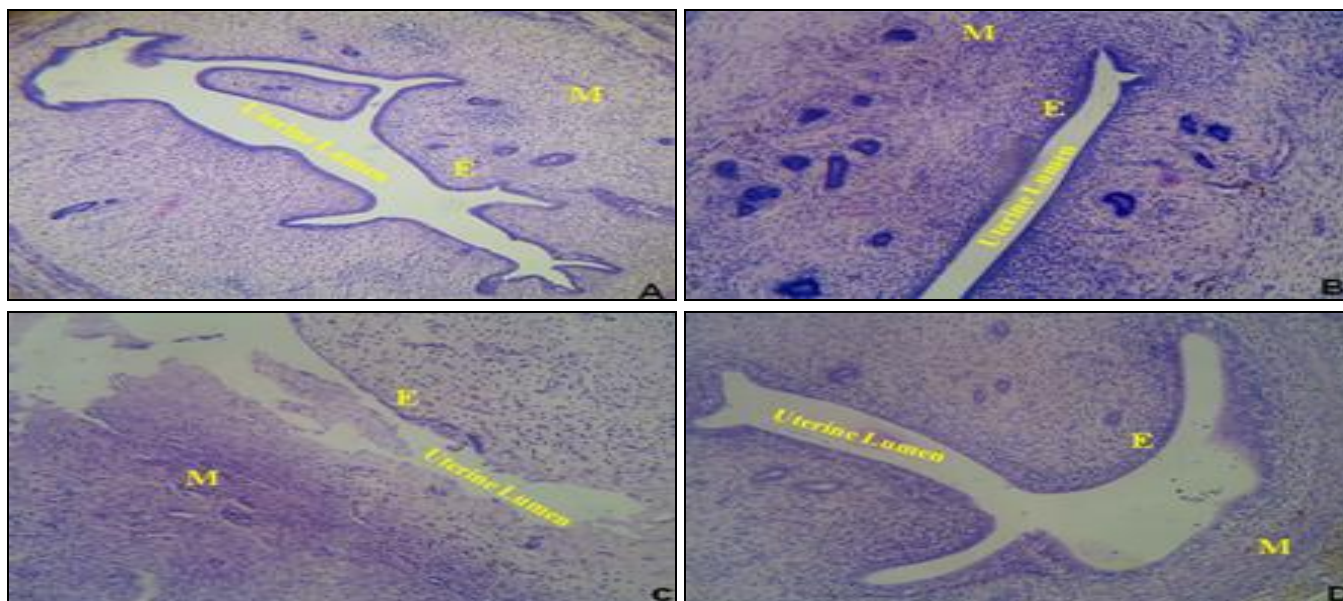


FIG. 7: A PHOTOCOPY SHOWN ARCHITECTURE EFFECT OF MICE UTERINE LUMEN

Normal uterine lumen (A) control vehicle uterine lumen (B) destructed uterine lumen in diseased group (C) MMP 2 & 9 inhibitor treated group (D) M- Myometrium E- Endometrium

Observation of Implantation: The results of implantation attaining groups such as normal, control vehicle and 15 μ g DES-treated with MMP inhibitor as attained implantation and produced young ones but not in other groups **Fig. 8**. Gelatinase A (MMP 2) mRNA has been involved in the extracellular matrix degradation by activating

the proMMP to active MMP and gelatinase B (MMP 9) mRNA detected only within the invading trophoblast cells^{32, 3, 34}, suggesting that it may be involved in tissue regression leads to trophoblast invasion. The pattern of similarities reported in mouse during pregnancy³⁵.



FIG. 8: OBSERVATION OF IMPLANTATION AND PRODUCTION OF PUPS IN ANIMAL GROUPS

Observation of implantation and production of young ones in normal (A) control vehicle (B) and MMP 2 & 9 inhibitors treated group (C) and DES induced endometriosis control group failure to produce young ones

CONCLUSION: The results of the present study indicated that among three different concentration of DES, 15 μg DES / kg / bw is the selective dose for endometrial lesion development and it treated by a 10 mM concentration of MMP 2 & 9 inhibitors-I, it may reduce the lesion development and it can initiate the implantation to produce young ones. This inhibitor selectively to target the implantation site through protease too activate proMMP to active MMP, which is degrades the extra cellular matrix degradation to prevent endometrial lesion development can recover from implantation failure.

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