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EVALUATION OF THE EFFECTS OF OXYTOCIN AND DIETHYLSTILBOESTROL ON MOUSE OESTROUS CYCLE USING AN INDEX

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ABSTRACT

The reproductive cycle depends on physiological changes which are controlled by the endocrine system and is therefore responsible for reproduction. The reproductive cycle and functions of female mammals can be altered by several drugs acting either directly or indirectly on activities of the female sex hormones. Oxytocin (OT) and Diethylstilboestrol (DES) are known to modulate the ovulation induction processes; however, their effects on cytological and morphological alterations of the reproductive organs (vagina, uterus and mammary glands) at different phases of the reproductive cycle are yet to be investigated. This study was therefore aimed at the comparative analysis of exogenously administered oxytocin at several concentrations and the simultaneous comparison with diethylstilboestrol on cytological and morphological parameters using modified methods of oestrous measurement in adult female mice. Evaluation was performed via macroscopic examination of animals as well as by microscopic examination of vaginal smears for cytological changes before and after drug administration. The treatment was carried out for 21 days at 4-5 day intervals. Diethylstilboestrol showed significant dose-related changes in both methods of measurement. DES (0.1 mg/kg) induced oestrous significantly ($p<0.05$) as observed in the increase in vaginal opening, colour, moisture, as well as abdominal distension and cornification of the epithelial cells while 0.2 mg/kg DES displayed a more significant oestrous induction ($p <0.01$) which resulted in full cornification of the epithelial cells. Oxytocin at doses of 12 IU/kg and 24 IU/kg did not induce statistically significant changes in this study. This study thus serves to update and contribute to current knowledge on the roles of oxytocin and DES on the reproductive cycle.

INTRODUCTION

The reproductive cycle constitutes physiological changes that occur in mature female mammals. These physiological changes are under the control of the endocrine system and therefore necessary for reproduction. The menstrual cycle is commonly grouped into three phases: the follicular (pre-ovulatory) phase, the ovulation phase and, the luteal (post-ovulatory) phase while the oestrous cycle on the other hand is divided into four phases: dioestrus, proestrus, oestrous, and metestrus [1-3].

Although oestrogen is involved in the regulation of the reproductive cycle also, there have been reports of the involvement of the neuropeptide, oxytocin in the regulation of the reproductive cycle[4]. Both the menstrual cycles and the oestrous cycles are stimulated by gradually increasing amounts of oestrogen in the follicular (pre-ovulatory) phase, and the lining of the uterus begins to thicken [5]. Follicles in the ovary begin developing under the influence of a complex interplay of hormones, and after which one or more become dominant (non-dominant follicles

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atrophy and die) [6]. This induces the Luteinizing Hormone (LH) surges and the dominant follicle releases the ovum or ova in an event called ovulation [7-8]. On the other hand, recent studies have shown that oxytocin is as well synthesized in the corpus luteum and endometrial cell and that oxytocin mRNA increases more than 150 times during gestation and at term[9-11], the presence of oxytocin receptor mRNA in granulose cells, before ovulation, indicate that oxytocin may be involved in follicular development[10]. There are also evidences that oxytocin has a role on the GnRH secretion, synthesis and release of luteinizing hormone (LH) and progesterone secretion [12].

Drug effect on reproductive function can manifest as a change in normal cytology and morphology of the reproductive tract or an alteration in the length of a particular phase of the oestrous cycle [13]. The cycle may be conveniently charted by examining vaginal smears. Cellular characteristics of vaginal smears reflect changes in the vaginal epithelium [14]. Available reports illustrating the normal appearance of the cells during mouse oestrous cycle have been somewhat ambiguous about distinct criteria for defining the end of one stage and the beginning of another [15-16]. These criteria are essential for consistent approach to staging of the cycle. Indeed, some workers [17-20] provide only a single description of regions of the reproductive tract at specific times daily, as typical of a particular phase.

In studies involving reproductive cycle, vaginal smear cytology has been described as one of the simplest and most reliable methods employed for the determination of stages of the cycle [14]. Vaginal smear cytology predicts the reproductive cycle according to the proportion of the three cell types observed in the vaginal smear: epithelial cells, cornified cells and leucocytes [15]. It was however considered necessary to interpret vaginal smear observation alongside physical changes in the female anatomy using an index to promote reliability of interpretation, a procedure which has been employed in this study.

This study is therefore aimed at updating and contributing to current knowledge on the roles of the neuro-peptide, oxytocin and oestrogen on the regulation of the reproductive cycle in mice.

MATERIALS AND METHODS

Animals: Adult female Swiss albino mice (25-30 g) aged 3-4 months were used. The animals were maintained at the Animal House Department of Pharmacology and Toxicology, University of Benin. Animals were handled according to standard guidelines for the care and use of laboratory animals as stated by the ethical committee, Faculty of Pharmacy, University of Benin, Nigeria as well as standard guidelines for use of laboratory animals (National Institute of Health, USA: Public Health Service Policy on Humane Care and Use of Laboratory animals, 2002). The animals were housed in plastic and stainless steel cages and were fed with mouse diet and water ad libitum.

Drugs: These include; oxytocin (Laborate, India), diethylstilboestrol (Sigma, Germany), 95% ethyl alcohol (Sigma, Germany), distilled water, gentian violet (GV).

Determination of Oestrous Cycle: The animals were acclimatized for 14 days after which the stages of the oestrous cycle for each mouse were determined daily. The daily determinations were performed in the early afternoon (12-1pm) for two weeks by

both the macroscopic (visual) method and the microscopic (vaginal smear swab) method. Both procedures were performed independently. Animals found to be pseudo-pregnant were excluded from the experiments.

The Macroscopic (Visual) Examination: A modified method of visual inspection was employed in this study [13-15]. This method utilized monitoring changes in the appearance of the genital tract, with respect to the degree of vaginal swelling (particularly the dorsal lip), the colour, and moistness of the vaginal dorsal lip. Also monitored was the degree of vaginal opening and the presence or absence of cellular debris.

Other anatomical areas also examined include the mammary glands and the degree of lower abdominal distension (which was also measured in order to estimate the possible size of the uterus).

The Microscopic (Vaginal Smear) Examination: The vaginal smear technique for determining the stages of the oestrous cycle was first studied by Stockard and Papanicolaou in 1917[13-15]. A slight modification of the technique was adopted for this procedure but it basically involved identification of cell types and their relative quantities present in the smear preparation obtained from the vagina by swabbing the vaginal walls.

Briefly, vaginal smears were collected daily via insertion of moistened cotton bud swabs. The swab tips were first moistened with distilled water and any excess was immediately removed. The mouse was held around the thorax, ventral surface uppermost while simultaneously providing lumbar support as much as possible. The tail of the animal was gripped slightly with the same hand to minimize the animal's movement. The tip of the swab stick was inserted carefully into the mouse vagina to a depth of approximately 0.1 cm, with rotating action of about 45° to the animal's body. The rotatory action was continued in the same direction until the swab stick was removed. The swab was held almost horizontally, to ensure cells from the full length of the vagina were transferred; the tip of the cotton swab was rolled gently onto a clean, pre-labelled glass slide, below the relevant animal number. The used swab stick was discarded and a new swab stick was used for each animal. Care was taken to reduce the risk of pseudo pregnancy. The smears were fixed with 95% ethyl alcohol and stained with gentian violet (GV) for 2 minutes. The GV was then gently rinsed off with water and the smears were observed under an Olympus Optical Binocular Microscope (model XS2-107BN) using x40 magnification.

Animal grouping: After complete staging of oestrous cycles of the animals were ascertained, the animals with similar oestrous cycles were allotted to the same group of 15 - 20 animals each. Two distinct phases of oestrous cycle were considered – the dioestrus and the metestrus phases corresponding to luteal phase of the reproductive cycle (since previous reports suggests low levels of oxytocin and oestrogen at these phases) [4, 14]. Each group was subdivided into three sub-groups (A, B and C) of five to six animals each. A total of six experimental groups (1A, 1B, 1C, 2A, 2B, and 2C) were created and all drugs were administered intraperitoneally i.p.

The treatment was carried out for 21 days at 4-5 day intervals and vagina smears were collected 24 hours each after drug administration. On the day of drug administration, animals on dioestrus phase

were placed in group 1 while animals on metestrus phase were placed on group 2.

- Group 1A was treated with a dose of 0.1 mg/kg DES;
- Group 1B was treated with a dose of 12 IU/kg OT.
- Group 2A was treated with a dose of 0.2 mg/kg DES.
- Group 2B was treated with a dose of 24 IU/kg OT
- To each of groups 1C and 2C was administered with normal saline and served as the negative control
- Groups 1A and 2A were used to study the effect of DES on various stages of the oestrus cycle and served as standards while groups 1B and 2B were used to study the effect of OT on various stages of the oestrus cycle.

Table 1:Scoring Macroscopic and Microscopic Parameters

% Occurrences	Cell Count within a square	Description		Score
		Microscopic (Visual)	Macroscopic (Vaginal smear)	
0	0	None	None	0
10	< 3	Very few	Light (Very low)	1
≈ 25	> 3	Few	Low (Slight)	2
≈ 50	> 5	Moderate	Moderate (Deep)	3
≥ 60	> 8	High	Prominent (Very deep)	4

Statistical Analysis: The parameters examined in the macroscopic examination were: vagina (colour, degree of opening, moisture content), mammary gland (appearance of the buds), uterus (degree of abdominal distension), and vaginal dorsal lip (degree of swelling). The parameters examined in the microscopically were the cell types– leucocytes, epithelial cells, and cornified cells found in the smear. The number of metestrus periods experienced was taken as a measure of the number of cycles completed during the observation. All the parameters examined were each ascribed a score (0-4). As shown in Table 1 which was a slight modification of the methods described in some novel works [2, 13]. An oestrous index was calculated by the addition of the scores ascribed (S_A) which are the scores attributed to each parameter examined divided by the overall total scores (S_T) which is the final score obtained upon addition of the maximum score for each parameter examined. The average of these scores was determined for each group and multiplying the result by 100. This was necessary in order to quantify the observations. These represented a progressive sequence in the intensity of changes observed within the various phases of the oestrous cycle between dioestrus and full oestrous. Statistical Analysis were

expressed as percentage of the oestrous index ± S.E.M and analysed using Student's t-test or One-way analysis of variance (one-way ANOVA) followed by Tukey's multiple range test. $P<0.05$ was considered statistically significant between compared data.

RESULTS

From the macroscopic and the microscopic methods, it was ascertained prior to drug administration that the animals had a range of 4-5 day oestrous cycles.

From the macroscopic examination, the uterus, the vagina as well as the mammary glands underwent a series of changes due to drug administration (Figure 1 and 3). The animals treated with DES (0.1 mg/kg) showed immediate responses with significant ($p<0.05$) increases in oestrous index for vaginal opening (83.3% ± 3.45%), colour (62.5% ± 2.98%), mammary gland (55% ± 2.54%), abdominal distension (83.2% ± 3.43%). while, the OT (12 IU/kg) treated group and the negative control group did not show any significant alteration in the morphology of the reproductive organs 24h after drug administration (Table 2; Figure 1).

Four types of cells were distinguished in the vaginal smears obtained, namely: leucocytes, epithelial cells, cornified cells, and cells intermediate in type between the epithelial and the cornified cells (Figure 5 and 6).

OT treated group and the negative control group followed the normal phases from dioestrus to proestrus (Figure 1 and 2). On the day of drug administration leucocytes and few epithelial cells were present but 24h after only epithelial cells were present. The oestrous index for epithelial cells was 75.5% ± 2.86% for OT (12 IU/kg) and 70% ± 2.67% saline control. With increased dose of OT to 24 IU/kg, there were no significant differences in oestrous index and cornification of epithelium between the negative control group and the experimental group (Figure 3 and 4). However, the DES (0.2 mg/kg) treated group showed significant increase of oestrous index ($p<0.05$) in experimental group when compared with the control (Table 2)

Smears taken from the animals treated with DES showed progressive changes in the cellular contents of the vagina as the intensity of oestrous reaction increased (Tables 2; Figure1-4) with 0.1 mg/kg DES produced sufficient cornification (Figure 2) and 0.2 mg/kg DES resulted in full cornification showing significant increase in the occurrence of oestrous of the animals in a dose-dependent manner (Figure 4). Significant increase in % oestrous index of approxiamtely 70% and 80% were observed with 0.1mg/kg and 0.2 mg/kg respectively when compared with the control.

DISCUSSION

The data presented here showed that both methods of assessment yielded essentially the same result (Table 2).

The oestrous index therefore provides a practicable method for estimating the intensity of oestrous changes (cytological and morphological), provided that the effect is not lower than ¾ of the maximum scores [12, 13-16].

Both the vaginal and uterine epithelial are influenced by hormonal changes and other activities during the oestrous cycle and in normal female mammals, the morphologic, cytologic, endocrine and secretory changes occurring in the vagina and uterus during oestrous cycle usually depict the stage of the cycle. In general appearance, the uterus and

the mammary gland like the vagina undergoes a series of anabolic and catabolic changes during oestrous cycle [3-5].

The result presented here showed that there was no significant oxytogenic effects on the cytologic as well as morphologic alterations of the reproductive organs at different phases of the reproductive cycle although, recent studies suggest that oxytocin may be involved in follicular development [21-24], possibly through regulation of GnRH secretion, synthesis and release of LH and progesterone secretion. Results from our study agree that a single injection of high dose OT had no effect on cycle length [25]. However, some authors reported that 10 days post slow-release injection of 10 I.U OT to Marino ewes increased ovulation rates; this has been suggested to occur through an indirect action on the hypothalamus [22]. This present study using oestrous index suggests that OT injection may have no immediate significant effect on the length of the oestrous cycle, supporting reports of either a gradual involvement by OT or a minor role of OT in the regulation of the reproductive cycle [20-21]. Notwithstanding, the possibility of OT shortening of the reproductive cycle still remains a possibility especially with the seeming irregular effects on the appearance of cornified cells.

These findings support the report that the vaginal epithelium is influenced by hormonal changes during the oestrous cycle [3, 6]. These changes have been associated with levels of steroid sex hormones (oestrogen and progesterone) [26-28]. These reports would be true for the observations that full vaginal cornification depend on circulating oestrogen levels [6]. This work suggests a possible direct action of exogenously administered oestrogen on the vaginal epithelium.

The gross changes observed in the appearance of the female reproductive canal during the oestrous cycle (Figure 1 & 3) were first mentioned in classic studies of the oestrous cycle of mouse [2, 15, 27]. They observed that in the absence of infections, the circulating levels of progesterone and oestradiol-17[] are the major determinants of the cytology pattern of the vagina. However it has been suggested that rising levels of peripheral oestrogen in the adult female goat causes thickening of the vaginal wall [28].

The present findings also agrees with a number of studies which have shown that intact mouse reproductive organs are highly responsive to oestrogen [27, 29]. Increasing the dose of DES elevated the oestrogen surge through activation of oestrogen receptors ($ER_{\square}$, and$ER_{\square}$). These in turn stimulate (through a positive feedback control on the hypothalamus) a large, pre-ovulatory surge of GnRH, FSH, and LH[30]. The pre-ovulatory surge of FSH stimulates more rapid growth of the follicle and more oestrogen secretion which is necessary to induce ovulation (priming of the pituitary)[29-30].

Concluding Statements

This investigation has shown correlations between cytological and morphological changes as direct consequences of changes in the reproductive cycle. This investigation has also shown the effect of DES on cytological changes at specific concentrations, and therefore makes it a useful tool in reproductive technologies and assays. This investigation has also shown that OT has no immediate stimulatory effect on ovulation and endometrial cornification. Additionally, this study has also shown specific cytological and morphological changes associated with specific doses of DES, an important requirement in some reproductive technologies which include

Table 2: Comparative occurrence of oestrous state with low dose of drugs as determined by the visual appearance of the vagina (Macroscopic examination) and by vaginal smear (Microscopic examination) n=6 mice.

GROUPS	DOSE	%OESTROUS INDEX ± SE	
		MACROSCOPIC	MICROSCOPIC
Control	-	22.5 ± 2.21	21.8 ± 3.25
OT	12 IU/kg	23.2 ± 2.43	20.4 ± 2.40
	24 IU/kg	24.2 ± 2.44	23.4 ± 1.92
DES	0.1 mg/kg	62.5 ± 2.21	75.4 ± 2.30
	0.2 mg/kg	80.4 ± 2.25	82.6 ± 2.74

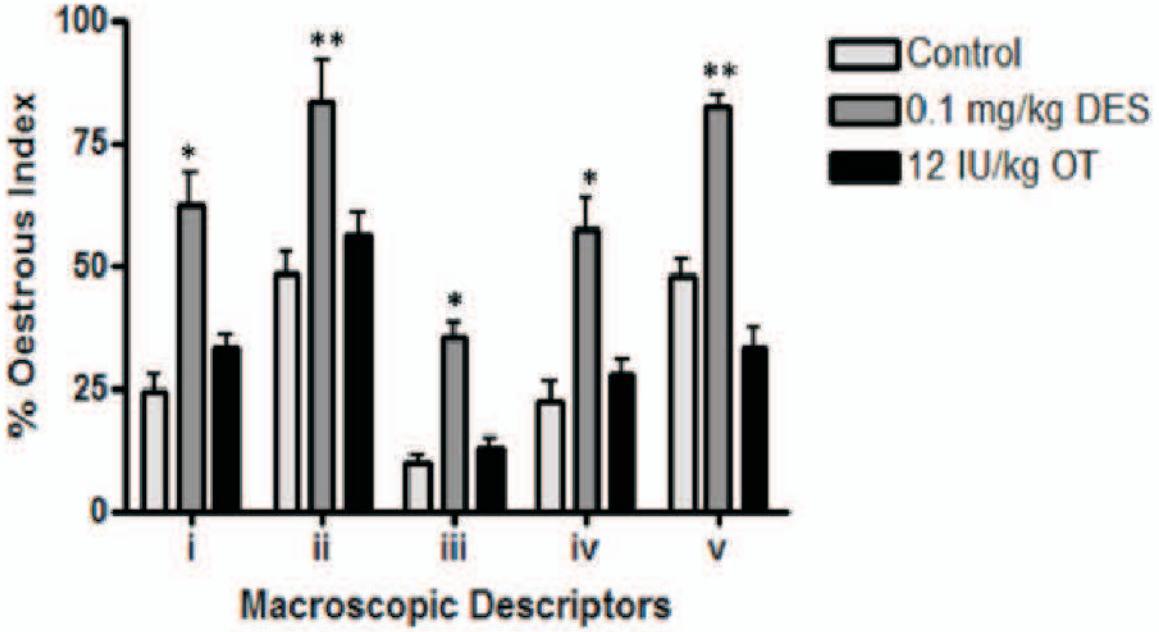

Figure 1: The effects of DES and OT on dioestrus phase of oestrus cycle 24h after drug administration (Macroscopic examination). DES significantly increased the occurrence of oestrous in animals. i = vulva colour; ii = vulva opening; iii = moisture; iv = mammary gland distension; v = abdominal distension. $*p < 0.05$ $**p < 0.05$ compared to the control; n = 6 mice.

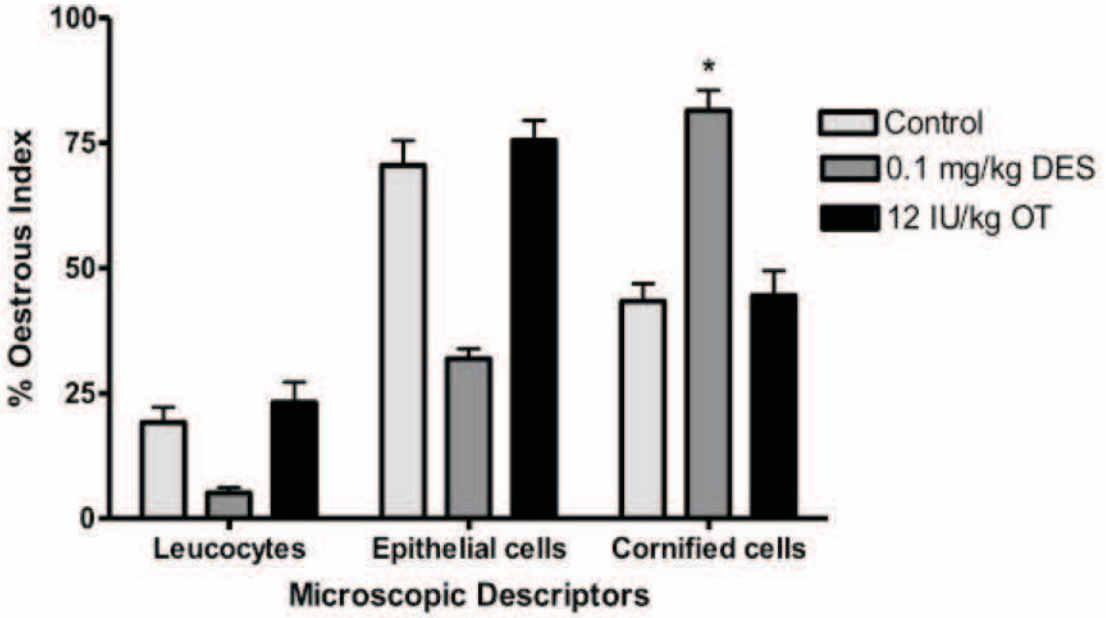

Figure 2: The effects of DES and OT on Dioestrus phase of oestrus cycle 24h after drug administration (Microscopic examination). DES significantly increased the occurrence of oestrous in animals. *P<0.05 compared to the control, n=6 mice.

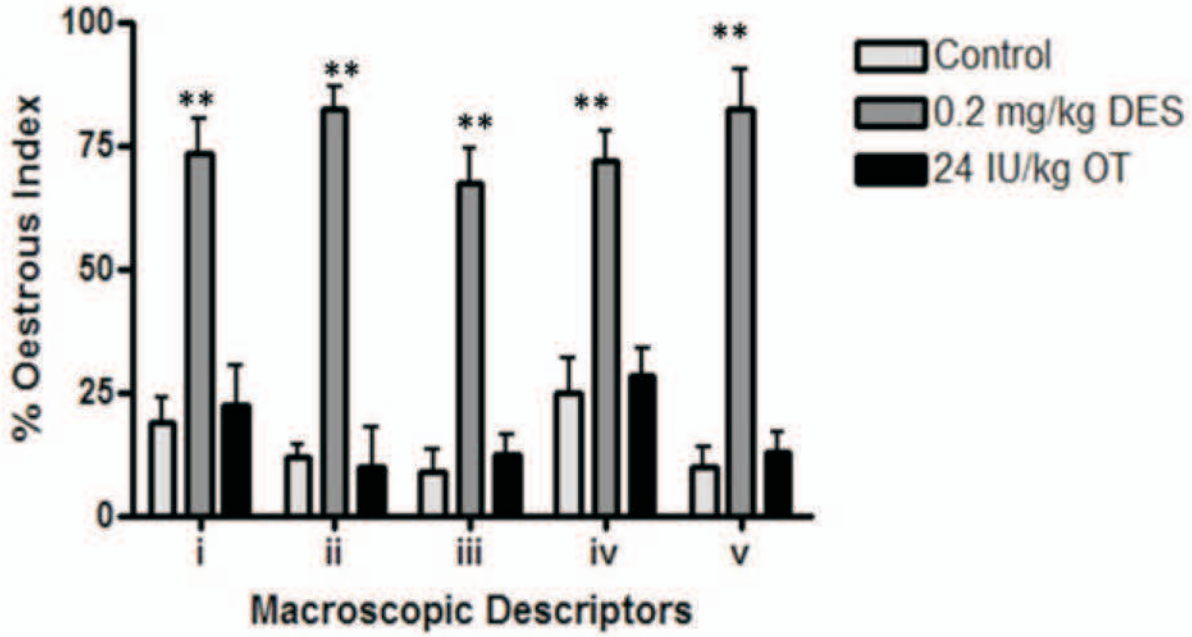

Figure 3: The effects of double dose of DES and OT on Metestrus phase of oestrus cycle 24h after drug administration (Macroscopic examination). DES significantly increased the occurrence of oestrous in animals. i = vulva colour; ii = vulva opening; iii = moisture; iv = mammary gland distension; v = abdominal distension ** $p<0.01$ compared to the control, n=6 mice.

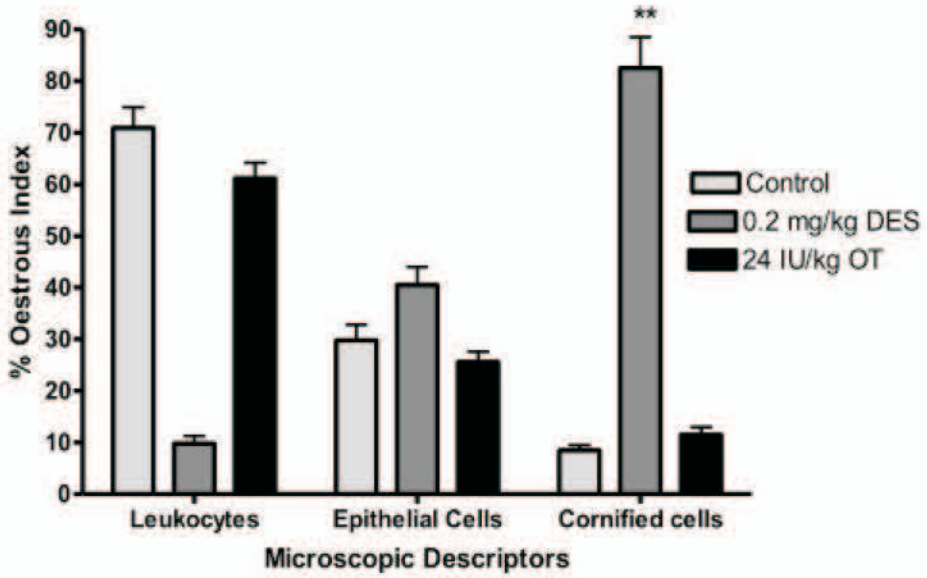

Figure 4: The effects of double dose of DES and OT on Metestrus phase of oestrus cycle 24h after drug administration (Microscopic examination). DES significantly increased the occurrence of oestrous in animals. ** $P < 0.05$ compared to the control, n=6.

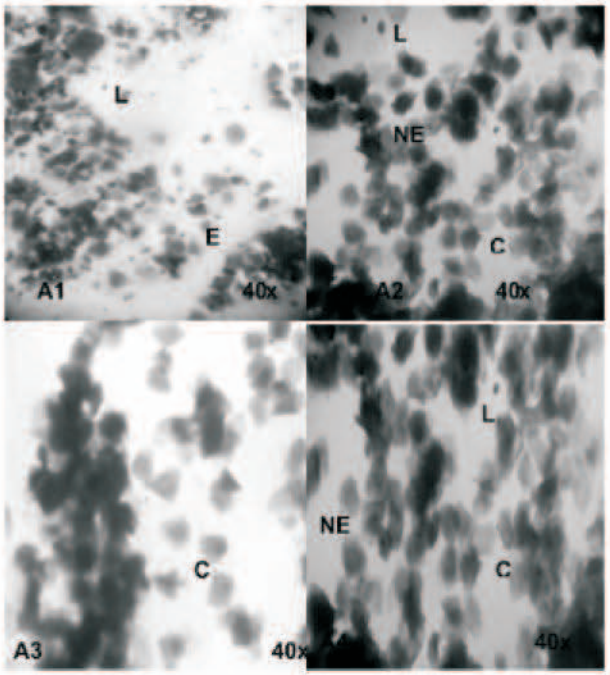

Figure 5: Representative of vaginal smears taken before and 24 h after administration (40x); Sections reveal gross changes in cytology. All drugs were administered at point **A1**, dioestrus phase; **A2**, OT (12 IU/kg) administration turned dioestrus to proestrus phase after 24 h; **A3**, DES (0.1 mg/kg) administstion turned dioestrus to oestrous phase after 24 h; **A4**, 0.03 ml normal saline turned dioestrus to proestrus phase after 24 h **NE**, Nucleated Epithelial cell; **C**, Cornified cell; **L**, Leucocyte; **E**, Epithelial cell.

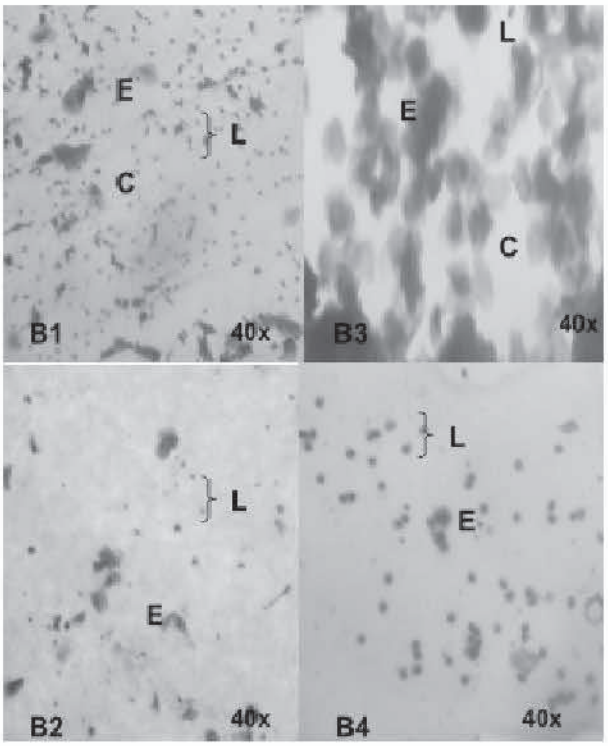

Figure 6: Representative of vaginal smears taken before and 24 h after drug administration (40x); Sections reveal gross changes in cytology. All drugs were administered at point **B1**, metestrus phase; **B2**, OT (24 IU/kg) administration turned metestrus to dioestrus phase after 24 h; **B3**, 0.2 mg/kg DES administstion turned dioestrus to oestrous phase after 24 h; **B4**, 0.03 ml normal saline turned metestrus to dioestrus phase after 24 h; **E**, Epithelial cells; **C**, Cornified cell; **L**, Leucocytes

ovulation induction and acceleration of endometrial maturation.

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