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Effects of opium alkaloid, noscapine in RU486 induced experimental model of polycystic ovary syndrome

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PCOS is a common endocrinopathy among premenopausal women, characterized by hyperandrogenism, anovulation, *hirsutism*. We evaluated credence of noscapine; in RU486 induced PCOS rat and compared with flutamide, a conventional drug. For 13 days, an oral dose of RU486 [20 mg/kg/day] was administered to Wistar rats exhibiting regular estrous cycle. Varying dosage of noscapine was given to PCOS rats and compared with rats administered flutamide. Cytology of estrous cycle and serum hormone levels (LH, FSH, PRL, estradiol, and testosterone) were measured. Histomorpho-metrical changes, events of apoptosis in theca and granulosa cells were observed using flow cytometry and the mode of cellular death was examined by TUNEL staining. Our findings suggested, normal folliculogenesis in PCOS rats, post noscapine administration (120 mg/kg) in 3-4 days with normal hormonal profile. Theca and granulosa cells undergo massive and marginal cellular degeneration respectively with no G_2/M arrest. TUNEL staining confirms the granulosa cells in follicles are major cell type undergo apoptosis in RU486 administered rats. However, low apoptotic DNA fragmentations were found in theca cells. We conclude that the RU486 model is suitable for studies of the metabolic features of PCOS and noscapine appears to be promising therapeutic modality for amelioration of PCOS induced condition.

Keywords: Anovulatory infertility, Hyperandrogenemia, Hypothalamic-pituitary-ovarian axis, Menstrual irregularity

PCOS is one of the most common endocrine disorders among premenopausal women. The clinical and biochemical features are heterogeneous and there has been much debate on the pathogenesis of the disease. Hyperinsulinemia and insulin resistance, independent of obesity, have been recognized in a large number of PCOS subjects[1-4] and have been implicated in the pathogenesis of PCOS. It is cofounded with *hirsutism* and anovulation in these patients. Addressing the symptom issues is not enough, but metabolic sequelae long-term consequences should be challenged and solved. Thus, PCOS management has to take account of the differential diagnosis and treatment of symptoms associated with it.

The anticancer and antitussive effects of noscapine have been well documented but its effects on other disease conditions like PCOS has not yet been explored. PCOS seems to stem from deranged hypothalamic-pituitary axis with ovary as the forerunner. But the drugs that are currently employed for managing PCOS are symptomatic in their approach, giving less importance to this fact. In this study, we have focused on the hypothalamic-pituitary intervention by an opium alkaloid, noscapine. This may be interesting in the light of the fact that noscapine is reported to have high affinity binding sites in brain[5,6]. Primarily, this drug is well-tolerated, non-sedative, non-habit forming opioid whose receptors are known to be located in hypothalamus.

Materials and Methods

Establishment of polycystic ovary syndrome (PCOS) in wistar rats

The laboratory rat has been repeatedly used as an animal norm to inspect continuous estrus associated with PCOS[7]. The establishment of the PCOS animal model was made by employing antiprogesterone RU486 with subtle transformations in the procedure engaged by Sanchez Criado[8]. Rats exhibiting at least three consecutive 4-5 day estrous cycles were orally aided RU486 (20 mg/Kg bwt/day) in olive oil customary for successive 13 days, commencing on day 1 of the estrous cycle. Polycystic ovary disease in rat samples portrays the induction of polycystic ovaries associated with PE, which symbolizes chronic anovulation[8]. Thus, we scanned the animals for vaginal cornification in vaginal smears. Further the changes in the rat models for the reproductive cycle, ovarian morphology, and hormonal parameters was

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also examined. The rats exhibiting arrest in the estrus phase (Persistent vaginal cornification) were taken for further studies and depicted the establishment of polycystic ovary syndrome.

Experimental protocol with different therapeutic modality

During the study period, the rats were ramified into distinct clusters, Group I (vehicle treated), Group II (RU486 administered), Group III (noscapine administered), Group IV (Flutamide treated) and Group V (autocorrection) according to the protocol (Table 1). The negative control was quantified by equal number of vehicle treated rats enduring 0.1 mL olive oil/100 g body weight/day (grp. I). The animals showing persistent vaginal cornification were subdivided into five groups. Noscapine was administered to the animals showing persistent vaginal cornification of vaginal epithelial cells (120 mg/Kg bwt.) (grp. III) and compared with flutamide (10 mg/mL in 70% ethanol) (grp. IV), the known drug for managing PCOS condition. RU486 was continuously given to the animals in the auto correction model (grp. V). Until the onset of regular cyclicity, they were checked for vaginal smear. The animals were laid down, blood collected, serum separated and deposited at −20°C till further use. Serum concentration of LH, FSH, testosterone, progesterone and estrogen were assayed.

Scrutiny of colpocytology

Vaginal smears were taken using cotton swabs soaked in saline solution throughout the analysis, further taken to histological slides, and stained[9]. We recognized the four phases of the estrous cycle of the rat (estrus, pro-estrus, meta-estrus, and diestrus). The cyclic span of 4-5 days was deemed typical. The adherence of cornified cells in the smears during a minimum of 10 serial days was described as persistent vaginal cornification (PVC). It could be considered as follicular cystic development articulation[10].

Hormone concentration assay in serum samples

Independent group of rats were checked for their estrous cycle, those with regular cyclicity (mentioned in parentheses of Table 2) were selected and treated according to dosing regimens. Blood was collected on their respective days and serum was separated. Testosterone, progesterone, and estradiol serum concentrations across groups' pre and post treatment

Table 1 — Animal Treatment Protocol. Collection of Blood samples and assaying different hormones by ELISA. (Number in parentheses, females monitored)

Set No.	Experimental Sets	Medicament	Administration course
I	Control (N= Ten)	For 13 days: 0.1 mL olive oil/100 g body weight/day	Lingual/ Oral
II	Induction of PCO (N= Nineteen)	20 mg/ Kg bwt/day of RU486 in 0.2 mL olive oil for 13 days	Lingual/ Oral
	Medicinal Modalities	Medicament	Administration course
III	Noscapine (N=Sixteen)	For 13 days RU486 in 0.2 mL olive oil with doses of 20 mg/ Kg bwt/day and administration of noscapine 120mg/Kg of bwt. with RU486	Oral
IV	Flutamide (N= Twelve)	For 13 days RU486 in 0.2 mL olive oil with doses of 20 mg/ Kg bwt/day, Persistent vaginal cornification commences and administration of flutamide in 70% ethanol 10 mg/Kg bwt (s.c.) continuing with RU486	Subcutaneous injection
V	Autocorrection (N=Eight)	For 13 days 20 mg/ Kg RU486 in 0.2 mL olive oil bwt/day, Persistent vaginal cornification begins and is stranded on its own for revision	Lingual/ Oral

Table 2 — Histomorphometric analysis of follicular wall of ovaries in control and treated rats groups. Values represent mean ± S.E.M (μM)

Parameter	Control	PCOS induced	Noscapine treated	Flutamide treated
Follicle Mean Diameter	517.28 ± 0.58	809.37 ± 0.68	569.39 ± 0.09	687.29 ± 0.45
Granulosa layer thickness	54.49 ± 0.07	32.47 ± .38	83.69 ± 0.07	66.61 ± 0.0
Theca interna layer thickness	11.42 ± 0.01	21.07 ± 0.0	10.80 ± 0.01	17.23 ± 0.0
Theca externa layer thickness	9.17 ± 0.03	14.82 ± 0.07	9.93 ± 0.01	13.08 ± 0.01
No. of healthy antral follicles	220	30	180	200
No. of atretic follicles	6	13	8	8

were measured by using a competitive ELISA KIT (Dia Metra). Non-competitive ELISA KIT (DRG) determined serum FSH and LH levels. The assay was done on duplicates for all samples.

Image analysis

Image analysis was performed using Image Pro-Plus 3.0.1® system (Media Cybernetics, Silver Spring, MA, USA). The analog-to-digital conversion was done by a CCD color video camera for the images (Sony, Montvale, NJ, USA) and Nikon (Japan) mounted on top of a conventional light microscope Olympus BH-2, Olympus Co., Japan) using ×4, ×10, and ×40 objective lenses. Infinitesimal domains shrouding the entire follicular wall area were digitized and stowed in a 24-bit true-color TIFF format. Follicular mean diameter and, granulosa and theca thickness were analyzed using specific tools of the software. Delineation of the cyst profile and calculation of the area, perimeter, and full diameter of the cysts was done.

Isolation of rat granulosa and theca cells

Ovaries were dissected free of fat, bursa, and oviduct from different experimental groups; control, RU486 administered, noscapine administered, flutamide administered, RU486 + noscapine, RU486 + flutamide administered. For the isolation of pure granulosa and theca cells, ovaries were collected in ice cold M199 medium with 25 mM HEPES, (pH 7.4) and 1 mg/mL BSA[11]. The ovaries were washed twice with ice cold M199 medium. Follicles were pierced with hypodermic needles (27-gauge), and granulosa cells were released carefully in ice cold M199 medium with 25 mM HEPES, pH 7.4 and 1 mg/mL BSA and transferred to ice cold M199 medium. An aliquot was diluted with trypan blue stain, and viable cells were counted on a hemocytometer. For isolating theca cells, ovaries were washed twice in the medium and incubated at 37°C for 90 min in 100 µL/ovary of collagenase-DNase solution (4 mg/mL of collagenase, 10 mg/mL of DNase, and 10 mg/mL of BSA in M199 medium). The dispersed cells were then centrifuged at 250 × g for 5 min, washed and the final pellet was resuspended in 500 µL of ice cold McCoy's 5a medium. Isolated theca cells were counted on hemocytometer, and the cell viability determined by trypan blue exclusion. The isolated thecal cells were then purified by an optimized discontinuous Percoll density gradient centrifugation procedure. Dispersed cells (0.5 mL, 10^7 cells) were carefully layered on top of the d =1.055 Percoll solution. For 20 min the tubes were centrifuged (48°C) at 400×g, and the purified thecal cells inside the d=1.055 Percoll layer were aspirated. The purified cells were washed and the pellet resuspended in ice cold McCoy's 5a medium. They were further counted with a hemocytometer and cell viability was determined by trypan blue exclusion.

Flow cytometry analysis of cell cycle progression in granulosa and theca cells with noscapine treatment

A quantitative measure of cell cycle distribution was obtained by flow cytometric analysis of DNA histograms[12]. Granulosa and theca cells isolated from different experimental groups were fixed in 70% ethanol and stored overnight at 4-8°C. Flow cytometric analysis of granulosa cells and theca cells isolated from normal (vehicle administered), RU486 administered, RU486 + noscapine administered and noscapine administered, RU486 + Flutamide and Flutamide administered rat ovaries.

Following fixation, cells were washed with PBS two times, and then incubated for 30 min with 200 µg/mL RNase-A at 37°C, stained using 50 µg/mL propidium iodide (Sigma, USA) in PBS at 4°C for 30 min. at a density of 0.5 million cells/mL. Cell cycle distribution was studied with the help of FACS Caliber (Becton-Dickinson & Co., USA) flowcytometer using the Cell Quest (version 3.0.1; Becton-Dickinson & Co., USA) and Mod fit LT (version 2.0; verify software house, inc., USA) software for acquisition and analysis. A minimum of 10000 cells/sample was taken. The results were expressed as the proliferative index (the sum of percentages of cells in S and G_2/M phases of the cell cycle obtained from DNA histograms). This procedure was carried out in at least four replicates.

Analysis of DNA cleavage by TUNEL

Apo-BdU-IHC^{TM} *in Situ* DNA fragmentation assay kit (Biovision, USA) was used to study DNA breaks occurring during the apoptotic process. Sections of ovary from different experimental groups, control, RU486 administered, RU486 + noscapine administered were cut at 5 µM. Labeling was performed with DAB as the chromogen and counterstain as methyl green. Minimum 100 cells of each cell type were counted on digital micrographs taken by assembly of CCD color video camera (Sony, Montvale, NJ, USA) affixed on top of a light microscope Olympus BH-2, Olympus Co., Japan)

with ×10, ×40 and ×100 objective lenses. If too many cells were present in one micrograph, cells were counted in four grids of 2500 μM^2 that were projected randomly over the micrograph. TUNEL staining intensity scores were in following range, 0 (no staining), 1 (weak staining), 2 (moderate staining) to 3 (strong staining). Apoptotic score (AS) was calculated using the formula: $AS = 0n_0 + 1n_1 + 2n_2 + 3n_3$ with n_0, n_1, $n_2$and $n_3$are the percentage of cells exhibiting a staining intensity 0, 1, 2 and 3, respectively[13].

Statistical analysis

All Experiments were run in replicate and were repeated 3 to 4 times. Statistical Analysis was performed using the unpaired Student's *t*-test. $P < 0.05$ were considered significant. Error bars represent ± SEM. Tukey test[14] was performed to check any significant differences $P < 0.05$ were considered Significant. Error bars represent ± SEM.

Results

Establishment of PCOS rat model

Vaginal lavage was taken from 90 young rats, 64 rats had regular estrous cycle (Fig. 1) while 26 had irregular estrous cycle. Rats with irregular cycles were excluded from the study. Remaining animals were used for the proposed study. All of them received an oral dose of 20 mg/Kg bwt/day of RU486 in 200 µL olive oil at estrus stage for consecutive 13 days for the induction of PCOS. Control rats showed normal 4-5 day estrous cycle with characteristic cytology in different stages of estrous cycle. The results of the present study demonstrates transitory estrus state in rats (subjected to RU486 administration) similar to human vaginal cornification as observed in PCOS. Treatment with RU486 from day 1 to day 13 caused persistent vaginal cornification from day 8, which remained uninterrupted through the remainder of the experiment (until day 13). It was further substantiated by assaying the endocrine profile of the rats which showed the biochemical features in accordance with the PCOS profile.

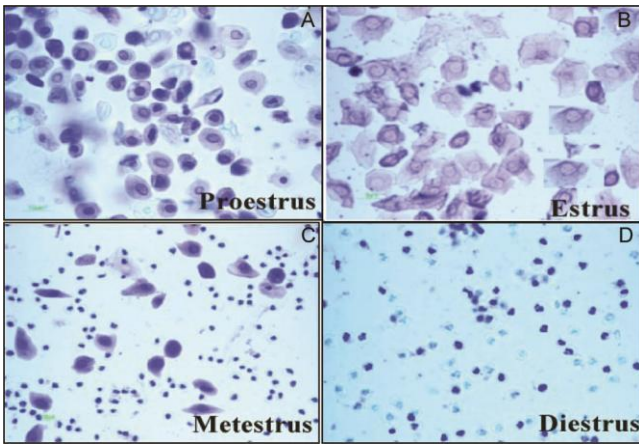

Fig. 1 — Representative photomicrographs at 10X showing characteristic cytology present in Vaginal smears obtained during four phases of the estrous cycle of the rat as mentioned in Table 1. Classified as Proestrus, Estrus, Metestrus and Diestrus

Effect of noscapine in PCOS induced rats

Cyclicity

PCOS induced rats administered with dose of 20 mg/Kg bwt/day and 80 mg/Kg bwt/day of noscapine did not reform the cycle arrested in 'Persistent estrus'. However, animals receiving noscapine (grp. III), flutamide (grp. IV) and auto correction (grp. V), reverted back to regular cyclicity in 3-4 days, 13-17 days, 17-21 days, respectively, starting from the day of treatment as observed by the resumption of regular estrous cycle.

Histopathology and morphometric analysis

Study of ovarian sections received from the control rats exhibited the presence of all stages of follicular development, including primary follicle, secondary follicle, antral follicle and corpora lutea (Fig. 2A). It undergoes luteinisation as evident by the formation by corpora lutea (Fig. 3A). Ovaries from rats receiving RU486 exhibited several ovarian alterations. Fluid filled cysts and few antral follicles were noted in the ovarian stroma (Fig. 2B). Cystic follicles formed under the influence of RU486 showed unusual thin granulosa layer with hypertrophied theca layer.

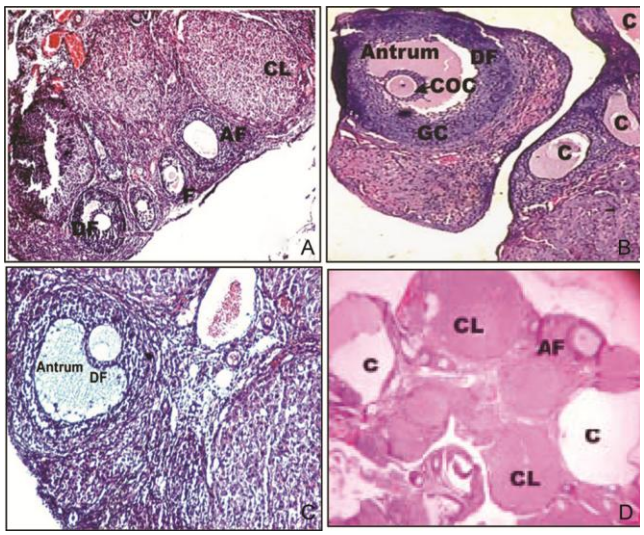

Fig. 2 —Hematoxylin and Eosin stained Representative section from the ovaries of Female Wistar rats receiving (A) olive oil; (B) RU486 (anti-progestin); (C) Noscapine ; and (D) Flutamide at 10X

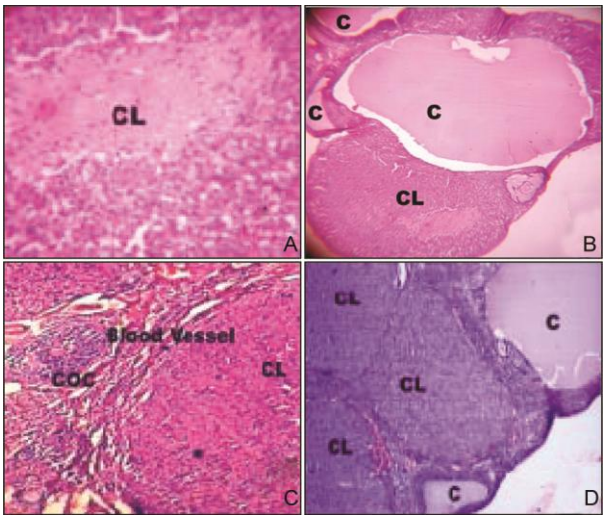

Fig. 3 — Hematoxylin and Eosin stained representative section from the ovaries of Female Wistar rats receiving (A) olive oil; (B) RU486 (anti-progestin); (C)Noscapine; and (D) Flutamide

Follicular cyst was completed formed when the entire cyst was lined by a single layer of transformed granulosa cells in contact *via* adhering gap, and tight junctions. Large cystic follicles (964 ± 37.43 µM) were found in abundance (Fig. 2B). Majority of the small follicles were atretic (about 87 %) with a thickened theca layer. Large cysts upto 1.7 ± 0.5 mM occupied the ovary in two of the experimental rats (Fig. 3B). Noscapine dosage brought noteworthy histopathological alterations in PCO like development of normal follicles in previously polycystic ovaries (Fig. 2C). Fig 3 Chighlights the healthy developing follicle and release of cumulus oocyte-complex between two corpora lutea respectively. Group IV rats *i.e*. flutamide treated rats underwent partial correction as observed in the micrographs. Figure 2D focuses on a pool of cysts, corpus luteum along with atretic follicles. Healthy developing follicles, corpus luteum were observed signifying follow up of regular ovulatory events (Fig. 3D). Table 2 reveals the histomorphometric analysis of follicles and cysts in different experimental groups.

Hormonal assays

Hormonal profile of RU486 administered PCOS induced rats was consistent with the hormonal profile reported in PCOS patients. Serum hormone levels ofrats administered with RU486when compared to control rats in estrus, had increased serum LH (6.88 ± 0.21 mIU/mL, Student's *t*-test; ***P*< 0.05) and decreased FSH (3.16 ± 0.64 mIU/mL, Student's *t*-test; **P*< 0.01) concentrations (Fig. 4D & E), and an increased LH/FSH ratio (2.17 ± 0.32). Raised serum concentration of estradiol (35.27 ± 5.49 pg/mL, Student's *t*-test; ***P*< 0.05) and testosterone (4.3 ± 0.261 ng/mL, Student's *t*-test; ***P*< 0.05) were also observed in PCOS mimicked rats when compared to control. Decreased LH concentrations was found in PCOS rats, post noscapine treatment (3.9 ± 0.17 mIU/mL, Student's *t*-test; ***P*< 0.05) and also in rats treated with flutamide (4.0 ± 0.21 mIU/mL, Student's *t*-test; ***P*< 0.05). In addition, LH/FSH ratiobecame comparable to normal controls (0.75 ± 0.21) against noscapine administered PCOS induced rats (0.9 ± 0.5) and post flutamide treatment (0.88 ± 0.08) (Fig. 4F). However, serum FSH levels in RU486 administered PCOS induced rats were not significantly different with FSH levels in noscapine and flutamide administered (4.3 ± 0.39 mIU/mL; 4.5 ± 2.6 mIU/mL respectively) rats. Administration of noscapine and flutamide significantly decreased serum testosterone concentration in PCOS induced rats (2.6 ± 0.06 ng/mL, Student's *t*-test; ***P*< 0.05, 2.8 ± 0.8 ng/mL, Student's *t*-test; ***P*< 0.05) (Fig.4C). Decreased sera levels of estradiol was observed in rats administered with noscapine (28.26 ± 3.4 pg/mL) and flutamide (30 ± 5.1pg/mL), which were not significantly different from PCOS induced rats (Fig. 4B). In addition, sera collected from autocorrection group were also assayed. As compared to the findings observed in other groups, serum levels of LH and FSH were found to show dissimilar profile in autocorrection group when compared to rats of control group (Fig. 4E). There were no differences between baseline levels for mean testosterone, estradiol and progesterone concentration against control group. However, LH and FSH levels were found to be significantly elevated (5 ± 0.31 mIU/mL, Student's *t*-test; **P*< 0.01, 2.4 ± 0.75 mIU/mL, Student's *t*-test; **P*< 0.01, respectively), than the control.

Isolation of granulosa and theca cells

To study cell cycle distribution by flow cytometry in different ovarian compartments, granulosa and theca cells were isolated from different experimental groups *i.e*. Control, RU486 administered, RU486 + noscapine administered, noscapine administered, RU486 + flutamide administered and flutamide administered rats. Granulosa cells were isolated and then theca cells micro-dissected from the ovary. Granulosa cells represented fairly homogeneous population interconnected by gap junctions. Pure

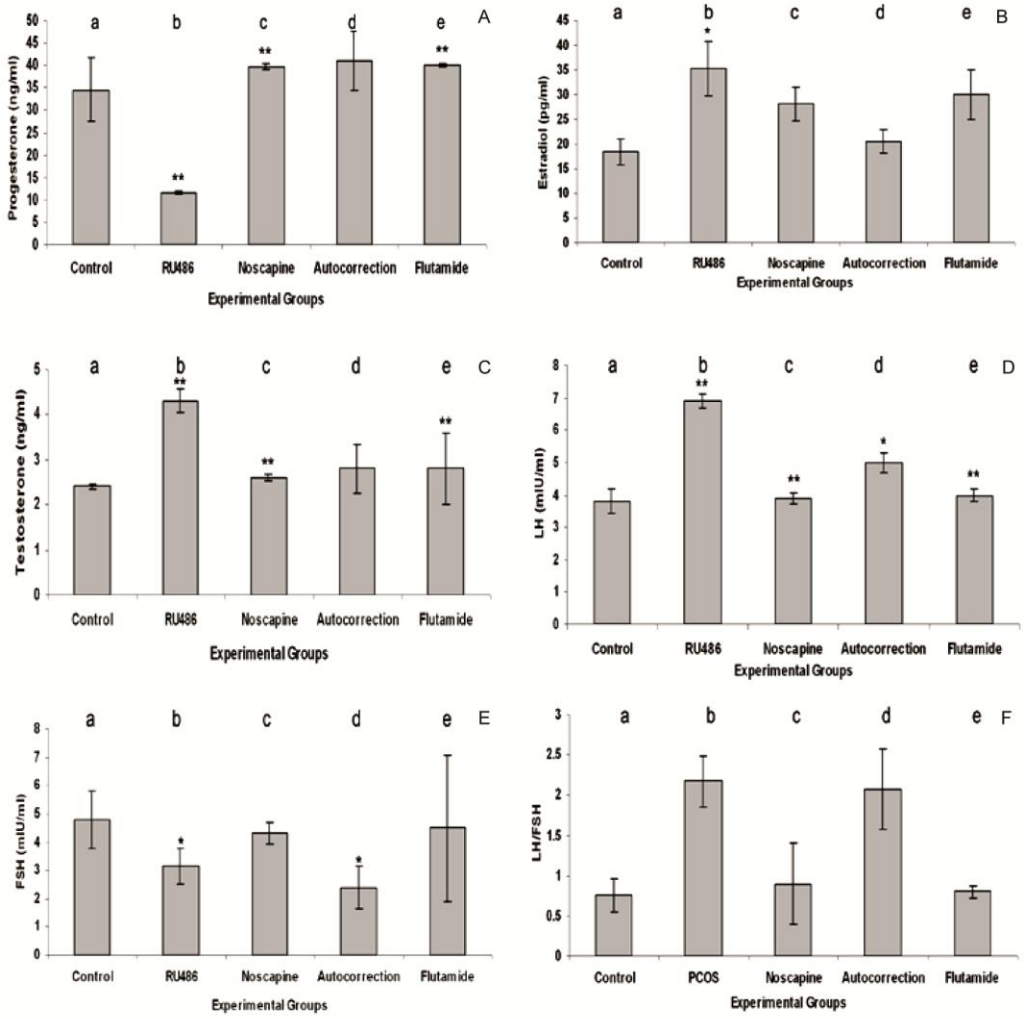

Fig. 4 — Hormonal evaluation of rat serum receiving different therapeutic modality according to Table 1. (A) Progesterone, (B) Estradiol; (C) Testosterone; (D) LH; (E) FSH; and (F) LH/FSH in (a) control (b) RU486 administered (c) noscapine administered (d) auto correction; and (E) flutamide administered rats. Values and error bars shown in the graphs represent the averages and standard error mean, respectively.Values are means ± SEM. Student's *t*-test was applied on following comparisons a *vs*. b, b *vs*. c, b *vs*. e, a *vs*. d ; ***P*< 0.05 and **P*< 0.01

granulosa cells were isolated by chelating the calcium (with EGTA), thereby dissociating gap junctions and leaving tight junctions intact. Theca cells and granulosa cells isolated were different in their morphology, theca being elongated rather than spherical. Granulosa cells shape was more oval than that of cells from the other culture groups.

Flow cytometric analysis of granulosa and theca cells

Granulosa cells

Fig 5A-D represents the granulosa cell-cycle distribution in different experimental groups and (Table3) tabulates cell-cycle distribution of granulosa population at various phases. Untreated cells (control) showed a normal progression of cells through G_1 to S transition wherein, 51 % cells in G_0/G_1 are with unduplicated (2N) DNA content and 8 % in G_2/M are with duplicated (4N) DNA content and 41% cells are in S phase (Fig. 5A). In contrast, RU486 treatment in rats resulted in an increase in cell population in G_2/M phase. Furthermore, a characteristic hypodiploid population, sub-G_1 is seen subsequent to the G_2/M block, and is comparatively prominent in the cells isolated from same group. The progressive accumulation of cells having hypodiploid DNA content (approximately 33%) reflects cellular degeneration (Fig. 5B). In group of rats administered RU486 followed by noscapine, G_2/M phase cell population increases from 8% in the control to 15 % with a small sub-G_1 peak indicating marginal cellular degeneration (Fig. 5C). However, the granulose cells

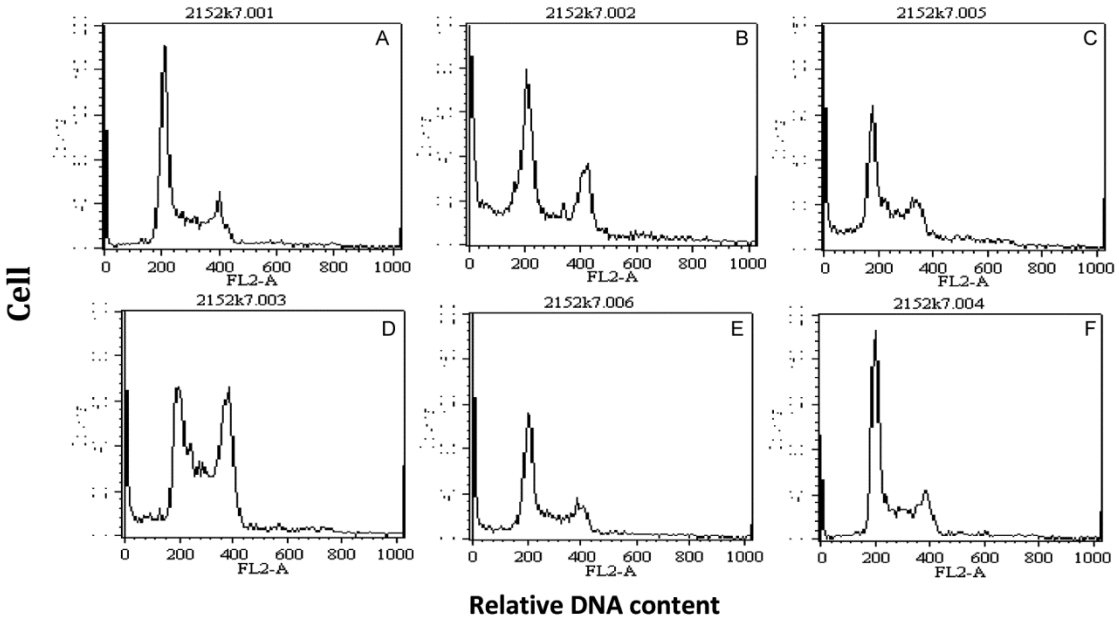

Fig. 5 — Flow cytometric DNA histograms showing treatment induced alterations in cell cycle distribution and cellular degeneration of granulosa cells. One representative experiment out of 3 is shown. (A) Control; (B) RU486; (C) RU486+Noscapine; (D) Noscapine; (E) RU486+ Flutamide; and (F) Flutamide

Table 3 — Cell Cycle progression of Granulosa cells from ovaries of various experimental groups as mentioned in Table 1

Groups	*Treatment*	Cell Cycle %G1	%S	%G2+M
A	Vehicle only	51	41	8
B	RU486	58	25	17
C	RU486+noscapine	43	42	15
D	Noscapine	19	52	29
E	Flutamide	52	39	9
F	RU486+ Flutamide	52	37	11

isolated from post noscapine administration to normal rats showed 29% of cells in G_2/M phase and only 19 % of cells in G_1 phase (Fig. 5D). Thus, we observed different behaviour of noscapine in presence and absence of RU486. In absence of RU486, noscapine arrested the granulosa cells in G_2/M phase but in the presence of RU486, the arrest was not observed. On flutamide administration to PCOS induced rats, more or less similar cell cycle profile to controls was observed signifying no effect of flutamide on PCOS induced rats (Fig. 5E) and also administration of flutamide alone to normal rats didn't alter the granulosa cell cycle distribution (Fig. 5F).

Theca cells

Fig 6A-D panel represents the theca cell-cycle distribution in presence of different drug modalities and (Table4) tabulates DNA histograms in different phases of cell cycle. Theca cells isolated from RU486 administered PCOS induced rats didn't show altered cell cycle profile w.r.t control group (Fig. 6B). However, there was significant alteration in the progression of theca cells through cell cycle post noscapine administration. Only 14% of cells were in G_1 phase while 79% of theca cells were arrested in G_2/M phase of cell cycle (Fig. 6C). A profound hypodiploid DNA content peak (sub-G_1) at the left side of G_1 peak in the panel was seen in the cells isolated from RU486 + noscapine administered rats. Cells isolated from rats administered noscapine only, showed similar profile to the noscapine + RU486 administration group with 78% of cells arrested in G_2/M phase (Fig. 6 D). Neither RU486 + Flutamide administration to PCOS induced rats nor flutamide administration to control rats altered the cell cycle profile w.r.t to control (Fig. 6E)

Taken together, both the results highlight different effects of noscapine in PCOS rats on cell cycle distribution of granulosa and theca cells. Theca cells undergo massive cellular degeneration with arrest in G_2/M phase whereas, granulosa cells showed marginal cellular degeneration without any arrest in G_2/M phase. But interestingly, we observed G_2/M block in granulosa cell population from normal rats, post noscapine administration.

Localisation of apoptosis by TUNEL assay in rat ovaries

The TUNEL method[13] was used to detect DNA fragmentation, which is one of the prominent

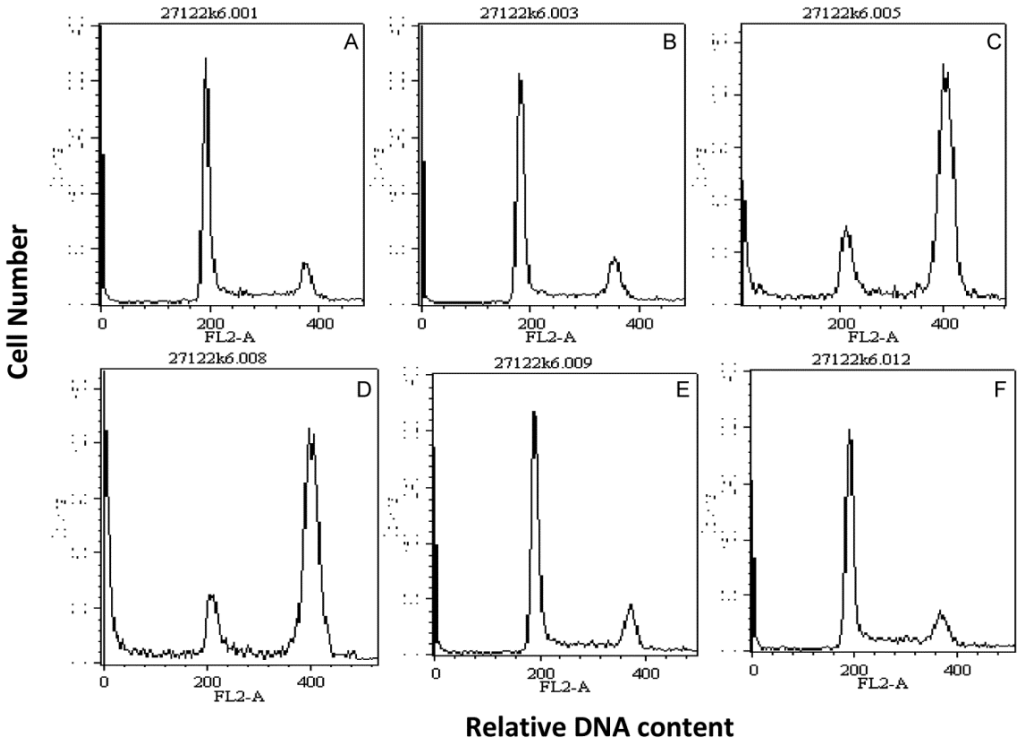

Fig. 6 — Flow cytometric DNA histograms showing treatment induced alterations in cell cycle distribution and cellular degeneration of theca cells. One representative experiment out of 3 is shown (A) Control; (B) RU486, (C) RU486+Noscapine; (D) Noscapine; (E) RU486 + Flutamide; and (F) Flutamide

Table 4 — Cell Cycle progression of Theca cells from ovaries of various experimental groups as mentioned in Table 1

Groups	*Treatment*	Cell Cycle %G1	%S	%G2+M
A	Vehicle only	63	29	9
B	RU486	67	27	11
C	RU486+noscapine	14	8	79
D	Noscapine	15	8	78
E	RU486+ Flutamide	67	29	5
F	Flutamide	64	26	11

morphological changes of the apoptotic cellular death. The dark brown stained cells signifies fragmented DNA, a hall mark of apoptosis whereas the methyl green is taken by TUNEL negative cells. As evident in (Fig. 7A, D, and G) control rat ovaries, exhibited high level of apoptosis in granulosa cell layer, low levels of apoptosis in theca interna and moderate levels in theca externa cells. This depicts death of granulosa or luteal cells in the CL is a natural fate at the end of each cyclical period. Follicles from ovaries of RU486 administered rats exhibited apoptosis which, was confined to granulosa cells, indicating it as a major cell type undergoing apoptosis. However, low apoptotic DNA fragmentation levels was noted in theca cells. The stroma, surface epithelium also contained apoptotic cells. Apoptosis was quite evident in cystic and atretic tertiary follicles with a high apoptotic score in the granulosa cells and a low score in the theca layer. These polycystic rat ovaries also displayed high number of apoptotic cells around cystic walls (Fig. 7B & E). In contrast to the control and noscapine administered rats, granulosa lutein cells showed low levels of apoptotic DNA fragmentation in corpus lutea of PCOS ovaries (Fig. 7H). However, unlike RU486 administered rats, differential effect of noscapine was observed in both the cellular compartments. Apoptosis was evident in capsular stroma cells and granulosa-lutein cells of corpora lutea. TUNEL staining revealed increasing number of apoptotic cells in follicles predominantly in the granulosa layer, and theca externa layer (Fig. 7C, F, and I). Thus besides granulosa layer, noscapine was shown to exert its apoptotic effect on the granulosa lutein and theca layer.

Discussion

Administration of anti-progestin RU486 for 13 days in rats shows hallmarks distinct from human PCOS. For instance, it includes ovulatory failure, persistent vaginal cornification, enlarged ovaries

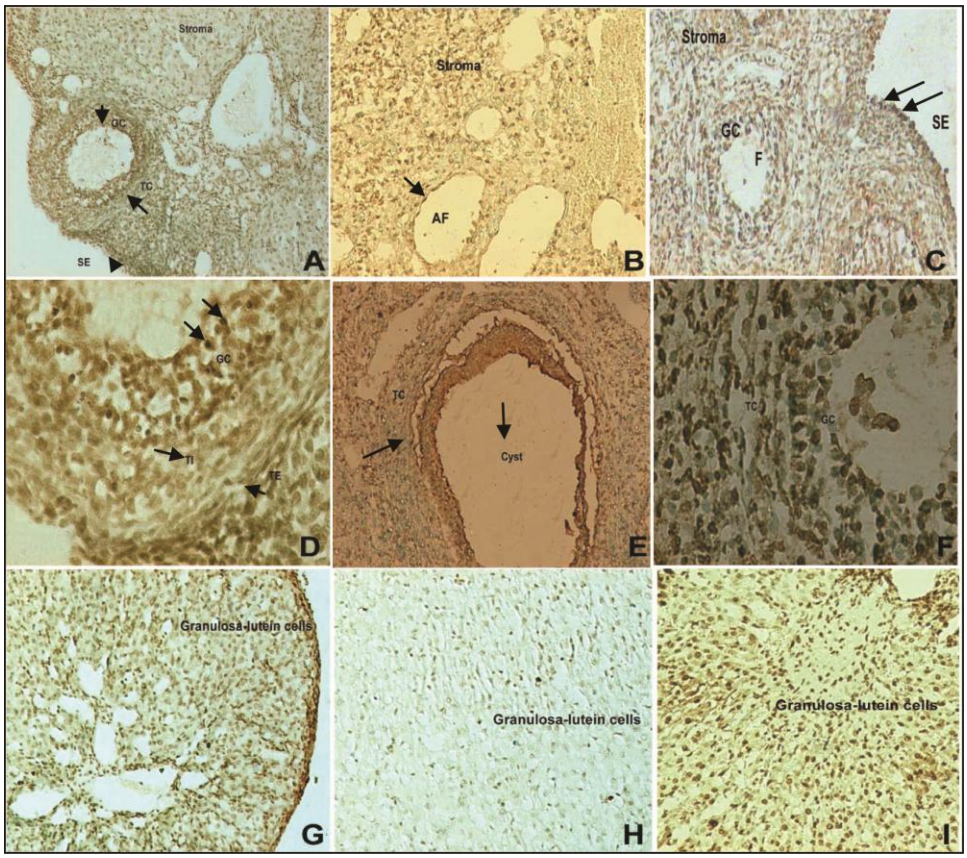

Fig. 7 — Apoptosis detection using TUNEL assay in ovarian tissue sections from control (A, D, G), RU486 administered (B, E, H) and noscapine-administered rats (C, F, I) as mentioned in Table 1(A) Overview of follicle with abundant TUNEL positive cells in the granulosa layer (GC), as compared to theca layer (TC), surface epithelium also showed significant number of apoptotic cells. (D) At higher magnification, 40X depicting differential DAB accumulation in granulosa, theca interna (TI) and theca externa (TE) cells. (G) TUNEL positivity can be observed in granulosa-lutein cells of corpus luteum (CL). (B) Cystic atretic follicle with high number of apoptotic cells in the granulosalayer. (E) Apoptosis in granulosa layer of cystic follicle and TUNEL negative cells in theca layer. (H) Comparatively very low level of apoptosis in lutein cells of corpus luteum (CL). (C) The surface epithelium (SE), stroma and granulosa layer show TUNEL positive cells. (F) At higher magnification 40X showing pronounced DAB accumulation in granulosa cells of follicle (f), (I) Overview of a corpus luteum with increased numbers of apoptotic cells in the lutein cells

containing atretic follicles, and follicular cysts. It is accompanied by increased serum LH, testosterone, and estradiol concentration and decreased FSH, progesterone levels. The rat's retort to therapies used in PCOS medicament is similar to humans. Therefore, theca interna RU486 administered rats are considered "fundamentally adequate" PCOS replica[14] for the therapeutic efficacies for various drug entities for treatment of PCOS condition. In an attempt to explore the potential effect of noscapine, an opium alkaloid, in the management of PCOS in non-human species, RU486 administered PCOS-induced rats were established in our laboratory with nuanced changeovers in the protocol mentioned by Sanchez-Criado and others in 1992. The animal model for PCOS was established by administering the rats orally for 13 consecutive days in olive oil with RU486 (20 mg/Kg bwt/day). In the PCOS animal model, RU486 being an anti-progestin blocks the progesterone receptors and brings about alterations in hypothalamic-pituitary secretions by increasing LH levels and influencing LH/FSH ratio. The underlying cause of PCOS induced by RU486 lies in programming LH secretion, which facilitates hyper-androgenemia. Also, the lack of progesterone triggers a deluge of endocrine events that creates an anovulatory cystic condition similar to human PCO prerequisites. This in turn disrupts the normal cyclicity of rats and arrests them at a stage known as 'Persistent vaginal cornification' (PVC). Thus, rat PCOD models characterize the polycystic ovaries formation with PE association, denoting chronic anovulation.

As continuing research illustrates the association of endogenous opioid compounds with various disease states, a possible therapeutic role of noscapine

couldn't be undervalued. The relationship between gonadal steroids and opioid peptides at the brain and peripheral level is well. More so the mechanism for the action of noscapine as an antitussive agent is believed to be mediated through the central nervous system as its receptors are predominantly braining specific (hypothalamus). Therefore, the hormonal profile in noscapine and flutamide administered PCOS-induced rats were monitored and compared with control rats. When hormonal profiling was performed on PCOS-induced rats, it was comparatively similar to the profile observed in PCOS patients. There was a substantial drop in serum testosterone and LH levels post noscapine treatment in PCOS rats when compared with PCOS-induced rats who were given flutamide and in cases obliterated for auto-correction. Flutamide treatment alone did not completely reverse the effects of RU486. Concerning this, noscapine is a better recourse that revamps and thoroughly boomerangs the RU486 effect on PCOS-induced rats. We believe, the elevated testosterone levels (4.3 ng/mL) in RU486 administered rats which was brought down to the level of 1.6 ng/mL by noscapine acts on granulosa cells to increase the FSH levels resulting in a concomitant decrease in LH levels. Thus, there is a comprehensive haul in the paradigm of hormones, which retrogresses the cystic and anovulatory conditions observed in PCO-inducted rats to normal. It is possible that a noscapine-induced decline of androgen levels may have led to an improvement of hypothalamic-pituitary-ovarian axis function because of its action on the hypothalamus.

A complex set of interactions between the theca and granulosa cells (GC) ensures that the ovarian follicle attains the capacity to secrete estrogen at the appropriate stage of development of follicles. Sections of ovary from rats receiving noscapine revealed reversal of thickened theca layer back to its normal range. Similar morphometric features were noticed in sections obtained from flutamide administered rat group. In light of ELISA results where LH and estrogen basal level increases and FSH level decreases in PCOS induced rats, this seems quite plausible. These findings support the concept that in humans, FSH stimulates the secretion of paracrine factors from granulosa cells, which in turn stimulate the production of androgens by thecal cells in response to LH.

Noscapine is well documented to induce apoptosis so we isolated pure population of granulosa and theca cells from ovaries of all experimental groups and evaluated their DNA content on stipulated days. Granulosa cells of PCOS induced rats showed that more than 51% of granulosa cells exposed to RU486 were unable to progress from the G1 to the S phase, exhibiting a block at the G_1/S transition. Additionally, we noted substantial cells in sub G1 phase (approximately 33%) in the granulosa cells isolated from RU486 administered rat ovaries indicating massive cellular degeneration. Our results go in concordance with earlier reports wherein, specifically, granulosa cell mitosis and apoptosis is inhibited by progesterone in a steroid-specific, dose-dependent manner. However, the mechanism through which progesterone regulates mitosis and apoptosis of granulosa cells is ill defined. In the PCOS model established in the study by antiprogestin RU486, progesterone is withdrawn from the rats thereby, making the cells susceptible to apoptosis. Following noscapine administration post RU486 treatment, granulosa cells were found predominantly in G1 phase, removing the G2/M block post RU486 treatment, and reverting back to their normal cell cycle status, a sign of physiological normalcy. However, theca cell cycle showed different cell cycle distribution in experimental groups w.r.t noscapine when compared to granulosa cells. Theca cells isolated from RU486 administered, PCOS rats didn't show altered cell cycle profile w.r.t control group. However, there was significant alteration in the progression of theca cells through cell cycle post noscapine administration. Only 14% of cells were in G1phase while 79% of theca cells were arrested in G2/M phase of cell cycle. Here, we also observed significant cells in sub G1 phase indicating massive cell death. Neither flutamide administration to PCOS induced rats nor administration of flutamide alone to normal rats altered the cell cycle profile w.r.t to control. Furthermore, to assess the mode of cellular degeneration in these cells in presence of noscapine and RU486, TUNEL assay was carried out. We observed that the cell death was apoptotic in nature as evident from the TUNEL-positive apoptotic cells in ovarian tissue sections from different experimental groups. The *in situ* analysis also showed that the granulosa cells are the chief cell population to undergo apoptosis, whereas theca cells show relatively less apoptosis. In contrast to the granulosa cell population of control rat section, which is entirely eliminated through apoptosis, not all follicular theca cells became apoptotic. RU486 administration

amplified apoptotic DNA fragmentation in granulosa cells of early antral and preantral follicles, but not in compartment of theca cells, when analyzed in situ. This is quite interesting and plausible finding in accordance with the flow cytometry data of same subset of cells where a profound sub G_1 peak was observed indicating cellular degeneration. Noscapine seems to act directly on hyper-proliferated theca layer in PCOS induced rat ovaries, thereby decreasing the production of testosterone that was aromatized to estradiol in PCOS induced rats. By balancing the estradiol to testosterone ratio, the normal functioning of ovary is achieved, healthy follicles are recruited and cystic follicles undergo regression, as evident in histopathological findings. But still we don't rule out the possibility that the effect of noscapine is also simultaneously centrally mediated through hypothalamo-pitiutary-ovarian axis.

Conclusion

Present results extend previous observations on the action of noscapine on different cancer cell lines as an antiapoptotic agent and apprise thriving action of noscapine in the amelioration of PCOS-induced rats as evident by physiological, gross morphological, histopathological and biochemical parameters. It alters the hormonal profile by meddling with feedback mechanisms in the hypothalamic-pituitary-ovarian axis. With the added advantage of being a safe oral therapy, and having relatively negligible side effects, noscapine (120 mg/kg bwt.) seems to be a promising PCOS medicament, as a putative drug. It not only acts centrally but also has the direct effect on the ovary by inducing apoptosis in hypertrophied theca layer. It was envisaged that noscapine can broaden its horizon from being an antimitotic and antitussive drug. It can treat specific pathological conditions associated with the hyperproliferation of cells involved in PCOS It is a preliminary report and a better understanding to mechanistic analysis of molecular mechanisms of effects of noscapine in PCOS induced rats can led to emergence of a potent therapeutic option.

Conflict of interest

All authors declare no conflict of interest.

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