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Short Communication

PHASE DEPENDENT DISCREPANCY IN MURINE VAGINAL MICRO-ENVIRONMENT: A CORRELATIVE ANALYSIS OF pH, GLYCOGEN AND SERUM ESTROGEN UPON EXPOSURE TO LAPATINIB DITOSYLATE

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ABSTRACT

Objective: This present investigation was designed to correlate the Hydrogen-ion concentration of vaginal fluid with glycogen content and serum estrogen level during different phases of mice reproductive cycle. These parameters were compared with the control and treated animals to set off this factor in the field of reproductive toxicological studies.

Methods: Female Swiss Albino mice [6-8 w] with regular estrous cyclicity were separated out into two groups as control [only HPMC] and treated [lapatinib in HPMC] animals. The treatment was carried out consecutively for 21 d via oral gavage at a dose of 20 mg/kg/d. Estrous cycle, pH and glycogen content of vaginal fluid was monitored on daily basis. At the end of the experiment period, serum estrogen level was quantified by ELISA method.

Results: Significant changes were observed in diestrus index, estrogen, and glycogen content with respect to their reproductive phases. In particular, the higher variations, seen within and between groups in estrous phase, suggest the maximum inhibitory potential exhibited by the drug used, was due to the elevated expression of its receptors [EGFR] at the time of oocyte maturation and ovulation.

Conclusion: Even, a change by one pH unit may illustrate the difference between healthy and heavily infected vaginal flora. Although, the pH value of vaginal secretions is not routinely used and compared with other parameters in the toxicological studies. This present study has become the trend set for considering pH as a valuable tool in *in vivo* studies.

Keywords: Estrous cycle, Vaginal pH, Phenol red, Glycogen, Estrogen, Correlation analysis

The vaginal tissue has been greatly used for demonstrating the estrus cycle of mice, rat, and even bovine species. This tissue is tubular in structure with an outer layer of loose connective tissue, a middle, smooth muscle, and an inner mucosal layer. In proestrus stage of mice/rat, all the four layers of the vaginal epithelium: the outermost is the *stratum mucification* [SM], followed by *stratum corneum* [SC], *stratum granulosum* [SG], and *stratum germinativum* [SGerm] are perceptible [1]. Each layer of cells was shed during every phase of mice estrus cycle. The most characteristic and consistent morphological alterations of vaginal tissue with respect to estrus cycle was determined using vaginal cytology [2]. Vaginal smears have repeatedly been taken by many ways without sacrificing the animal. Thus, many published literature adapt this technique.

Relatively, only limited number of studies on the pH values of vaginal environment and its secretions in rodent species exist. Variations in the pH value of certain tissue environment or body fluids may influence the physiological and/or pathological functions [3]. During the menstrual cycle, the vaginal pH varies across pre-ovulatory [follicular phase], ovulatory, and post-ovulatory period [luteal phase]. Endocrine functions such as the involvement of hormones affect the sexual cycle and vaginal pH of an organism undoubtedly [4]. Therefore, several vaginal parameters and its biological roles are still in evident.

Potential of hydrogen ion [pH] was measured by various methods such as usage of electrodes, pH indicator strips, and chemical indicators. Commercial ready-to-use pH indicator rolls or solutions are available with standard color-coded pH key. Chemical indicators like phenol red, methyl orange, cresol red, thymol blue, phenolphthalein, etc., have been in the market for years. In particular, phenol red is extensively used in cell culture medium. In 2000, scientists from Uganda, come up with a 'swab technique' for assessing the vaginal pH in a woman using a four-inch pH strip attached to a pediatric depressor to give observable color change in the acidic environment [5].

The optimum condition, existing in the vaginal environment, has been altered due to external and/or internal factors. Vulvovaginitis is a classic example that occurs during or after chemotherapeutic treatment showing a symptom of loss of ovarian function, which leads to a decrease in vaginal epithelium glycogen content and an increase in the vaginal pH [6]. Hence, the present investigation was aimed to establish a relationship between the three parameters [vaginal pH, glycogen content, and serum estrogen] across different phases of estrus cycle and to compare these multi-factorials with that of lapatinib-treated animals.

To unresolve these objectives, lapatinib ditosylate [>99% purity] was purchased from Abmole Bioscience Inc. [Houston, USA] and stored at-20 °C until use. Female Swiss albino mice exhibited 4–5 d estrus cycle was segregated into two groups and designated one as vehicle control (group 1) and the other as treatment group (group 2). Group 1 mice received only HMPC in tween 80, whereas group 2 mice were orally gavaged with lapatinib [prepared in 0.5% HPMC in 0.1% tween80] at a dose of 20 mg/kg/d for 21 consecutive days. All procedures performed were in accordance with the Institutional Animal Ethical Committee [IAEC] approval [BDU/IAEC/2015/NE/30/Dt. 17.03.2015]. Vaginal cells were collected twice a day in 10 µl of 0.05 M phosphate buffer for the determination of diestrus index from day 0 to 21 [7].

The mouse vaginal pH was determined by adopting two methods: one with pH indicator strips at a range of 6.5–10 & 4–7 [Merck, Darmstadt, Germany] and the other by using 25 µM aqueous solutions of phenol red dye. Briefly, the pH strip was cut into small pieces with reduced diameter that was inserted into the mice vagina [8]. The latter method involved rinsing the mouse vagina internally with 10 µl of phenol red solution and the chromo solution thus obtained was spotted in a petri dish. The remnant phenol red in the

vaginal orifice was immediately swiped off with tissue paper. This procedure was carried out throughout the study period in both the experimental groups. Confounding spots, obtained from the previous phenol red experiment, was confirmed by analyzing the maximal spectral absorbance [λ_{max}] using UV-visible spectrophotometer [Synergy HT multimode reader, BioTek Instruments, Inc., Winooski, Vermont, USA]. The maximum absorbance spectrum of phenol red flushed into the mice vagina was analyzed at a wavelength ranged from 400 to 700 nm. The resultant peak and the absorbance per sample were correlated with the control graphs for determining the acidic/basic nature of the vaginal sample.

Glycogen content of vaginal flushing was measured by adopting the previous method [9] using 96-well microtitre plate. The final reddish-yellow colored complex was measured at 460 nm, which was extrapolated using the standard curve equation and was expressed in µg of glycogen/swab. At the end of the experiment, serum estrogen level was quantified using a competitive Pathozyme® oestradiol EIA kit [Omega Diagnostics Ltd., Scotland, United Kingdom]. All quantitative data were expressed in mean±SD and a *P* value<0.05 was considered to be significant. Correlation of estrogen with pH values, glycogen with pH values and estrogen with glycogen levels were statistically measured by Pearson's R value.

Vaginal tissue and its secretion have a significant contribution to successful fertilization to occur. There exists a prominent association between vaginal pH and reproductive lifecycle of humans [10]. Several other biochemical parameters in that microenvironment and the tissue itself have been prone to many exogenous chemicals. Chemotherapy given to younger age group women are highly vulnerable to those changes that happen in their reproductive tract. To emphasise this issue, mice fed with lapatinib, an oral drug prescribed for metastatic breast cancer, was considered as the treatment group. Unlike the human menstrual cycle, rodent's estrus cycle is primarily comprised of proestrus, estrus, and diestrus characterized by the presence of nucleated epithelial cells [fig. 1A], cornified cells [fig. 1B], and polymorphonuclear cells [PMCs; fig. 1D] respectively [8]. Metestrus is considered as the transition period between estrus and diestrus phases. Hence, this phase has all the three cell types in the vaginal smear [fig. 1C].

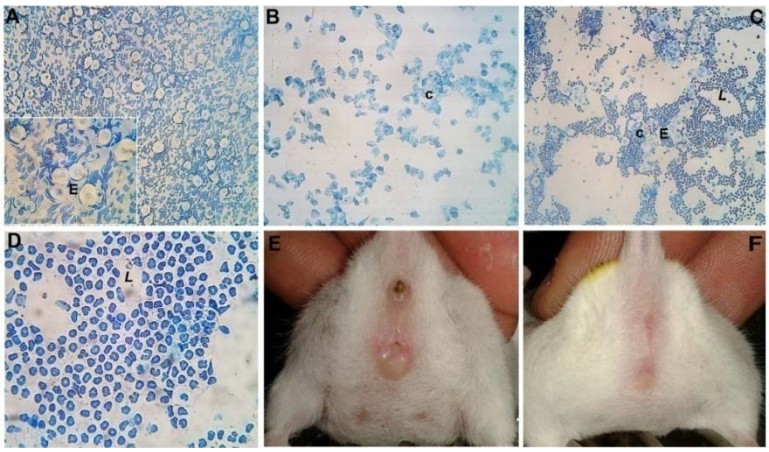

Fig. 1: Physiological and cytological characterization of different phases of mouse estrus cycle

The time of estrus phase in the murine reproductive cycle has been easily identified by the appearance of the vaginal orifice as it is the external sign for pubertal onset. This postnatal apoptotic tissue remodeling at the distal end of vaginal cavity occurs at regular intervals of estrus phase, which indicates the animal's receptive period to male [11]. In addition, the vagina orifice swells appeared pinkish in color and found to be opened at the time of estrus [fig. 1E] but not at all other stages [fig. 1F].

Mice exhibiting prolonged PMCs or cornification of cells in vaginal smears was classified as anestrus [12] which was evidenced with the diestrus index of 61 in the group 2; whereas, it was 48.38 in group 1 animals. This was followed by the onset of acyclicity of estrous cycle in treated animals with extended overall duration of the cycle.

Any such alterations observed in estrus cycle reflect the change in the functional integrity of the hypothalamic–pituitary–gonadal axis [13]. The results depicted in table 1 indicates that the magnitude of estrogen in mice serum varied in the order of estrus> proestrus>diestrus. The maximal concentrations of this steroid hormone in individual animals varied between 51.50±0.71 and 44.98±0.99 pg/ml. A significant change [p<0.05] was observed in estrus phase of mice between control and treated groups. But, such significant difference was not seen in either of the two phases.

Table 1: Comparison of biochemical parameters of mice vaginal micro-environment

Estrous cycle	pH		Estrogen [pg/ml]		Glycogen [µg/swab]	
	Control	Treated	Control	Treated	Control	Treated
Proestrus	5.57±0.16	5.64±0.20	40.85±0.95	37.17±0.71	4.48±0.34	3.90±0.80
Estrus	4.53±0.12	4.70±0.14	51.50±0.71*	44.98±0.99**	12.40±0.70*	8.55±0.15**
Diestrus	7.52±0.16	7.39±0.21	18.00±0.36	18.35±0.07	2.71±0.25	3.39±0.49

Where, * denotes significant difference between phases of estrous cycle and ** denotes *P*<0.05 between the two groups.

Steroidal hormone fluctuations are having an indirect effect on vaginal pH in mammalian species [14, 15] because it creates an optimum room for the growth of various microorganisms [16]. In this present study, vaginal pH was well correlated with the different phases of estrous cycle [fig. 3]. The overall pH range in vaginal microenvironment across the mice estrous cycle was between 5.57±0.15 and 7.50±0.10. Accordingly, the pH of vaginal secretions varied with the reproductive phases of mice. In control mice, the values were 5.57±0.16, 4.53±0.12 and 7.52±0.16 for proestrus, estrus and diestrus respectively. The acidic pH of mice vaginal fluid as denoted in fig. 2[E] was also reported in 2013 [17], which further strengthen the present finding. In women, this acidic environment [≤

5] was validated by the anaerobic degradation of sugars by lactobacilli that results in the lactic acid production [18]. Very few articles reported that the vaginal pH of female mice varied between 6.5 and 8.0 [8] irrespective of their phases.

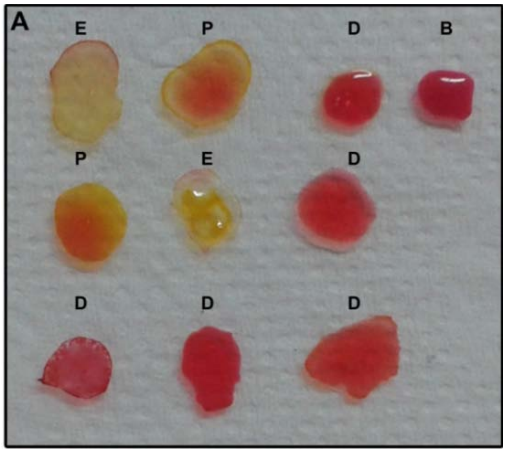

Fig. 2: Identification of each phases of estrous cycle using phenol red dye

No significant changes in the vaginal pH were observed between control and treated animals throughout the three phases of the estrous cycle. Even though, on the 12th d, prolonged diestrus phase in treated group animals was evidenced with the vaginal pH as compared with the control mice [fig. 3]. Results obtained using phenol red solution was confirmed by the pH indicator strips. Furthermore, the absorption maximum [λ_{max}] at 440 nm and 560 nm was validated as acidic and basic pH by comparing it with known pH spectrums. Phenol red used in this protocol has found to be less estrogenic in nature [19] and was used for assessing the normal renal function in humans [20].

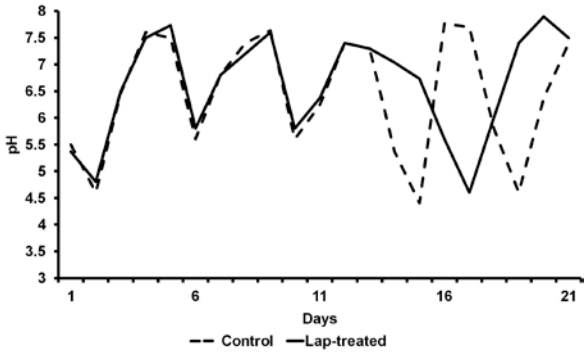

Fig. 3: Comparative illustration of mouse vaginal pH throughout estrous cycle

As mentioned earlier, the types of organisms that are colonizing the vaginal flora fully depends on two factors: pH and the availability of glucose. Glycogen, synthesized by the epithelial cells under the influence of estrogen, is crucial for the establishment of pH at the respective phases of the reproductive cycle. To view in detail, table 1 clearly depicts the glycogen concentration in the vaginal flushing of control and treated animals. In animals exhibited normal estrous cycles, glycogen in vaginal secretions differ significantly [P<0.001] between the ovulatory and preparative stages. During estrus period, group 2 animals had decreased glycogen content [8.55±0.15] compared to that of control mice [12.40±0.70]. At the end of 21st d, the vaginal glycogen was significantly less [P<0.01] in group 2 mice than the animals showed a regular estrous cycle.

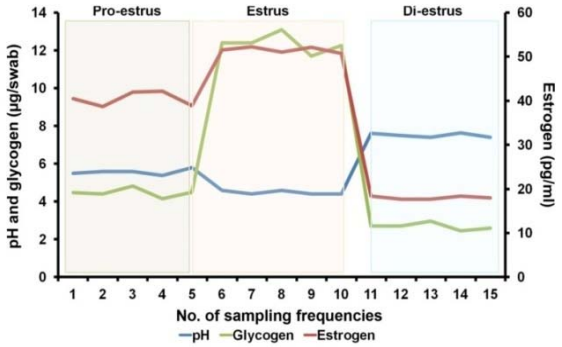

Fig. 4: Correlation analysis of vaginal H-ion concentration, glycogen, and serum estrogen level

Differences in the individual parameters were not observable, every so often, in control as well as treated animals. In the present study, a correlative analysis between the parameters was calculated to elucidate the variations among different groups. Fig. 4 represents the overall relationship existed between these three factors in a control group of animals. There was a significant positive relationship found between the concentration of estrogen and glycogen; r [df=3] = 0.99, P<0.05, whereas. it was negative in the case of pH with estrogen [r=-0.996], and pH with glycogen [r=-0.865]. Unlike in humans, the amount of glycogen synthesized and glycogen-synthesizing enzymes are directly proportional to the release of estrogen in rat [21] and hamster [22]. There are few studies, which concluded that the amount of glycogen-containing cells [glycogen-index] has been applied for evaluating the signs of estrogenic activity [23, 24]. This kind of correlation was disrupted in animals treated with lapatinib. It clearly indicated that these parameters may occupy a prime position in the field of toxicology especially in studying the vaginal tissue.

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CONFLICTS OF INTERESTS

All authors have none to declare

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