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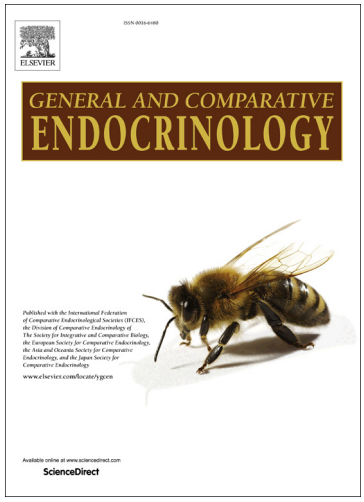

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Expression of ODC1, SPD, SPM and AZIN1 in the hypothalamus, ovary and uterus during rat estrous cycle

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Running title: Expression of ODC1, SPD, SPM and AZIN1 in rat

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Abstract: The aim of the present study was to investigate variation in the expression pattern of ornithine decarboxylase (ODC1), spermine (SPM), spermidine (SPD) and antizyme inhibitor (AZIN1) in hypothalamus, ovary and uterus during the estrous cycle of rats. Further, to understand any correlation between polyamines and GnRH I expression in hypothalamus; effect of putrescine treatment on GnRH I expression in hypothalamus and progesterone and estradiol levels in serum were investigated. The study also aims in quantifying all the immunohistochemistry images obtained based on pixel counting algorithm to yield the relative pixel count. This algorithm uses a red green blue (RGB) colour thresholding approach to quantify the intensity of the chromogen present. The result of the present study demonstrates almost similar expression pattern of polyamine and polyamine related factors, ODC1, SPD, SPM and AZIN1, with that of hypothalamic GnRH I, all of which mainly localized in the medial preoptic area (MPA) of the hypothalamus, during the proestrus, estrus and diestrus. This suggest that hypothalamic GnRH I expression is under regulation of polyamines. The study showed significant increase in hypothalamic GnRH I expression for both the doses of putrescine treatment to adult female rats. Further, it was shown that in ovary expression pattern of ODC1, SPM, SPD and AZIN1 were similar with that of steroidogenic factor, StAR during the estrous cycle, and putrescine supplementation increased significantly estradiol and progesterone levels in serum, all suggesting ovarian polyamines are involved in regulation of ovarian steroidogenesis. Localization of these factors in the theca and granulosa cells suggest involvement of polyamines in the process of folliculogenesis and luteinization; and ODC1, SPD, SPM and AZIN1 in oocyte further suggests polyamine role in maintenance of oocyte physiology. Finally, in uterus SPM and AZIN1 were localized throughout the estrous cycle, being comparatively more during the metestrus phase. There was intense immunostaining of SPD in the luminal and glandular epithelium during the metestrus and diestrus phases of the estrous cycle suggesting these all the three polyamines as such play important role in regulation of uterine physiology.

Keywords: polyamine, ovary, hypothalamus, uterus

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Introduction:

Estrous cycle, the reproductive cycle of adult female rodents, is characterized to have the following phases: proestrus, estrus, metestrus and diestrus (Long and Evans, 1922; Marcondes, 1988). During different phases of the estrous cycle there are distinct morphological, hormonal and biochemical changes occurring in the ovaries, uterus and in the hypothalamus (Goldman et al., 2007). A key regulator of the estrous cycle in mammal is the gonadotropin releasing hormone (GnRH) that mainly acts through the hypothalamo-hypophyseal axis. Hypothalamic GnRH neurons are regarded as the final common pathway in the brain in the control of reproductive function. In this context, various investigators attempted to demonstrate the changes in GnRH gene expression in female rats during estrous cycle (Park et al., 1992; Marks et al., 1993 Gore and Roberts, 1995), however these results were controversial. A study on adult female rats during estrous cycle disclosed a significant increase in GnRH gene expression during proestrus and estrus phases also suggesting this increase is estrogen dependent (Suzuki et al., 1995). GnRH has an intraovarian regulatory function which had been well documented (Leung et al., 2003; Metallinou et al., 2007). Further this is supported by the observation on direct effects on steroidogenesis (Andreu et al., 2001; Jones and Hsueh 1981), folliculogenesis (Srivastava et al., 1995), and meiotic maturation of the oocyte (Hillensjo and LeMaire 1980). Three different forms of GnRH exist, as evident from molecular phylogeny, GnRH I, GnRH II and GnRH III respectively; most vertebrates have the GnRH I form (Chen and Fernald, 2008). Recently, a study showed immunolocalization of GnRH I in mice ovary during the estrous cycle. The study showed relative abundance of GnRH I in the ovary during proestrus and estrus suggesting a role of ovarian GnRH I in regulation of ovarian steroidogenesis (Singh et al., 2011). Studies of novel factors which undergo variation in expression in the hypothalamus/ other reproductive tissues like ovary and uterus during the estrous cycle would widen the scope of better understanding the significance of these factors in regulation of reproductive physiology of the female rodents.

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Polyamines, a class of low molecular weight aliphatic compound having an amino group present ubiquitously in living organisms have been shown to widely regulate various physiological processes including reproduction (Lefevre et al., 2011). In mammals, there are four types of polyamines present, putrescine, spermine, spermidine (Tabor and Tabor, 1964; Lefevre et al., 2011; Hussain et al., 2016) and agmatine was described in mammalian brain (Li et al., 1994, Raasch et al., 1995). Spermine and spermidine were initially found in human semen as a volatile compound responsible for the odour in semen, putrescine can result from the putrefying flesh of cadavers (Kusano et al., 2008). Polyamines are present in food in variable amount and can be obtained from various edible sources. These can be absorbed from digested food in the small intestine through carriers and cellular absorption which is then distributed throughout the various organs of the body (Hussain et al., 2016). Polyamines in mammals can be synthesised *de-novo* from amino acids such as methionine, proline and arginine (Kusano et al., 2008, Tabor et al., 1964, Tabor et al., 1984) which can be absorbed by intestines and then released into the blood circulation (Milovic, 2001). Although many of their functions remain unclear, polyamines are essential for cell proliferation and differentiation (Tabor et al., 1984, Pegg, 1986). The intercellular levels of these polyamines are tightly regulated by the cells growth status which is dependent on the metabolic pathways that regulate their cell synthesis, degradation and/or excretion (Asotra et al., 1988). Ornithine decarboxylase (ODC1) the rate limiting enzyme has been the enzyme of intense scrutiny in these last few years. ODC1 catalyses the decarboxylation of L-ornithine to yield putrescine; putrescine in turn combines with S-adenosylmethionine and is transformed to spermidine and spermine in the presence of spermidine synthase and spermine synthase (Lefevre et al., 2011). Polyamine catabolism principally functions by back conversion mechanisms, such as spermine can back convert to spermidine and putrescine by the combination of spermidine/spermine N 1 -acetyltransferase (SAT1) and polyamine oxidase (Shappell et al., 1993). Further, to regulate the activity of ODC1 there is availability of the antizyme 1 (AZ1) which degrades ODC1; also, there is antizyme inhibitor (AZIN1) having high affinity towards AZI to rescue ODC1 in tissues (Tang et al.,

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2009; Greenwood et al., 2015). There is synthetic inhibitor of ODC1, difluromethyl ornithine (DFMO), whose treatment results in decrease in the cell proliferation (Takigawa et al., 1990, He et al., 1994).

Reports also suggest that majority mice transgenic for polyamines are infertile (Pietilä et al., 1997; Kilpeläinen et al., 2001) thereby emphasising the involvement of polyamines in regulation of normal reproductive function. It has been shown that ovarian ODC1 activity is elevated during the prepubertal period in rabbit and mouse (Bastida et al., 2005, Johnson et al., 1977). It has been observed in adult cycling mouse, hamster, and rat the ovarian ODC1 activity is elevated during late proestrus, the time when the LH surge initiates the ovulatory process (Persson et al., 1982, Icekson et al., 1974, Kobayashi et al., 1971, Kogo et al., 1993). Steroid hormones modulate the polyamines in the reproductive tract by extracellular signals in particular it was shown that estrogen and catecholamine administered to immature rats increased ODC1 activity in the uterine horns (Zhao et al., 2008).

Numerous studies exist related to effect of polyamines on embryogenesis and implantation. Studies showed ODC1 activity in the embryo likewise increases between the two-cell and early blastocyst stages in the mouse (Alexandre, 1978 and 1979) similar increase have also been recorded in pigs (Cui and Kim 2005) and *Xenopus* (Russell, 1971 and Bassez et al., 1990). Polyamine-related genes, such as ODC1, SAT1, and AZI, and uterine polyamine contents are significantly up-regulated in the uterus during the early stages of embryo reactivation (Lefevre and Murphy 2009; Lefevre et al., 2011). Studies also showed that polyamines are necessary for placenta formation (Lopez-García et al., 2008; Guha and Janne 1976). ODC1 activity was lower in fetal tissues, compared with the placenta, but polyamine content was higher in the fetus and yolk sac relative to the placental compartment (Lopez-Garcia et al., 2009).

With all this above information, it is evident that polyamines play an important role in regulating a number of events in ovarian and uterine physiology, having more insight into the cross talk between polyamines and the hypothalamic pituitary gonadal axis would be an interesting event to explore. However, till date there is no information available about the

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variation in the expression profile of the polyamine related factors in the reproductive tissues of female rodents. Thus, the aim of the present study was to immunolocalize ODC1, SPM, SPD and AZIN1 in the hypothalamus, ovary and uterus; and relating these factors with GnRH I in the hypothalamus; StAR in the ovary during the estrous cycle of adult rat. Further, the effect of putrescine treatment on estradiol and progesterone serum levels and GnRH I expression in hypothalamus were also investigated. The study also aims in quantifying all the immunohistochemistry images obtained based on pixel counting algorithm to yield the relative pixel count.

Materials and Methods*:*

Animals and treatments*:* Adult female Wistar rats (10-12 weeks old) were caged in a controlled environment with a 12-h light, 12-h dark cycle and at 22°C ambient temperature. The adult rats were housed in polypropylene cages (430mm270mm50mm) and fed a standard pellet diet with access to water ad libitum. General health condition and body weight of the animals were monitored regularly during the entire tenure of the experiment. All animal procedures were approved by the Institutional animal care and animal Ethical Committee of Birla Institute of Technology and Science (BITS) Pilani Rajasthan. Estrous cycles were determined through daily examination of vaginal cytology and further were placed in groups as per their cyclicity. Rats (n=20) with at least two consecutive regular estrous cycles were selected for the experiment. Animals were sacrificed by decapitation under mild anaesthesia (anaesthetic ether) in accordance to their estrous cycle.

Further, to examine effect of putrescine on ovarian steroidogenesis, adult cyclic rats (n=15) (showing proestrus phase) were divided into three groups: Group 1 was treated with distilled water (controls); Group 2 was treated with Putrescine low dose (150ug/day); Group 3 was treated with Putrescine high dose (250ug/day) for 5 consecutive days after which they were sacrificed by supplementation of ether before cervical dislocation. All treatments including vehicle and putrescine supplementation was given during the morning 10.00h of proestrus. The dose was selected in accordance with (Rehman et al., 2011). Ovaries, uterus and hypothalamus

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were immediately dissected out and one side of the ovaries, one uterine horn and hypothalamus were snap frozen and kept at -20°C for future studies, and the contra lateral ovaries; uterus and hypothalamus were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight for immunohistochemistry. The fixed tissues were dehydrated in ethanol, embedded in paraffin wax and sectioned at 5 µm. Blood samples were collected under light ether anesthesia by retro-orbital plexus. Serum was centrifuged at 4°C and was kept frozen in -80°C until further analysis.

Antibodies and chemicals: Ornithine decarboxylase is a goat polyclonal antibody (cat no sc-21515), spermidine (cat no ab7318) and spermine (cat no ab26975) both rabbit polyclonal antibodies, purchased from Peninsula laboratory, San Carlos, CA, USA. GnRH I antibody (cat no ab189878) a rabbit polyclonal antibody was purchased from Abcam, Cambridge, United Kingdom. Steroidogenic acute regulatory protein (StAR) and antizyme inhibitor (AZIN1) were kind gifts from Prof. Douglas M Stocco (Texas Tech University Health Sciences Center, USA) and Prof. David Murphy (University of Bristol, United Kingdom) respectively.

Immunohistochemistry: Standard protocol of immunohistochemistry was followed to localize the proteins on tissue sections (Banerjee et al., 2014). Sections of 5µm thick hypothalamus, uterus and ovary were deparaffinized; endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes. The slides were subsequently washed in 0.01M PBS and further blocked by 5% bovine serum albumin and then incubated overnight at 4°C with rabbit polyclonal antibodies against spermidine (1:100), spermine (1:100), antizyme inhibitor (1:100), ornithine decarboxylase (1:100) using a goat antiserum, StAR (1:500) or GnRH I antibody (dilution 1:1500). As negative controls, some sections were incubated with PBS in place of any antiserum. Sections were then incubated at RT for 60 min with a biotinylated mouse anti-rabbit IgG (diluted 1:500) and rabbit anti-goat IgG (diluted 1:500) streptavidin–biotin horseradish peroxidase tagged complex. The bound complex was made visible by reaction with 3,30-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemicals Co., St Louis, USA) in 0.05 M Tris pH 7.6 and 0.1% $H_2O_2$for 7-8 min at RT. A minimum of five sections was examined for

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each sample. Representative tissue sections were viewed, analysed and photographed under light microscope with the nucleus counterstained by Elrich's haematoxylin.

Digital Image Capture

All the specimens were evaluated using Leica microscope, DM2000LED. Images were acquired at 40X and 100X using a Leica microscope, DM2000LED. For all the cases, a tagged-image file format (TIFF) was used which has the advantage of being LZW compressible over the more commonly used joint photographic experts group JPEG format.

Determination of Relative Pixel Count (RPC) using Photoshop and MATLAB

Earlier methods to find pixel counts include the magic wand tool in photoshop but it is tedious and inaccurate in the case of a range of pixel intensities (Matkowskyj et al., 2000). A modified form of RGB (Red, Green, Blue) color thresholding approach (Raof et al., 2008; Matkowskyj et al., 2000; Varghese et al., 2014) was used, which includes a certain pre-processing of the image in question and then separating out the three (Red, Green, Blue) channels to yield relevant pixels in the desired range. The desired range here is given as a function derived from a set of points (15-20) manually selected from the pre-processed image.

Algorithm and workflow for determination of RPC

1. In Photoshop

a. Open Image (test_image.tiff)
b. Define the size argument for Quick Selection Tool
c. Use the tool to define the area that is not relevant for analysis. (Easier to spot area that is not relevant as opposed to relevant area)
d. Fill the extracted area using Shift+F5. Use #000000 as the fill color with 100% opacity. (Here we are basically filling the non-relevant area with #000000 so that it doesn't interfere with subsequent analysis)
e. Save as new image. (test_image_E.tiff) in the MATLAB work path.

In MATLAB

. Write the MATLAB script that is given at the end and save it as rpc.m
a. Run it (using F5) and it'll show the negative of the original image in a window that allows one to choose relevant pixels manually.
b. Choose about 15-20 data points for the algorithm to threshold the entire image optimally. The data points are used as a reference for the range of the desired values.
c. Press enter at the impixel window and the results of image thresholding appear as a binary image with relevant pixels appearing as white in the first image. The second image is the original image for visual comparison.
d. The relative pixel count (RPC) appears at the command window.

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```
%Beginning of the Algorithm
%Loading the image
TestImg = imread('test_image.tiff');  %Path of the image file


%Creating a complement of the image for better visualization.
%Negatives of original images help for bright-field images
TestImg = imadjust(TestImg,[0 1],[1 0]);


%Separating out the three RGB channels
TestImg_R = TestImg(:,:,1);
TestImg_G = TestImg(:,:,2);
TestImg_B = TestImg(:,:,3);


%Using impixel to choose data points
chosen_points = impixel(TestImg);


%Setting MAXC and MINC as threshold references
MAXC = max(chosen_points);
MINC = min(chosen_points);
%Including references points themselves
r_max = MAXC(1) + 1;
r_min = MINC(1) - 1;
g_max = MAXC(2) + 1;
g_min = MINC(2) - 1;
b_max = MAXC(3) + 1;
b_min = MINC(3) - 1;


%Choosing relevant pixels based on threshold values
OutImg = TestImg_R<r_max & TestImg_R>r_min & TestImg_G<g_max & TestImg_G>g_min
& TestImg_B<b_max & TestImg_B>b_min;


%OutImg is a binary image
%Showing extracted images and original image
subplot(1,2,1);
imshow(TestImg);
subplot(1,2,2);
imshow(OutImg);


%sum(A(:)) works for a 2D binary image A
%it gives the required relative pixel count
fprintf('\nRelative Pixel Count : %d units',sum(OutImg(:)));
%END of the Algorithm
```

Serum estradiol assay: ELISA kit for estradiol assay was purchased from diagnostics Biochem Canada Inc. (# CAN-E-430). To each well of ELISA plate 50µl of standard, control, 50µl samples were added. After this 100µl enzyme conjugate solution was added. The wells were

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then incubated with mild shaking (500-700rpm) at room temperature for one hour. The wells were then aspirated and washed three times with wash solution. Then 150µl of the TMB chromogen solution (substrate) was added to each well and plate was incubated at room temperature for 10-15 minutes in dark. Finally, 50µl of stop solution was added in each of the well and absorbance was taken at 450 nm. Intra assay variations was less than 5 % respectively.

Serum progesterone assay: ELISA kit for progesterone assay was purchased from DRG Diagnostics. (# EIA-1561). To each well of ELISA plate 25µl of standard, control, 25µl samples were added and incubated for 5 minutes at room temperature. After this 200µl enzyme conjugate solution was added. The wells were then incubated with mild shaking (500-700rpm) at room temperature for one hour. The wells were then aspirated and washed three times with wash solution. Then 200µl of the TMB chromogen solution (substrate) was added to each well and plate was incubated at room temperature for 15 minutes in dark. Finally, 100µl of stop solution was added in each of the well and absorbance was taken at 450 nm. Intra assay variations was less than 5 % respectively.

Statistical analysis:

Data are expressed as mean ±SEM. For IHC image analysis significance was determined by one way analysis of variants (ANOVA) followed by Tukey's range test. The data was considered significant if $P<0.001$ for the immunohistochemistry images, and for variation in serum estradiol and progesterone levels and for the correlation study, significance was considered at $P<0.05$.

Results:

Digital analyses of the immunohistochemistry images: All the images obtained are pre-processed and analysed based on the algorithm described earlier.

Immunolocalization of ODC1, SPD, SPM, AZIN1 and GnRH I in the hypothalamus:

Presence of the various polyamines (SPD and SPM) and other related factors (ODC1 and AZIN1) were demonstrated immunohistochemically in the hypothalamus of rats during the estrous cycle and showed similar expression pattern of hypothalamic GnRH I. It was observed that localization of ODC1, SPD, SPM and AZIN1 proteins were cytoplasmic and mainly localised in the medial preoptic area (MPA) of the hypothalamus. ODC1 showed intense staining during estrus and diestrus and moderate staining during proestrus and mild during metestrus (Figure 1). ODC1 expression was significantly ($p<0.001$) more during the estrus and diestrus in comparison to the expression during other phases as evident from relative pixel values of images. SPD showed intense staining during estrus and mild to moderate staining during proestrus, metestrus and diestrus (Figure 2). SPD expression during estrus is

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significantly higher (P<0.001) than its expression during other phases. SPM showed intense staining during estrus and diestrus, and showed moderate staining during proestrus and mild staining during metestrus (Figure 3). Expression of SPM during estrus and diestrus is significantly higher (P<0.001) than its expression during other phases. AZIN1 showed intense staining during proestrus and diestrus, and showed moderate staining during estrus and metestrus (Figure 4). Expression of AZIN1 during proestrus and diestrus is significantly higher (P<0.001) than its expression during other phases.

GnRH I showed intense staining during proestrus and diestrus, moderate staining during estrus and mild staining during the metestrus. GnRH I was expressed from rostral to caudal regions in the rat hypothalamus (Figure 5). GnRH I immunoreactivity was detected mostly in the cytoplasm and scattered throughout the MPA. Expression of GnRH I during proestrus, estrus and diestrus is significantly higher (P<0.001) than its expression in metestrus.

Effect of putrescine on hypothalamic GnRH I expression:

The present study showed significant (P<0.01) increased hypothalamic GnRH I expression for both the doses of putrescine treatment groups in comparison with control group, immunostaining was particularly observed in the MPA area (Figure 6).

Immunolocalization of ODC1, SPD, SPM, and AZIN1 in the ovary:

Presence of the various polyamines (SPD and SPM) and other related factors (ODC1 and AZIN1) were demonstrated immunohistochemically in the ovary of rats during the estrous cycle (Figure 7). ODC1 was cytoplasmic and showed intense staining during proestrus and diestrus, moderate staining was observed during estrus and mild staining during the metestrus. Proestrus showed intense immunostaining in the ovarian surface epithelium, stroma, theca cells (TC) and granulosa cells (GC), moderate staining was also observed in the oocyte (Oo). Estrus showed moderate immunostaining in the ovarian surface epithelium, stroma, theca cells (TC) and the oocyte (Oo) and mild staining was also observed in the granulosa cells (GC). Metestrus showed mild immunostaining in the ovarian surface epithelium, moderate in the stroma and theca cells (TC), mild staining was observed in the granulosa cells (GC) and the oocyte (Oo). Diestrus phase showed intense staining in the corpus luteum (CL), ovarian surface epithelium, moderate staining was observed in the stroma, theca cells (TC) and the oocyte (Oo), mild staining was observed in the granulosa cells (GC). Expression of ODC1 during proestrus and diestrus is significantly higher (P<0.001) than its expression during estrus and metestrus.

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SPD and SPM showed a similar expressional pattern like ODC1. Intense staining was observed during proestrus and diestrus, moderate staining was observed during estrus and mild staining during the metestrus (Figure 8 and Figure 9). Expression of SPD and SPM during proestrus and diestrus is significantly higher ($P<0.001$) than its expression during estrus and metestrus.

Intense staining of AZIN1 was observed in the ovary during proestrus and diestrus, moderate staining was observed during estrus and metestrus (Figure 10). Expression of AZIN1 was during proestrus and diestrus is significantly higher ($P<0.001$) than its expression during estrus and metestrus.

Immunolocalization of StAR in the ovary:

Immunolocalization of StAR was performed in the various phases of the estrous cycle (Figure 11). Immunoreactivity of StAR showed intense staining during proestrus and diestrus while moderate staining was observed during estrus and metestrus phases. StAR was mainly localized in the theca cells (TC) and the interstitial cells of the antral follicles and mild staining in the oocyte (Oo) of maturing follicles. StAR expression during diestrus is significantly higher ($P<0.001$) than its expression during the other phases.

Serum estradiol (E2) and progesterone (P4) levels during each stage of the estrous cycle:

The levels of E2 and P4 in the serum of the adult female rats vary throughout the estrous cycle (Figure 12a and 12c). There were two peaks ($P<0.05$) observed in the serum estradiol level, first during the proestrus and the second peak during the estrus phase compared to metestrus and diestrus. Metestrus and diestrus showed basal levels of estradiol during the estrous cycle. P4 showed higher ($P<0.05$) levels during metestrus and diestrus compared to the proestrus and estrus levels.

Serum E2 and P4 levels after putrescine treatment:

The levels of E2 and P4 in the serum were assessed after the treatment with putrescine (doses of 150ug and 250ug) in the adult female rats. Rats treated with low dose of putrescine showed significant ($P<0.05$) decrease in the circulating E2 level and rats treated with higher dose of putrescine showed significant ($P<0.05$) rise as compared with the control rat sera (Figure 12b). Whereas rats treated with both low and high doses of putrescine showed significant ($P<0.05$) increase in the circulating P4 level as compared with the control rat sera (Figure 12d).

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Immunolocalization of ODC1, SPD, SPM, and AZIN1 in the Uterus:

Presence of the various polyamines (SPD and SPM) and other related factors (ODC1 and AZIN1) were demonstrated immunohistochemically in the uterus of rats during the estrous cycle. Intense immunoreactivity of ODC1 was observed during metestrus and diestrus phases while moderate staining was observed in proestrus and estrus (Figure 13). Expression of ODC1 during metestrus and diestrus is significantly higher ($P<0.001$) than its expression during other phases. ODC1 during proestrus and estrus showed moderate staining in the stromal cells (ST), luminal epithelium (LE) and glandular epithelium (GE). (C) metestrus showed intense staining in the luminal epithelium (LE), stromal cells (ST) and myometrium (MYO) region. During diestrus there was intense staining in the luminal epithelium (LE) and glandular epithelium (GE).

Immunoreactivity of SPD and SPM (Figure 14 and 15) showed a similar pattern. Intense immunoreactivity of SPD and SPM were observed during proestrus and metestrus phases while moderate staining was observed in estrus and mild staining of SPD was observed in the diestrus phase whereas moderate staining of SPM was observed during diestrus phase. Expression of both SPD and SPM during proestrus and metestrus is significantly higher ($P<0.001$) than its expression during other phases.

Intense immunoreactivity of AZIN1 (Figure 16) was observed during estrus and metestrus phases while moderate staining was observed in proestrus and diestrus. Expression of AZIN1 during estrus and metestrus is significantly higher ($P<0.001$) than its expression during other phases.

Correlation study

Table 1 shows the relative intensity of immunolocalization of various antibodies in hypothalamus, ovary and uterus during various phase of estrous cycle. Further, correlation coefficients between polyamine associated factors with GnRH I expression in the hypothalamus and with StAR expression in the ovary during the estrous cycle is shown in Table 2. There was a significantly strong ($P<0.05$) correlation between ODC1 and SPM with hypothalamic GnRH I expression suggesting putrescine as well as SPM might regulate hypothalamic GnRH I expression. Also there was a significantly strong ($P<0.05$) correlation between ODC1, SPD and SPM with ovarian StAR expression suggesting all the three polyamines are potent in regulating ovarian steroidogenesis.

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Discussion:

Role of polyamines is essential during the development and differentiation of various cells and other vital cellular processes. There is a constant search to study the involvement of polyamines in other metabolic and physiological processes. The current work provides evidence of expression pattern of the polyamines and their associated factors in the hypothalamus, ovary and uterus during the various phases of the estrous cycle in adult female rat.

ODC1, SPD, SPM and GnRH I proteins were mainly cytoplasmic and localized in the medial preoptic area (MPA) of the hypothalamus particularly during the proestrus, estrus and diestrus phases. Earlier studies have shown cytoplasmic localization of ODC1 in brain (Junttila et al., 1993) and other polyamines in kidney cell lines (Huang and Moczydlowski 2001). This is the first study demonstrating the distribution of polyamines and polyamine related factors in the hypothalamus of adult female rats during estrous cycle. Increased levels of polyamines have been reported in adult brain and these levels decrease with aging (Jasper et al., 1982). SPD and SPM had been shown to be distributed in adult rat brain particularly in the paraventricular nucleus and the supra optic nucleus of the hypothalamic hypophyseal system (Fujiwara et al., 1997; Laube et al., 2002).

The study further showed cytoplasmic localization of AZIN1 in the hypothalamus being high during the proestrus, and diestrus. Increased AZIN1 expression during these phases might be required to facilitate increased ODC1 expression during estrus and diestrus as AZIN1 provides additional regulation to ODC1 by neutralizing ODC1 inhibitor AZ1. There is a report suggesting an increase in AZIN1 expression preceding the increase in the expression of ODC1 mRNA (Nilsson et al., 2000). Previous studies have also reported that overexpressing AZIN1 in cells and animal model, increases polyamine synthesis (Tang et al., 2009; Greenwood et al., 2015). Further, a recent study has shown expression of AZIN1 in the rat supraoptic and paraventricular nuclei suggesting a role in regulation of the arginine vasopressin in male rats (Greenwood et al., 2015).

The present study shows hypothalamic GnRH I immunostaining during proestrus and diestrus, moderate staining during estrus and mild staining during the metestrus, being mostly localized throughout the MPA. Earlier reports suggested the localization of GnRH I in the hypothalamus of mouse, rat, guinea pig, lamb and other mammals by light and electron microscopy (Silverman et al., 1985, 1987, 1990; Yin et al., 2007, Shirasawa et al., 2007). GnRH I immunoreactivity was detected in the cytoplasm and dendrites of neurons scattered throughout the medial preoptic area (MPA) and septohypothalamic nucleus (SHy) (Dorfman et al., 2011). A recent report suggests that hypothalamic GnRH acts on its receptor on the neurons and triggers burst firing during late proestrus and estrus (Schauer et al., 2015). Earlier report suggests that ovarian GnRH I have similar pattern of expression being high during proestrus, estrus and diestrus (Singh et al., 2011).

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Overall, the pattern observed is that polyamine and polyamine associated factors showed intense to moderate staining in the hypothalamus during the proestrus, estrus and diestrus, a pattern of expression which was also showed by the hypothalamic GnRH I. Correlation studies showed that ODC1 and SPM showed significant correlation with hypothalamic GnRH I expression. This suggests that hypothalamic GnRH I might be under regulation of putrescine and SPM in adult female rats.

The present study for the first time showed a significant increase in GnRH I expression in the MPA area of the hypothalamus after putrescine treatment in adult female rats. Till date there is only one report which suggests that hypothalamic GnRH I is regulated by polyamines in developing female rats. Polyamine treatment to hypothalamus and anterior pituitary obtained from six and fifteen day old rats *in vitro*, was able to regulate GnRH, FSH, and LH secretion (Thyssen et al., 2002). The same group had demonstrated that DFMO treatment induced delayed puberty of female rats with high FSH levels during the infantile period (Thyssen et al., 2002).

The present study showed intense immunoreactivity of ODC1 in the ovary of female rats during proestrus and diestrus phases. Relatively, immunoreactivity of ODC1 was mainly in the ovarian surface epithelium, granulosa cells, theca cells and interstitial cells and moderate staining was observed in the oocyte antral follicles during proestrus; and in the corpus luteum during the diestrus. Like ODC1, SPD and SPM showed intense staining pattern in the oocyte, theca cells and interstitial cells in the maturing follicles, during proestrus and diestrus phases. Earlier studies have showed that ODC1 was expressed in the ovarian theca cells after administration of human chorionic gonadotropin (Persson et al., 1982, 1986; Bastida et al., 2005). ODC1 activity was shown to be high during the prosestrus phase coinciding with the LH surge, in the adult cycling hamster (Persson et al., 1982), and rat (Icekson et al., 1974 Kobayashi et al., 1971).

In addition to ovarian ODC1, SPM and SPD expression, the present study showed ovarian AZIN1 expression which was similar to that ovarian ODC1; thereby suggesting that in the ovary there is AZIN1 activity in regulating ODC1 expression by neutralizing ODC1 inhibitor, AZ1, similar to the hypothalamus. There is no report till date about ovarian AZIN1 expression or activity; however, there exist a report of AZI expression to be high in ovarian tumours (Schaner et al., 2003). This is the first report demonstrating the cellular distribution of ODC1, SPM, SPD and AZIN1 in the ovary during estrous cycle.

ODC1, SPD and SPM, expressed in the ovarian granulosa and theca cells, suggest that polyamines might be involved in the regulation of ovarian folliculogenesis and luteinization. There are many reports suggesting polyamines role in cell proliferation and differentiation (Heby, 1981; Kusano et al., 2008; Lefevre et al., 2011), and ovarian folliculogenesis involves cell proliferation while luteininzation involves differentiation of existing theca cells (Young and McNeilly, 2010; Voronina et al., 2005). An earlier study in mice had reported that ODC1

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expression being confined to theca and granulosa cells might implicate its role in ovarian folliculogenesis and luteinization of the theca cells (Bastida et al., 2005). The present study strengthens the hypothesis that not only putrescine but even spermine and spermidine in the ovarian granulosa and theca cells might play a crucial role regulating folliculogenesis and luteinization by promoting cell proliferation and differentiation. This action of polyamines might be under regulation of FSH and estradiol as it is well documented that estradiol and FSH act on developing ovarian follicles stimulating proliferation of both granulosa and theca cells (Rao et al., 1978) and gonadotropins and steroid up regulates ovarian ODC1 activity (Osterman et al., 1978; Bastida et al., 2002).

Further, presence of ODC1, SPM and SPD in oocyte of ovary implicates its role in maintenance of oocyte physiology. Recently, it was shown that rise in ovarian ODC1 during late proestrus is essential for proper chromosome segregation during oocyte maturation and periovulatory putrescine supplementation might reduce the risk of aneuploidy among older women (Tao and Liu, 2013).

The present study showed a variation in the steroid levels during the estrous cycle and also showed the effect of putrescine on the ovarian steroidogenesis. Serum estradiol levels showed two peaks during the estrous cycle once in proestrus and once in estrus, whereas serum progesterone levels showed peaks at metestrus and diestrus phases. Earlier studies have showed the levels of P4 and E2 during the estrous cycle in rodents which suggested an increase in E2 levels during the estrus phase and increase in P4 during diestrus phase (Wood et al., 2007; Butcher et al., 1974). Butcher et al., (1974) had further reported there was a similar rise in serum level of estradiol in evening of estrus.

Intense expression of ovarian polyamines and polyamine related factors in the theca and the corpus luteal cells further suggest polyamines might regulate ovarian steroidogenesis. To substantiate it, ovarian expression of polyamine related factors were correlated with ovarian steroidogenesis (P4 and E2 levels) and steroidogenic factors (StAR expression). It was shown in the present study that the ovarian polyamine and polyamine related factors had similar expression pattern as that of ovarian StAR, suggesting a crosstalk between the ovarian polyamines and the steroidogenic factors in regulating ovarian steroid synthesis. Correlation studies revealed that ODC1, SPD and SPM had a significant positive correlation with ovarian StAR expression, suggesting all the three polyamines, putrescine, SPD and SPM, might be positively regulating ovarian steroidogenesis. The present study further showed the effect of putrescine on progesterone and estradiol levels in adult cyclic rats. The levels of P4 significantly increased after the treatment of both the doses of putrescine as compared with control group, whereas E2 level significantly decreased with the low dose while showed slight increase in the high dose group. The increased progesterone level after putrescine treatment in the present study supports the earlier finding of increased ovarian StAR expression and

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increased serum progesterone level during the diestrus phase after putrescine treatment (Bastida et al., 2005). An earlier study showed that in adult cyclic female rats, there is a preovulatory rise of ODC1, and DFMO treatment to these rats on the evening/night of proestrus significantly decreased plasma progesterone levels and ovarian StAR at diestrus (Bastida et al., 2005).

The study further shows immunolocalization of ODC1, SPD and SPM in the uterus during estrous cycle. Expression of ODC1 was shown to be high in the stromal cells, myometrium, and luminal epithelium and in glandular epithelium during the metestrus and diestrus, emphasizing role of all putrescine in regulating uterine physiology particularly during pregnancy. Expressional pattern observed in the uterine epithelium suggests possible role of polyamines during implantation which is in accordance with earlier studies where polyamines had been shown to be essential for implantation (Kogo et al., 1993). Rat uterine ODC1 and SAMDC activities were shown to be increased within a few hours in response to estradiol-17β injection in both immature and non-pregnant adult rats (Kaye et al., 1971; Wing, 1988). Recently, it has been shown that uterine ODC1 gene expression is increased at implantation sites in the mouse uterus after estrogen induction of nidation (Zhao et al., 2008). An intense expression of ODC1 in the uterine luminal epithelium and glandular epithelial cells during diestrus suggests role of putresine, among the other polyamines, in secretion of histotrophs by endometrial cells during early diestrus. Importance of histotrophic nutrition from uterine glands forms the initial forms of nutrition for the developing embryos in almost all species before placenta formation (Bazer et al., 2011; Spencer, 2014). Increased AZIN1 expression during the metestrus phase coincides with increased ODC1 expression during the same phase, suggesting a regulation of ODC1 expression by AZIN1 in uterus too. Uterine tissues also showed increased SPD and SPM expression during proestrus and metestrus, thus suggesting all the three polyamines are important in maintenance of uterine physiology.

In conclusion, the present study highlights an algorithm using a red green blue (RGB) colour thresholding approach to quantify the immunohistochemistry images. Using that quantitative immunohistochemistry approach the present study demonstrates similar expression pattern of polyamine and polyamine related factors, ODC1, SPD, SPM and AZIN1, with that of GnRH I in the hypothalamus during the estrous cycle. This study shows that putrescine treatment to adult female rats, significantly increased hypothalamic GnRH I expression, suggesting hypothalamic GnRH I is regulated by polyamines in adults. The study showed putrescine supplementation increased significantly GnRH I expression in the hypothalamus. Further, in ovary ODC1, SPM, SPD and AZIN1 expression pattern were similar with that of steroidogenic factor, StAR during the estrous cycle, and putrescine supplementation increased significantly estradiol and progesterone levels in serum, all suggesting ovarian polyamines are involved in

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regulation of ovarian steroidogenesis. Expression of these factors in the granulosa and theca cells suggest involvement of polyamines in the process of folliculogenesis and luteinisation; and ODC1, SPD, SPM and AZIN1 in oocyte further suggests polyamine role in maintenance of oocyte physiology. Finally, in uterus, SPM and AZIN1 were localized primarily throughout the estrous cycle, being more comparatively during the metestrus phase; ODC1 was intense during metestrus and diestrus phases. Intense immunostaining of ODC1 in the uterine luminal epithelium and glandular epithelial cells during diestrus suggest that putrescine might play an important role in secretion of histotrophs by endometrial cells. There was intense immunostaining of SPD in the luminal and glandular epithelium during the metestrus and diestrus phases of the estrous cycle suggesting these all the three polyamines as such play important role in regulation of uterine physiology.

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Conflict of Interest:

The authors declare that there is no conflict of interest that would prejudice the impartiality of their scientific work.

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Fig. 1. Immunohistochemical localization of ODC 1 in the hypothalamus of adult female rats: Immunolocalization of ODC 1 mainly in the medial preoptic area (MPA) of the hypothalamus during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. ODC 1 showed intense staining during estrus and diestrus and moderate staining during proestrus and mild during metestrus phase. (E) shows higher magnification of the medial preoptic region during diestrus (F) Negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ± S.E.M. ODC1 expression was significantly (*, P<0.001) more during the estrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 2. Immunohistochemical localization of SPD in the hypothalamus of adult female rats: Immunolocalization of SPD mainly in the medial preoptic area (MPA) of the hypothalamus during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. SPD showed intense staining during estrus and mild to moderate staining during proestrus, metestrus and diestrus. (E) Shows higher magnification of the medial preoptic region during estrus. (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. SPD expression was significantly (*, P<0.001) more during the estrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 3. Immunohistochemical localization of SPM in the hypothalamus of adult female rats: Immunolocalization of SPM mainly in the medial preoptic area (MPA) of the hypothalamus during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. SPM showed intense staining during estrus and diestrus, and showed moderate staining during proestrus and mild staining during metestrus. (E) shows higher magnification of the medial preoptic region during estrus. (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. SPM expression was significantly (*, P<0.001) more during the estrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 4. Immunohistochemical localization of AZIN1 in the hypothalamus of adult female rats: Immunolocalization of AZIN1 mainly in the medial preoptic area (MPA) of the hypothalamus during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. AZIN1 showed intense staining during proestrus and diestrus, and showed moderate staining during estrus and metestrus phases. (E) Shows higher magnification of the medial preoptic region during proestrus. (F) Negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. AZIN1 expression was significantly (*, P<0.001) more during the proestrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

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Fig. 5. Immunohistochemical localization of GnRH I in the hypothalamus of adult female rats: Immunolocalization of GnRH I mainly in the medial preoptic area (MPA) of the hypothalamus during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. GnRH I showed intense staining during proestrus and diestrus, moderate staining during estrus and mild staining during the metestrus. (E) Shows higher magnification of the medial preoptic region during proestrus. (F) Negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. ODC1 expression was significantly (*, P<0.001) more during the proestrus, estrus and diestrus in comparison to the expression in metestrus. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 6. Immunohistochemical localization of GnRH I in the hypothalamus after putrescine treatment in adult female rats: Immunolocalization of GnRH I mainly in the medial preoptic area (MPA) of the hypothalamus after low (B) and high dose (C) of putrescine treatment. GnRH I showed increased immunostaining in both the treated groups i.e. in the low dose (B) of putrescine (150µg/250gm body weight) as well as with the higher dose of putrescine (250µg/250gm body weight) (C) in comparison to the control (A). (D) Shows higher magnification of the medial preoptic region. (E) Negative control showing no immunoreactivity. (F) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. putrescine dose of 150µg/250gm and 250µg/250gm body weight showed significant higher (*, P<0.001) expression value compared to the control. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 7. Immunohistochemical localization of ODC 1 in the ovary of adult female rats: Immunolocalization of ODC 1 in the ovary of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. ODC 1 showed intense staining during proestrus and diestrus, moderate staining was observed during estrus and mild staining during the metestrus phase. (A) Proestrus showed intense immunostaining in the stroma, theca cells (TC) and granulosa cells (GC), moderate staining was also observed in the oocyte (Oo). (B) Estrus showed moderate staining in the stroma, theca cells (TC) and the oocyte (Oo), mild staining was also observed in the granulosa cells (GC). (C) Metestrus showed moderate staining was observed in the stroma and theca cells (TC), mild immunostaining was observed in the granulosa cells (GC) and the oocyte (Oo). (D) Diestrus phase showed intense staining in the corpus luteum (CL), moderate staining was observed in the stroma, theca cells (TC) and the oocyte (Oo). (E) shows higher magnification of immunolocalization of ODC1 during estrus. (F) Negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. ODC1 expression was significantly (*, P<0.001) more during the estrus and metestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 8. Immunohistochemical localization of SPD in the ovary of adult female rats: Immunolocalization of SPD in the ovary of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. SPD showed a similar expressional pattern like ODC1. Intense staining was observed during proestrus and diestrus, moderate staining was observed during estrus and mild staining during the metestrus. (A) Proestrus showed intense immunostaining in the stroma, theca cells (TC), granulosa cells (GC) and oocyte (Oo). (B) Estrus showed moderate immunostaining in the stroma, theca cells (TC) and the oocyte (Oo), mild staining was observed in the granulosa cells (GC). (C) Metestrus showed mild immunostaining in the stroma and theca cells (TC). (D) Diestrus phase showed intense staining in the corpus luteum (CL), (E) shows higher magnification of immunolocalization of SPD

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during proestrus. (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. SPD expression was significantly (*, P<0.001) more during the proestrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 9. Immunohistochemical localization of SPM in the ovary of adult female rats: Immunolocalization of SPM in the ovary of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. SPM showed a similar expressional pattern like ODC1 and SPD. Intense staining was observed during proestrus and diestrus, moderate staining was observed during estrus and metestrus phase. (A) Proestrus showed intense immunostaining in the stroma, theca cells (TC) and granulosa cells (GC), moderate staining was observed in the oocyte (Oo). (B) Estrus showed moderate immunostaining in the stroma, oocyte and the theca cells (TC) and mild staining was observed in the granulosa cells (GC). (C) Metestrus showed mild immunostaining in the stroma and theca cells (TC). (D) Diestrus phase showed intense staining in the corpus luteum (CL). (E) shows higher magnification of immunolocalization of SPM during proestrus. (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. SPM expression was significantly (*, P<0.001) more during the proestrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 10. Immunohistochemical localization of AZIN1 in the ovary of adult female rats: Immunolocalization of AZIN1 in the ovary of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. Intense staining was observed during proestrus and diestrus, moderate staining was observed during estrus and metestrus phase. (A) Proestrus showed intense immunostaining in the stroma, theca cells (TC), granulosa cells (GC) and the oocyte. (B) Estrus showed moderate immunostaining in the stroma and theca cells (TC) and the oocyte (Oo). (C) Metestrus showed mild immunostaining in the stroma, theca cells (TC), granulosa cells (GC) and the oocyte (Oo). (D) Diestrus phase showed intense staining in the corpus luteum (CL). (E) shows higher magnification of immunolocalization of AZIN1 during proestrus. (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. AZIN1 expression was significantly (*, P<0.001) more during the proestrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 11. Immunohistochemical localization of StAR in the ovary of adult female rats: Immunolocalization of StAR in the ovary of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. Intense staining was observed during proestrus and diestrus while moderate staining was observed during estrus and metestrus phases. (A) Proestrus showed moderate immunostaining in the stroma, theca cells (TC), granulosa cells (GC) and the oocyte (Oo). (B) Estrus showed moderate staining in the pre-ovulatory follicles and theca cells (TC), and stroma. Mild staining was observed in granulosa cells (GC). (C) Metestrus showed moderate immunostaining in the stroma and theca cells (TC). (D) Diestrus phase showed intense staining in the corpus luteum (CL). (E) shows higher magnification of immunolocalization of StAR during diestrus. (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. StAR expression was significantly (*, P<0.001) more during the proestrus and diestrus in comparison to the expression during other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

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Fig. 12. Serum E2 and P4 levels: Comparison of serum estradiol and progesterone levels in adult female rats during estrous cycle and after putrescine treatment. Figure 12a and 12c shows the serum estradiol and progesterone levels during estrous cycle, figure 12b and 12d shows estradiol and progesterone levels after treatment with two doses of putrescine (150ug and 250ug respectively). The serum values (n=5) are represented as mean ± S.E.M and values (n=5) are significantly (*, $P<0.05$) different from control group.

Fig. 13. Immunohistochemical localization of ODC1 in the uterus of adult female rats: Immunolocalization of ODC1 in the uterus of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. Intense immunoreactivity was observed during metestrus and diestrus phases while moderate staining was observed in proestrus and estrus phases. (A) and (B) proestrus and estrus showed moderate staining in the stromal cells (ST), luminal epithelium (LE) and glandular epithelium (GE), intense staining was observed in the myometrium (MYO) region. (C) metestrus showed intense staining in the luminal epithelium (LE), stromal cells (ST) and myometrium (MYO) region. (D) diestrus showed intense staining in the luminal epithelium (LE) and glandular epithelium (GE). (E) shows higher magnification of myometrium cells (MYO). (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. Metestrus and diestrus expressional value is significantly higher (*, $P<0.001$) than the other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 14. Immunohistochemical localization of SPD in the uterus of adult female rats: Immunolocalization of SPD in the uterus of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. Intense immunoreactivity was observed during proestrus and metestrus phases while moderate staining was observed in estrus and mild staining was observed in the diestrus phase. (A) proestrus showed intense staining in the stromal cells (ST) and myometrium (MYO) region. (B) estrus showed moderate staining in the luminal epithelium (LE), glandular epithelium (GE), stromal cells (ST) and myometrium (MYO) region. (C) metestrus showed intense staining in the luminal epithelium (LE) and glandular epithelium (GE). (D) diestrus showed intense staining in the luminal epithelium (LE) and glandular epithelium (GE). (E) shows higher magnification of myometrium cells (MYO). (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. Proestrus and metestrus expressional value is significantly higher (*, $P<0.001$) than the other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Fig. 15. Immunohistochemical localization of SPM in the uterus of adult female rats: Immunolocalization of SPM in the uterus of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. SPM showed a similar staining pattern as SPD. Intense immunoreactivity was observed during proestrus and metestrus phases while moderate staining was observed in estrus and diestrus phases. (A) proestrus showed intense staining in the stromal cells (ST) and myometrium (MYO) region. (B) estrus showed moderate staining in the luminal epithelium (LE), stromal cells (ST) and myometrium (MYO) region. (C) metestrus showed intense staining in the luminal epithelium (LE) and glandular epithelium (GE). (D) diestrus showed intense staining in the luminal epithelium (LE) and glandular epithelium (GE). (E) shows higher magnification of myometrium cells (MYO). (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. Proestrus and metestrus expressional value is significantly higher (*, $P<0.001$) than the other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

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Fig. 16. Immunohistochemical localization of AZIN1 in the uterus of adult female rats: Immunolocalization of AZIN1 in the uterus of adult female rats during proestrus (A) estrus (B) metestrus (C) and diestrus (D) phases. Intense immunoreactivity was observed during estrus and metestrus phases while moderate staining was observed in proestrus and diestrus phases. (A) proestrus showed intense staining in the stromal cells (ST) and myometrium (MYO) region. (B) estrus showed intense staining in the luminal epithelium (LE), stromal cells (ST) myometrium (MYO) and glandular epithelium (GE). (C) metestrus showed intense staining in the luminal epithelium (LE) and glandular epithelium (GE). (D) diestrus showed moderate staining in the luminal epithelium (LE) and glandular epithelium (GE). (E) shows higher magnification of myometrium cells (MYO). (F) negative control showing no immunoreactivity. (G) Histogram showing relative pixel values among the four phases: Values are mean ±S.E.M. Estrus and metestrus expressional value is significantly higher (*, $P<0.001$) than the other phases. Scale bar of A-D and F, 50µm; and scale bar E, 20µm.

Table 1. The relative intensity of immunolocalization of various antibodies in hypothalamus, ovary and uterus during various phase of estrous cycle. Intensities of signals indicated above represent a subjective consensus of sections examined from the hypothalamus, ovary and the uterus collected from the all the animals over the estrous cycle (N=20) and also after putrescine treatments (N=15). The signals were estimated as score intensity of immunoreactivity on a scale of mild (+); moderate (++) and intense (+++), heterogeneity was observed in the signals, as in some showed mild, some moderate where as others showed intense signals in the sections.

Table 2. Correlation coefficients for polyamine associated factors with GnRH I expression in the hypothalamus and with StAR expression in ovary during the estrous cycle. *Significant correlation between parameters (*, $P<0.05$).

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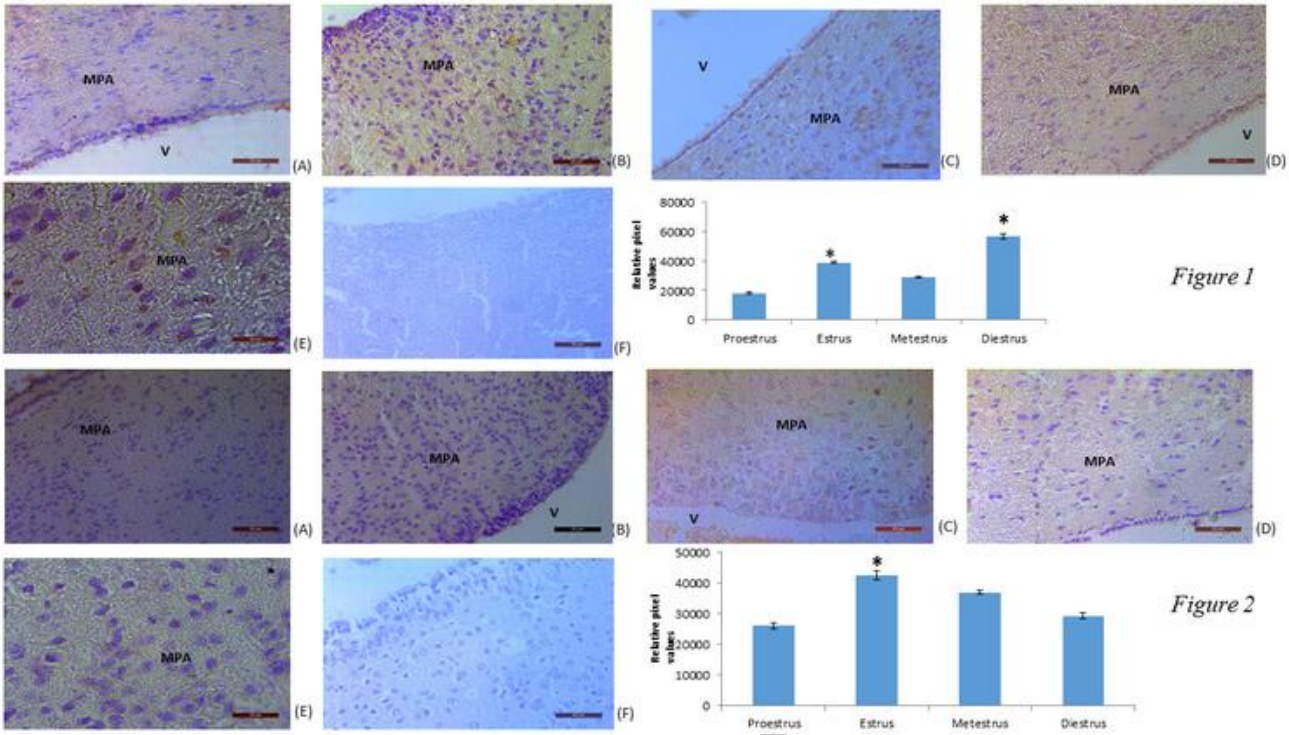

Figure 1

Figure 2

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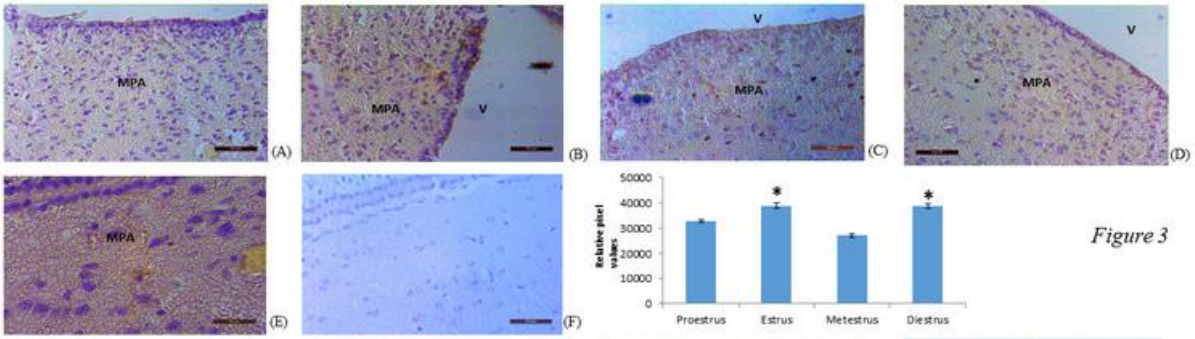

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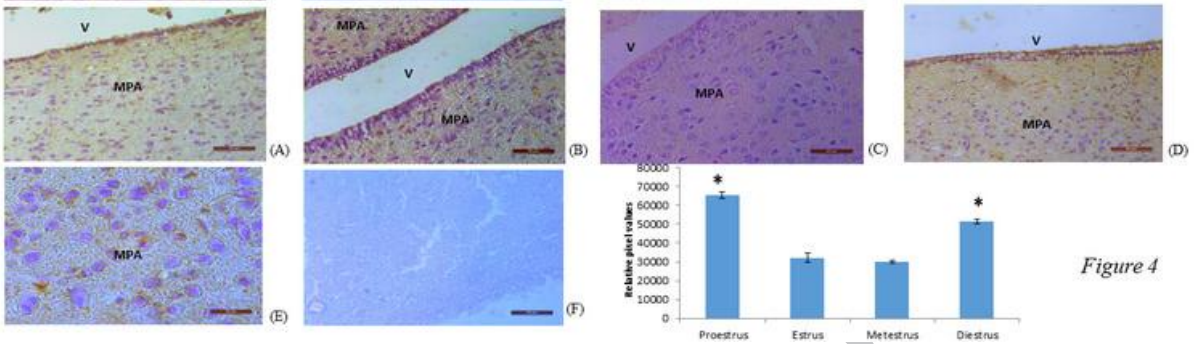

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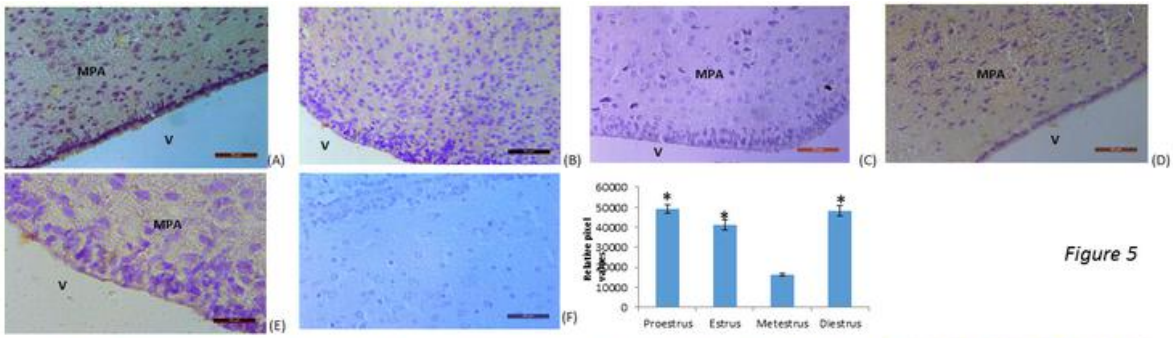

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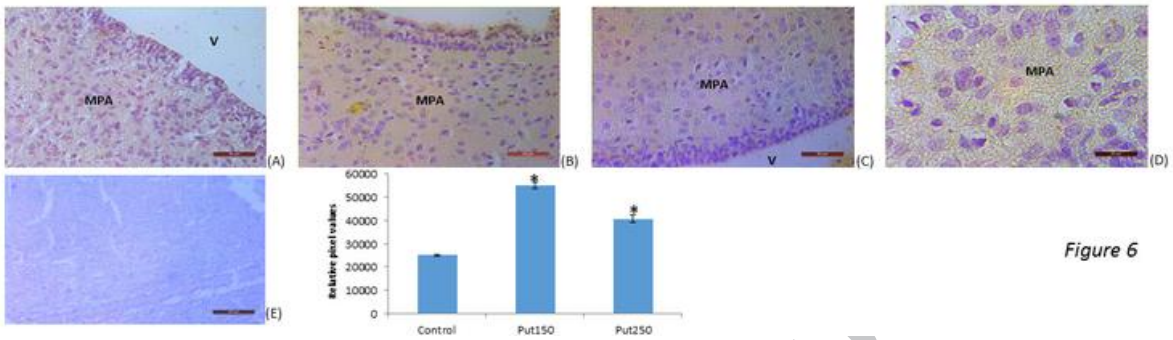

Figure 6

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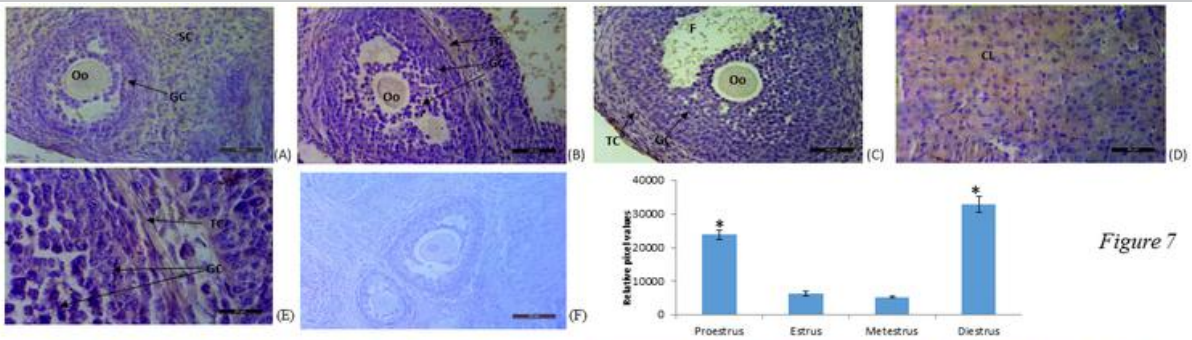

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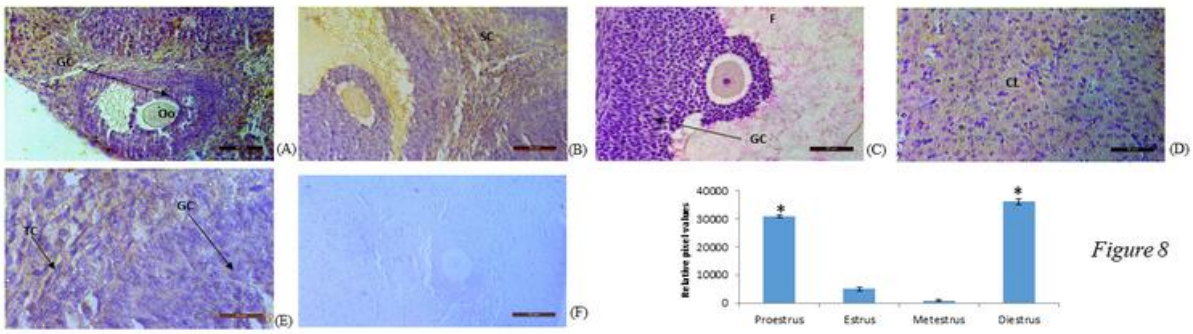

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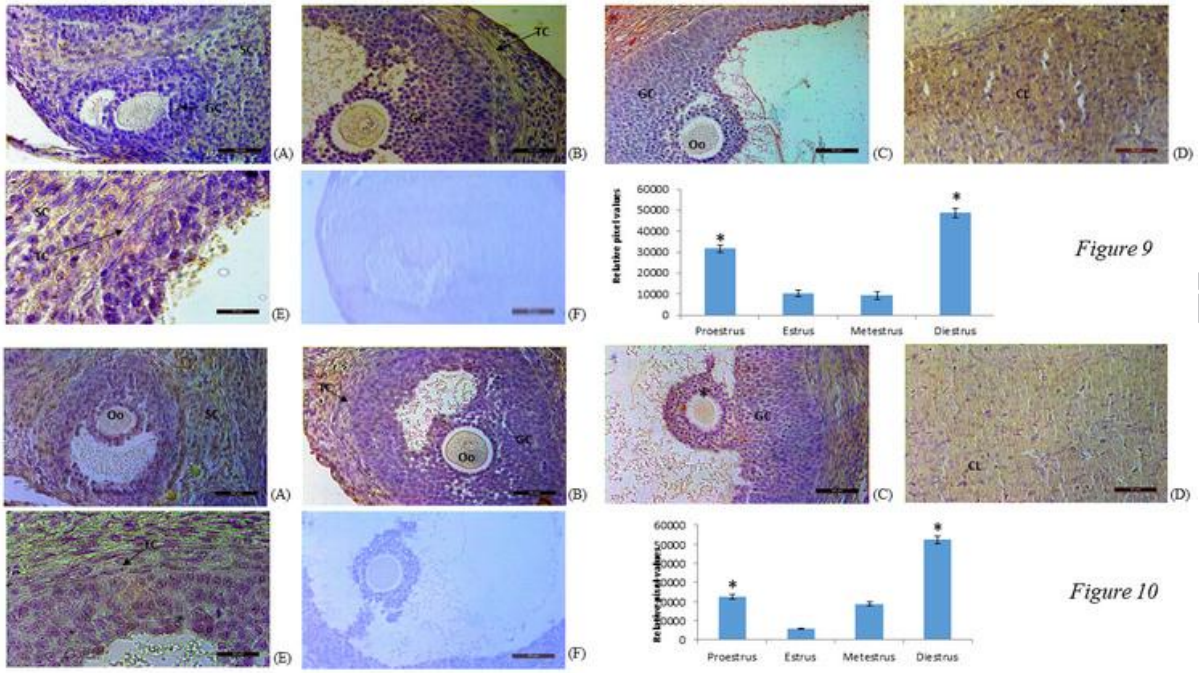

Figure 9

Figure 10

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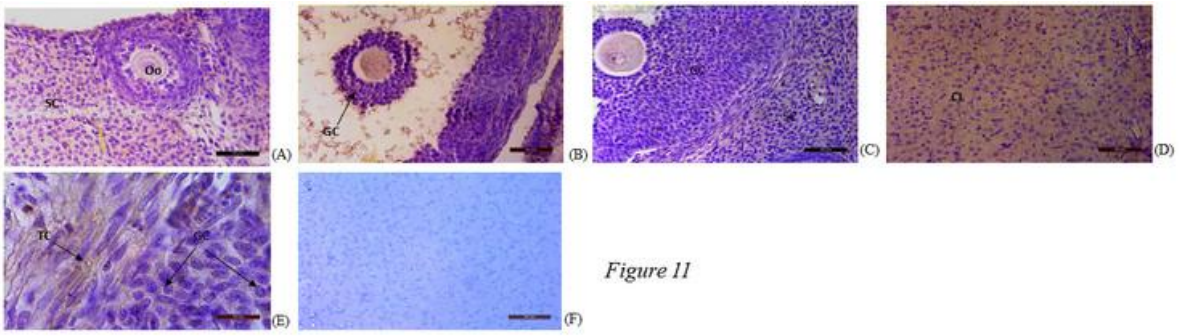

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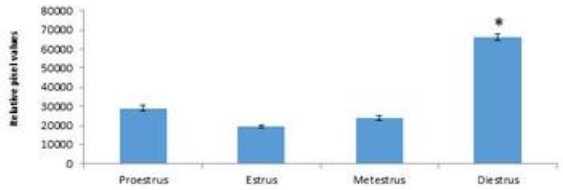

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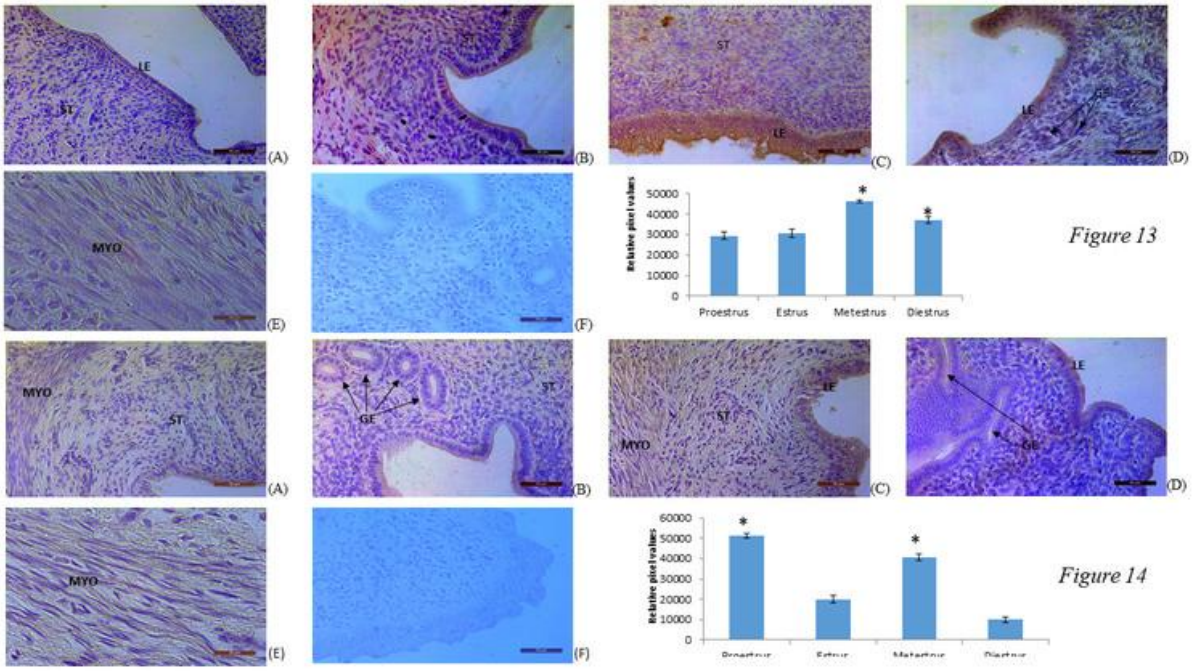

Figure 13

Figure 14

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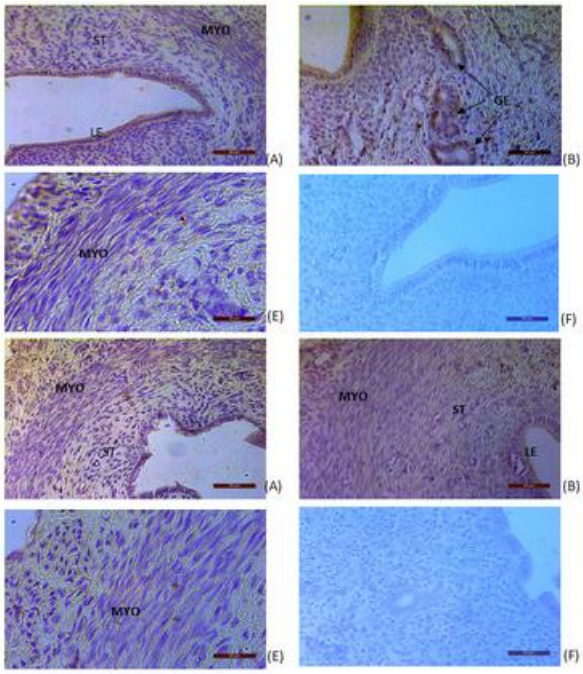

Figure 15

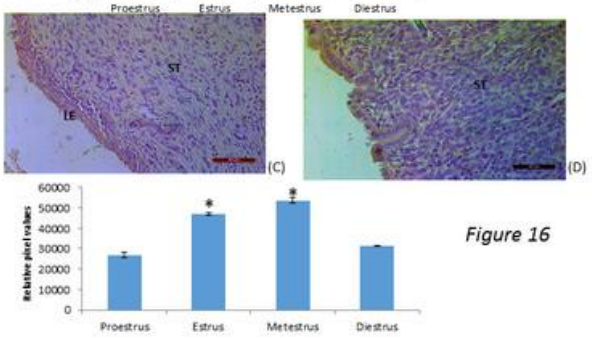

Figure 16

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HYPOTHALAMUS	Proestrus	Estrus	Metestrus	Diestrus
ODC1	++	+++	+	+++
SPD	++	+++	++	++
SPM	++	+++	+	+++
AZIN1	+++	++	++	+++
GnRH I	+++	++	+	+++
Putrescine treatment	**Group 1- Control**	**Group 2-Putrescine 150/250gm**	**Group 3-Putrescine 250/250gm**	
GnRH I	++	+++	+++	
OVARY	**Proestrus**	**Estrus**	**Metestrus**	**Diestrus**
ODC1	+++	++	+	+++
SPD	+++	++	+	+++
SPM	+++	++	+	+++
AZIN1	+++	++	++	+++
StAR	+++	++	++	+++
UTERUS				
ODC1	++	++	+++	+++
SPD	+++	++	+++	+
SPM	+++	++	+++	++
AZIN1	++	+++	+++	++

Table 1

Table 2:

Hypothalamus

Correlation	GnRH I
ODC1*	0.600
SPD	0.403
SPM*	0.730

Ovary

Correlation	StAR
ODC1*	0.833
SPD*	0.755
SPM*	0.878

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Highlights:

1) Investigated the variation in the expression pattern of ornithine decarboxylase (ODC1), spermine (SPM), spermidine (SPD) and antizyme inhibitor (AZIN1) in hypothalamus, ovary and uterus during the estrous cycle of rats.
2) Expression of ODC1, SPD, SPM and AZIN 1 were further related to steroidogenesis in ovary and GnRH I expression in the hypothalamus.
3) Putrescine treatment increased the GnRH I expression in the hypothalamus also alleviated serum progesterone and estradiol levels.
4) Used a modified algorithm of red green blue (RGB) colour thresholding approach to quantify the intensity of the chromogen present.

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