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Determine Cyp17a1 and Ki67 Expressions in PCOS Induced Rat Model Treated with *Sepia pharaonis* Ink Extract Proves Effective

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ABSTRACT

Background: The present study aims to evaluate the therapeutic action of *Sepia pharaonis* ink extract in experimentally induced polycystic ovary syndrome (PCOS) rat model and perform Immunohistochemistry to confirm the findings

Methods: The female rats were grouped into 5 consisting of six animals each. Only water was given to the control group. For inducing PCOS, Letrozole 1mg/kg.b.wt was administered to the subsequent four groups for 21 days. To the third group, an allopathic drug, clomiphene citrate 1mg/kg was given for 19 days after PCOS induction. The SPIE was subjected to lyophilisation to make a black powder and next two groups were administered orally with two different dosages of SPIE (100 mg and 200 mg respectively) mixed with water after PCOS induction. Vaginal smears were taken from all groups till 21 days and then treatment was started with clomiphene citrate (CC) and SPIE. After 21 days, vaginal smears were taken from all groups till ovulation was induced. The test drug effects were studied using ovary weight both right and left, body weight, hormonal levels, histopathology of ovary and Immunohistochemistry.

Result: PCOS group showed rapid increase in the ovarian weight when compared to control and was reduced by CC and SPIE treatment. The levels of progesterone and estradiol were found lower and testosterone levels were found higher in PCOS rats which were favourably altered. Vaginal smears coincided with the histopathology and immunohistochemistry findings. SPIE can be used as a therapeutic drug for PCOS in future, reduce infertility complaint of females and prevent early inception of diabetes mellitus.

Key words: Histopathology, Immunohistochemistry, Polycystic ovary syndrome, *Sepia pharaonis* ink extract.

INTRODUCTION

Marine environment includes complex ecosystems and various organisms that are branded to possess bioactive substances as a general means of self-defence or for self-protection. Recently, many bioactive compounds have been taken out, categorized and purified from diverse marine animals such as cephalopods (Nair *et al.,* 2011) Sepia pharaonis, is one among the most significant cephalopod fishery species in south-eastern Asia (Lee *et al.,* 2016). *Sepia pharaonis* ink extract is known to have several beneficiary effects. It has been used for treatment purposes. It is to be noted that endocrine metabolic dysfunction affecting 5%-20% women of reproductive age is polycystic ovary syndrome (PCOS). According to the older data, identification of PCOS in women was ascertained with irregular periods, presence of polycystic ovaries and the hyperandrogenism, increased insulin level, hormonal dysfunction, stressand increased stimulation of adrenals are measured to be most important risk factors in the expansion of PCOS (Hamza *et al.,* 2019).

Recent studies show that PCOS influences 10% of women of reproductive age and is most common in developed countries. The PCOS aetiology is not fully understood (Rodgers *et al.,* 2019). PCOS is considered as the most common women endocrinopathy. Clomiphene citrate, which is widely used for treating PCOS condition

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(Sushma *et al.,* 2016). The cuttlefish ink extracts have good antibacterial activities also. Using agar well diffusion technique, when *Sepia pharaonis* was tested against human pathogens, it showed an inhibitory effect against these pathogens (Senan *et al.,* 2015).

Different species of squid and cuttlefish also contain nutraceutical properties together with the use as a food item. Astoundingly, the inks of these different species contain different nutraceutical properties. This ink is a great source for reducing various health problems (Hossain *et al.,* 2018). Additionally, Quercetin, a flavonoid, had shown effects by

diminishing body weight, diameter of ovary, cysts and repairing healthy follicle against the positive control. The effects of quercetin are fairly similar to metformin. Therefore, quercetin also has potentials to ease the metabolic and hormonal disturbances resulting in PCOS (Jahan *et al.,* 2018).

Histopathological analysis of liver treated with Letrozole illustrated moderate degeneration of hepatocytes. Thus, the Sepia ink provides a healing effect and an antioxidant capacity on BDL rats that could restructure the complications of liver cholestasis (Saleh *et al.,* 2015).

Even though, the ink extract possesses various pharmacological properties such as antioxidant, anticancer, antimicrobial and antiretroviral properties it could not be used as a pharmacological agent (Anisuzzaman *et al.,* 2001). This present finding helps us to select this therapeutic study of SPIE on PCOS experimental model. Thus, the present study measured certain hormonal parameters to discover the action of *Sepia pharaonis* ink extract in female wistar albino rat model of polycystic ovary syndrome (PCOS). Moreover, it also discussed the histological and Immunohistochemistry changes in the ovaries in response to the respective treatment (Fig 1).

MATERIALS AND METHODS

Test drug

Sepia pharaonis fishes were obtained from seashore, Tamil Nadu. It was informed by the Zoological Survey of India that these species belonged to the *Sepia pharaonis* (Ehrenberg, 1831). Fishes transferred to the laboratory and the ink was squeezed out from the ink sac by milking procedure and taken in a sterile glass ware. Then lyophilisation was done to make the ink into a black powder. The lyophilized black powder of SPIE was kept in sterile condition.

Experimental animals

Virgin female wistar albino rats aged three to four months were selected for the present study. The weights of the rats ranged from 200-240 gms and were allowed to get adapted to the condition for about a week. Under hygienic condition, the rats were kept in the cage and the temperature varied from (22 ± 3°C). They were kept in the artificial light and ensured 12 hours' day and 12 hours' night cycle. They had unrestricted access for food and water. The experiment was done in Biomedical Research Unit and Laboratory Animal Centre (BRULAC), Saveetha Dental College and Hospital, Velappanchavadi, Chennai-600077, Tamil Nadu, India in the year 2018 conferring to the strategies of Institutional Animal Ethical Committee (BRULAC/SDCH/SIMATS/IAEC/09-2018/012) of CPCSEA.

Experimental design

The female rats were categorized into five groups with 6 animals in each group. Only distilled water was given to the control group. For 21 days, letrozole 1mg/kg b.wt was administered to the subsequent groups to induce PCOS for other four groups. Subsequently, the PCOS model evaluation with an anovulatory estrous cycle was verified by analysis of vaginal smear, for which the first one-third of the vaginal wall was taken daily to evaluate the stage of the 4-day ovarian cycle during the examination of the study period. Group II is only PCOS induced. For 19 days, an allopathic drug, clomiphene citrate 1mg/kg was given to the

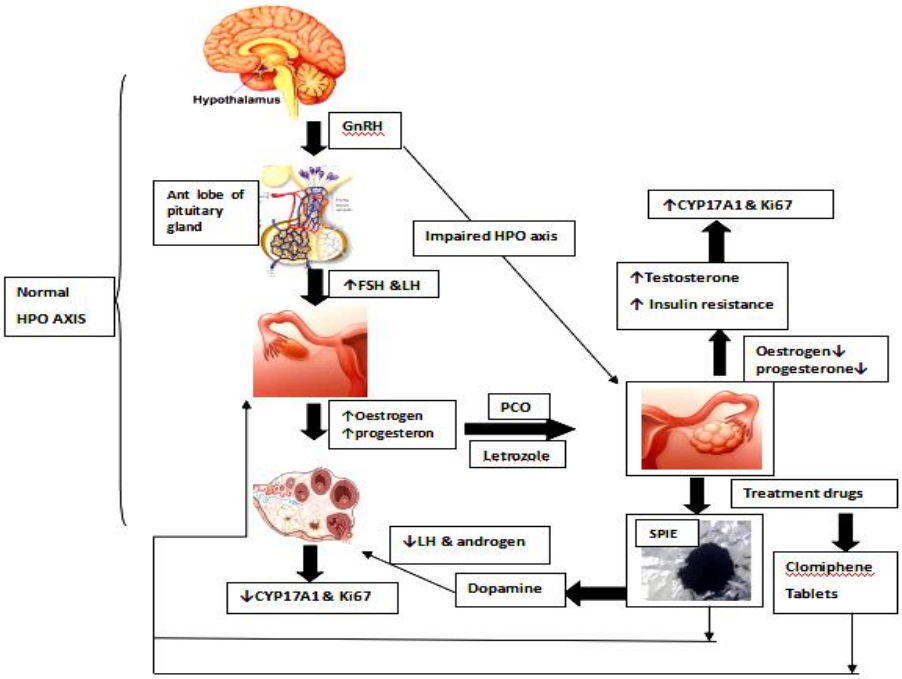

Fig 1: The action of treatment drug SPIE on the target region "the ovaries". HPO-Signifies-Hypothalamo-pituitary-ovarian axis, SPIE-*Sepia pharaonis* ink extract, CYP17A1 and Ki67-Pcos specific Immunohistochemical protein expression.

standard treated group III. The different doses of SPIE (100 mg and 200 mg respectively) were administered to the next two groups along with water to test its beneficial action.

Collection of tissue and blood samples

All animals were weighed at the beginning and end of study. The rats were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg) and xylasine (12 mg/kg). The blood samples were obtained by retro-orbital puncture and transferred to EDTA-containing tubes. Plasma was separated by cold-centrifugation (4°C) at 3,000 rpm for 10 min. Ovarian tissues were excised for histological analysis and fixed in 10% formaldehyde (PBS 10 mM, pH 7.4). Plasma samples were stored in a freezer at -80°C for biochemical analysis.

Assay of biochemical parameters

Serum levels of estradiol, testosterone and progesterone were determined using a competitive enzyme-linked immunosorbent assay using commercially available ELISA kits (Elab Bioscience).

Histopathological study

Ovaries were fixed in 10% formalin at RT for a week. In a graded series of ethanol, the specimens were dehydrated, cleared in xylene and fixed in paraffin wax. Using a rotary microtome, tissue blocks were partitioned into 5 µm thickness. Sections were stained and viewed under the light microscope by a qualified pathologist.

Immunohistochemistry study

Immunohistochemically analysis was performed on cytochrome P450, 17alpha-hydroxylase protein staining in ovary tissues. The tissues of each group were immersed in the fixative solution for 4 h. The tissues were cryoprotected in 30% sucrose, embedded in tissue-freezing medium with liquid nitrogenand cut into frozen sections (3-5 µm) using a cryostat. Sections were stored under anti-freeze buffer. Parallel free-floating sections were subjected to endogenous peroxidase quenched with 1% H_2O_2 in PBS, followed by treatment with blocking buffer (5% normal chicken serum in PBS and 0.3% Triton X-100 for overnight at 4°C) and incubated with CYP17A1 primary antibody (dilution 1:500). After washing with PBS, tissues were incubated with a biotinylated goat anti-mouse secondary antibody (dilution 1:200). The tissues were subsequently exposed to an avidin-biotin peroxidase complex for 2 h. The peroxidase activity was visualized using a stable diaminobenzidine solution. All immunoreactions were observed using a compound light microscope.

The tissue sections were subjected to endogenous peroxidase quenched with 1% H_2O_2 in PBS, followed by treatment with blocking buffer (5% normal chicken serum in PBS and 0.3% Triton X-100 for overnight at 4°C) and incubated with ki67 primary antibody with the dilution 1:200. After washing with PBS, tissues were incubated with a biotinylated goat anti-mouse secondary antibody 1: 100 for 8hrs at 4°C. The tissues were subsequently exposed to an avidin-biotin peroxidase complex for 2 h. The peroxidase activity was visualized using a stable diaminobenzidine solution. All immunoreactions were observed using a compound light microscope.

RESULTS AND DISCUSSION

Effect of SPIE on reproductive hormone levels

The data on the serum levels of progesterone, estradiol and testosterone in control and different treatment groups are presented in Fig 2. Data discovered a significant reduction

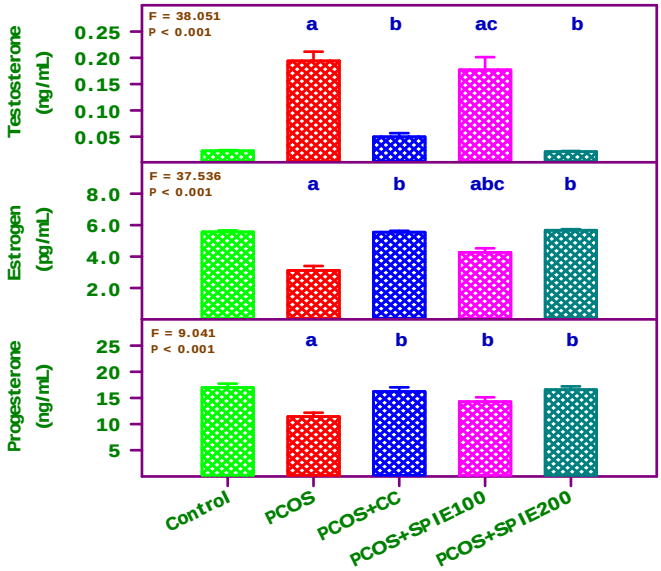

Fig 2: Effect of SPIE on the level of progesterone, estrogen and testosterone (E2) in control and other treatment group. Values are mean±SE (n=6 each). The 'F' and 'P' values are by one way ANOVA with student Newman Keul's multiple comparison test. Progesterone: F value= 9.041 and P value<0.001, Estrogen: F value= 37.536 and P value<0.001, Testosterone: F Value= 38.051 and P value<0.001. A significantly different from control group, b significantly different from PCOS group, c significantly different from SPIE+CC group.

in progesterone, estradiol accompanied by a rapid increase in the testosterone levels in rats with PCOS when compared to the healthy control group. Diseased rats treated with clomiphene citrate showed a remarkable decrease in the testosterone levels and significant increase in the estradiol and progesterone levels when compared to PCOS group. Consistently, a minor regression in the levels of testosterone accompanied by a rapid increase in estradiol and levels of progesterone were noticed in PCOS rats treated with SPIE 100 mg compared to PCOS rats. Similar findings were observed in diseased rats treated with SPIE 200 mg with a major regression in the levels of testosterone. Moreover, the reproductive hormone levels in SPIE treated PCOS rats were comparable to that of control, in which SPIE 200 mg treated PCOS rats exhibited good results when compared.

Effect of SPIE on ovary weight of rats in PCOS model

The ovary (Left and right) weight of test animals of both control and different treatment groups were depicted in Fig 3. PCOS group exhibited significant increase in ovary weight when compared with the control. Whereas clomiphene citrate treated PCOS rats showed a significant reduction in the ovary weight in contrast to PCOS group. SPIE treated PCOS rats were comparable to that of healthy rats. It has been shown from the results that SPIE with a dose of 200 mg considered to be safer than other treatment groups.

Effect of SPIE on ovary histology study

Fig 4A and 4C, healthy tertiary follicle in the normal control and standard drug treated rats. The theca layers and granulosa layers appear normal. Fig 4B follicle in the early process of atresia with apoptotic granulosa cells, most of which are in the inner parts of the granulosa layer in a polycystic ovary syndrome (PCOS) rat. Thin and elongated epithelioid cells form the inner surface of the wall. The cyst fluid contains macrophages. Fig 4 D and E from a SPIE (100 and 200 mg) treated PCOS rat with normal tertiary follicles. (H & E staining; index bars, 50 µm)

Effect of SPIE on CYP17A1 and Ki 67 protein expression in immunology-assay

In Fig 5 the level of CYP17A1 and Ki 67 expression in control group was low compared to PCOS group. PCOS+100 mg expressed moderately reduced level when compared to control and PCOS. PCOS+CC and PCOS+200 mg expressed good reduced levels of protein expression when compared to control and PCOS group.

Immunohistochemistry of CYP17A1 and Ki 67 pictures is depicted in Fig 6 and 7(A-E). 6 and 7A-Signifies control group showing decreased level of CYP17A1. 6 and 7B-Signify the PCOS group with increased levels of CYP17A1. 6 and 7C and 6 and 7E Signify the treatment groups PCOS+CC and PCOS+SPIE 200 mg highlights the reduced levels of CYP17A1. 6 and 7D-signify the PCOS+SPIE100 mg with slight reduction in CYP17A1 levels.

Effect of SPIE on body weight of rats in PCOS model

The body weight of PCOS was significantly rising compared to control group. PCOS+CC group also showed significant weight reduction compared to PCOS group. PCOS+SPIE 100 mg and PCOS+SPIE200 mg showed very progressive weight reduction compared to PCOS group as in Fig 8.

The most prevalent hormonal disorder found in reproductive aged women is PCOS. The weight of ovary gets decreased in PCOS rats (Song and Tan, 2016). On the contrary, a rapid increase in the ovary weight of PCOS group was observed in our study which was confirmed with the study of De Leo *et al*. (De Leo *et al*., 2016). This was

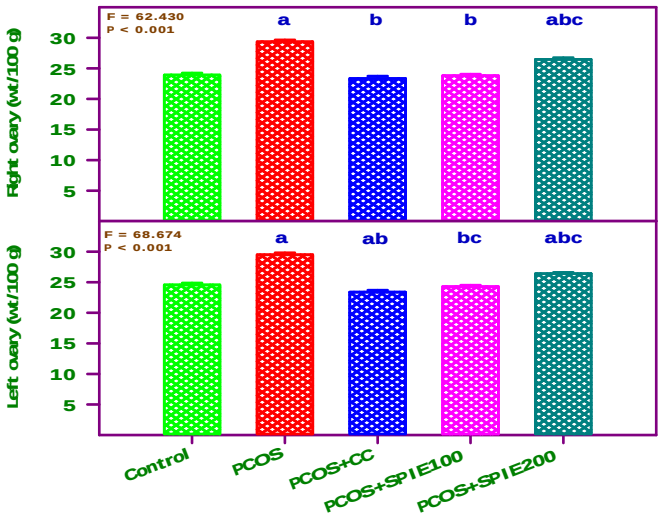

Fig 3: Effect of *Sepia pharaonis* fish ink extract (SPIE), 100 mg/kg and 200 mg/kg, compared with clomiphene citrate (CC) on ovary weight (both right and left) of poly cystic ovarian syndrome (PCOS) in rats. Values are mean±SE (n=6 each). The 'F' and 'P' values are by one way ANOVA with student Newman Keul's multiple comparison test right ovary- F= 62.430; P<0.001, Left ovary- F= 68.674; P<0.001. A significantly different from control group, b significantly different from PCOS group, c significantly different from SPIE+CC group.

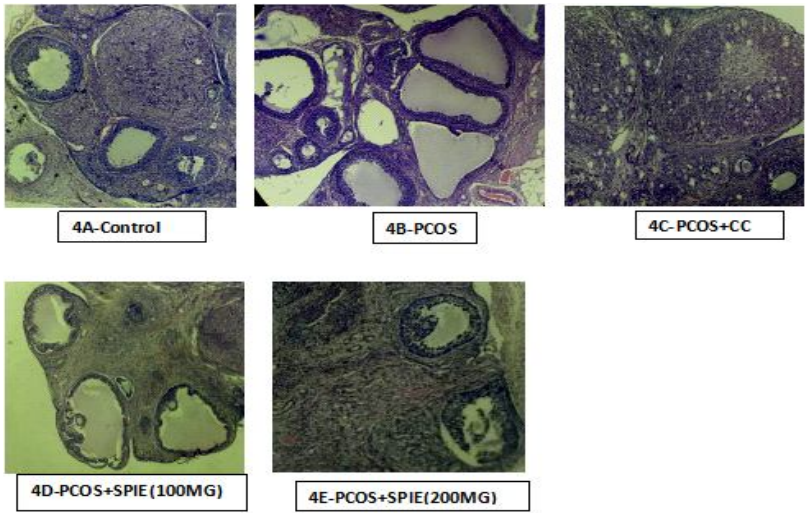

Fig 4(A-E): Histopathological pictures (40× magnification) of the wistar albino rat ovaries (H & E staining) of control, PCOS, PCOS+CC, PCOS+SPIE 100 mg and PCOS+SPIE 200 mg.

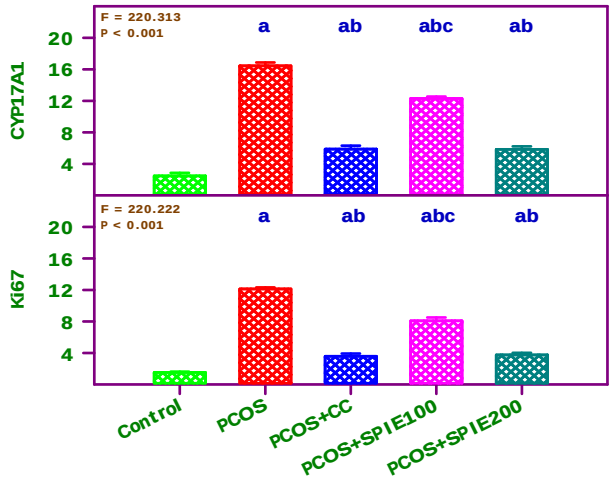

Fig 5: Effect of *Sepia pharaonis* fish ink extract (SPIE), 100 mg/kg and 200 mg/kg, compared with clomiphene citrate (CC) on CYP17A1 and Ki67 protein expression of poly cystic ovarian syndrome (PCOS) in rats. Values are mean±SE (n=6 each). The 'F' and 'P' values are by one way ANOVA with student newman Keul's multiple comparison test CYP17A1- F= 220.313; P<0.001, Ki67- F= 220.222; P<0.001. a significantly different from control group, b significantly different from PCOS group, c significantly different from SPIE+CC group.

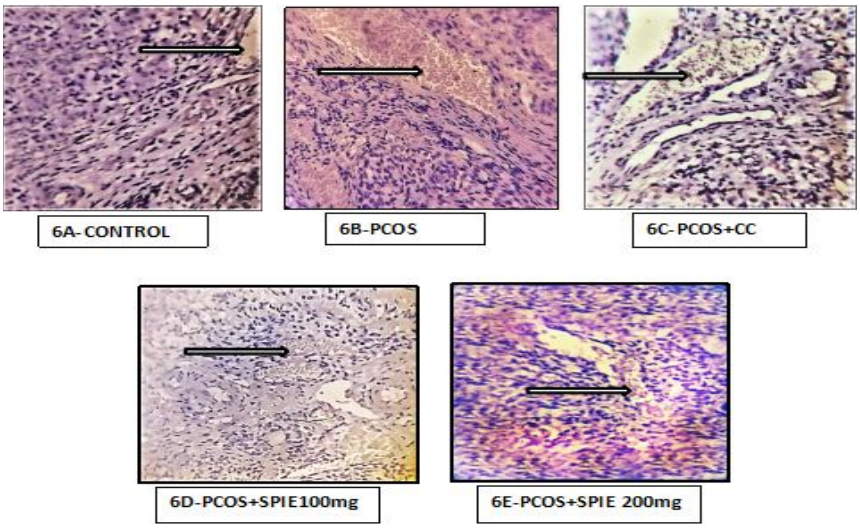

Fig 6(A-E): Immunostaining of CYP17A1 protein expression in ovary tissue (200x magnification)

supported by the study of Louise *et al* (2007) which showed an increase in ovary weight of Letrozole rats. Reduced aromatase effect might increase ovarian production of androgen and reduce oestrogen production, resulting to PCOS. Ovary weight found to be slightly higher in SPIE treatment rats when compared to the control in the present study. The present study also confirmed that SPIE treatment was mildly effective in normalizing the ovarian weight of rats when compared with PCOS group.

Many people with PCOS have higher insulin levels that can excite the ovaries to produce testosterone. Hyperan drogenism, particularly unbalanced testosterone levels are a key disturbance noticed in PCOS (Azziz *et al*., 2006). Testosterone has relatively strong bond with globulin (66-78%) and weak bond with albumin (20-32%). Only 1-2% of the total pool of testosterone is not protein-bound and thus biologically active (Mroczko and Medras, 2003). In PCOS women, free testosterone and free androgen index (FAI) are the markers of hyperandrogenism (Rotterdam, 2003; Conway *et al*., 2014). The levels of total testosterone in PCOS women might be normal or somewhat increased (maximal 5.2 nmol/L) (Sheehan, 2004). The present study confirmed higher testosterone levels in PCOS rats which was reduced by an allopathic drug and further normalized with SPIE treatment. Higher levels of total testosterone are typical for tumours that synthetize androgens (Conway *et al*., 2014).

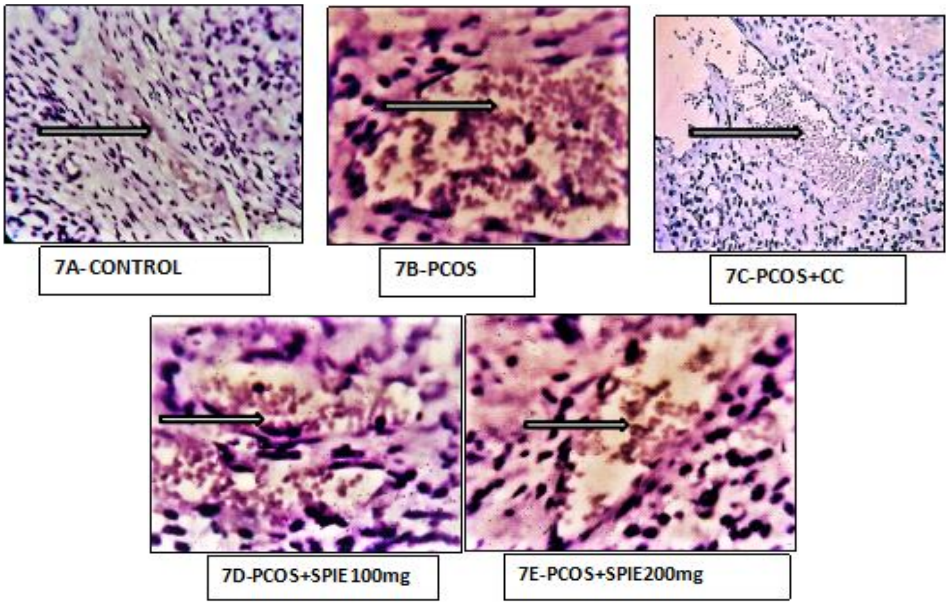

Fig 7(A-E): Immunostaining of ki67 protein expression in ovaries of different study groups (200× magnification).

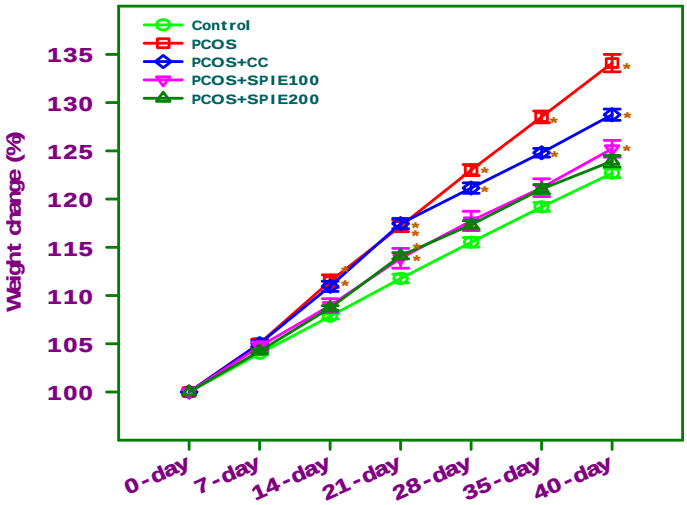

Fig 8: Effect of *Sepia pharaonis* fish ink extract (SPIE), 100 mg/kg and 200 mg/kg, compared with clomiphene citrate (CC) on body weight of poly cystic ovarian syndrome (PCOS) in rats. Values are mean±SE (n=6 each). The 'F' and 'P' values are by one way ANOVA with student newman Keul's multiple comparison test. For 7-day - F= 1.854; P=0.150. For 14-day - F= 8.979; P<0.001. For 21-day - F= 14.763; P<0.001. For 28-day - F= 22.716; P<0.001. For 35-day - F= 37.037; P<0.001, For 40-day - F= 43.316; P<0.001. [a]Significantly different from control group of the respective day.

It was exposed that augmented levels of total testosterone in PCOS women, participates in the pathogenesis of atheroslerosis (Hak *et al*., 2002). Progression of several reproductive defects related with PCOS, with disruption of cyclicity, higher levels of testosterone levelsand expression of enzymes involved in androgen biosynthesis, as well as ovulatory dysfunction, were found in a rat model with targeted deletion of Pcos particularly in ovarian theca cells (Lan *et al*., 2017). NKB receptor antagonism in PCOS females were found to reduce LH pulse frequency and decrease serum LH as well as testosterone levels (George *et al*., 2016).

Progesterone is considered to be the primary GnRH pulsatility regulator. The present investigation confirmed a reduction in the levels of progesterone in PCOS rats further increased with standard drug and SPIE treatment. It is identified that PCOS patients exhibit a GnRH-generating pulse resistance to negative feedback by progesterone, which leads to higher LH pulses frequency and/or amplitude (Eagleson *et al*., 2000). Besides, LH excess is a variable PCOS feature, thus, it is hard to point the primary role to LH excess in the disease progression of all PCOS patients.

The effect of E_2 on granulosa cell GC function are not being clinically tested in PCOS women. Preclinical studies exhibited that higher estradiol concentration after ovarian stimulation, (Homer *et al*., 2014), negatively affects endometrial receptiveness. The current investigation showed reduced levels of estradiol in PCOS rats can be increased by different treatment. In contrast, studies have connected higher levels of estradiol with altered gene expression during implantation and asynchrony among endometrial gland as well as stroma development. Data from clinical observations recommend that higher concentration of estradiol has an adverse action on pregnancy outcomes, after fresh embryo transfer. Though, PCOS women usually have a greater ovarian response for the stimulation and usually have more oocytes, along with a higher estradiol level, than ovulatory women (Wei *et al*., 2018). The body weight of PCOS rats was significantly higher compared to control and treatment group (Karatekea *et al.,* 2018). Similar results were found in our study.

The melanin in the sepia ink, a carrier of dopamine prevents excess dilution of ink after ejection in water. (Gabriella Fiore *et al* 2004). Further studies implicit that Dopamine agonist effectively reduces the levels of Luteinizing hormone and testosterone levels (Dirami and Cook 1998). The SPIE also contains melanin and dopamine that must have acted on the PCOS ovaries to reduce the levels of androgenic hormone and induced ovulation.

Dopamine agonist cabergoline, a safe and well established medicine was useful in prevention of ovarian hyperstimulation syndrome in PCOS women undergoing assisted reproduction (Alvarezc *et al* 2007). In the PCOS induced group the numbers of preantral follicles, antral follicles and corpus luteum decreased compared to the control groups. Treatment by Clomiphene citrate and SPIE increased the number of maturing ovarian follicles. Histological evaluation of ovaries revealed that thickness of granulosa and tunica albuginea increased, while thickness of theca layer deceased in the Pcos group compared to the control groups; however, consumption of SPIE and standard showed normal thickness of ovary layer. Immunoexpression of Ki-67 was higher in the theca interna cells and CYP17A1 in the interstitial and granulosa cells in the PCOS group (Leonardo Augusto Lombardi *et al* 2014). Similarly, in our study PCOS group had more Ki-67 and CYP17A1 Immunoexpression compared to control and treatment group.

CONCLUSION

PCOS is a multifaceted disorder for which several treatment approaches are needed, depending on the reason for treatment. SPIE has exposed the best outcome study in treating infertility and also the data are limited concerning the treatment on PCOS. In the present treatment SPIE proved to be effective in controlling the hormones levels especially testosterone more effectively than standard drug treatment and oestrodiol and progesterone levels similar to treatment drug. Further confirmation by histopathological and Immunohistochemistry confirms that SPIE can be used as a drug of choice to effectively control PCOS in women of the present world and facilitate the healthy reproductive process. Advance studies are required to examine the mechanism of action and the effect of SPIE treatment.

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Conflict of interest

All authors declare that they have no conflict of interest.

REFERENCES

Alvarez, C., Marti-Bonmati, L., Novella- Maestre, E., Sanz, R., Gomez, R., Fernandez Sanchez, M., Simon, C., Pellicer, A. (2007). Dopamine agonist cabergoline reduces hemo concentration and ascitis in hyperstimulated women undergoing assisted reproduction. The Journal of Clinical Endocrinology and Metabolism. 92: 2931-2937.

Anisuzzaman, A.S.M., Sugimoto, N., Bhuiyan, M.S.A., Sadik, G., and Gafur, M.A. (2001). Toxicological studies of two novel compounds isolated from Streptomyces species. The Sciences. 1(6): 368-371.

Azziz, R., Carmina, E., Dewailly, D., *et al.* (2006). Criteria for defining polycystic ovary syndrome as a predominantly hyper and rogenic syndrome: An androgen excess society guideline. J. Clin. Endocrinol Metab. 91: 4237-45.

Conway, G., Dewailly, D., Diamanti-Kandarakis, E., *et al.* (2014). The polycystic ovary syndrome: A position statement from the European Society of Endocrinology. Europ. J. Endocrinol. 171: 1-29.

De, L.V., Musacchio, M.C., Cappelli, V., Massaro, M.G., Morgante, G., Petraglia, F. (2016). Genetic, hormonal and metabolic aspects of PCOS: An update. Reproductive Biology and Endocrinology. 14(1): 38.

Dewailly, D., Robin, G., Peigne, M., Decanter, C., Pigny, P., Catteau-Jonard, S. (2016). Interactions between androgens, FSH, anti-Müllerian hormone and estradiol during folliculo--genesis in the human normal and polycystic ovary. Human Reproduction Update. 22(6): 709-24.

Dirami, G., Cooke., B.A., (1998). Effect of dopamine agonist on Leutinizing hormone receptors, Cyclic AMP production and steroidogenesis in rat Leydig cells. Toxicology and Applied Pharmacology. 50(2): 393-401.

Eagleson, C.A., Gingrich, M.B., Pastor, C.L., Arora, T.K., Burt, C.M., Evans, W.S., *et al.* (2000). Polycystic ovarian syndrome: Evidence that flutamide restores sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by estradiol and progesterone. J. Clin. Endocrinol Metab. 85(11): 4047-52.

Fiore, G., Poli, A., Cosmo, A.D., Ischia, M.D., Plumbo, A., (2004). Dopamine in the ink defence system of *Sepia officinalis*: Biosynthesis, vesicular compartmentation in mature ink gland cells, nitric oxide(NO)/cGMP-induced depletion and fate in secreted ink. Biochemical Journal. 378: 785-791.

George, J.T. *et al.* (2016). Neurokinin B receptor antagonism in women with polycystic ovary syndrome: A randomized, placebo controlled trial. J. Clin. Endocrinol. Metab. 101: 4313-4321.

Hak, A.E., Witteman, J.C., de Jong, F.H., Geerlings, M.I., Hofman, A., Pols, H.A. (2002). Low levels of endogenous and rogens increase the risk of atherosclerosis in elderly men: The Rotterdam study. J. Clin. Endocrinol. Metab. 87: 3632-9.

Hamza, A.H., AlBishri, W.M. and Alfaris, M.H. (2019). Effect of Vitex agnus-castus plant extract on polycystic ovary syndrome complications in experimental rat model. Asian Pacific Journal of Reproduction. 8(2): 63.

Homer, M.V., Rosencrantz, M.A., Shayya, R.F., Chang, R.J. (2014). The role of estradiol (E2) on granulosa cell (GC) function in women with polycystic ovary syndrome (PCOS): Evidence suggesting that E2 facilitates GC response to FSH. Fertility and Sterility. 102(3): e264.

Hossain, M.P. *et al.,* (2018). Medicinal and therapeutic properties of cephalopod ink: A short review. Food Research. 3(3): 188-198.

Jahan, S., Abid, A., Khalid, S., Afsar, T., Shaheen, G., Almajwal, A., and Razak, S. (2018). Therapeutic potentials of quercetin in management of polycystic ovarian syndrome using Letrozole induced rat model: A histological and a biochemical study. Journal of Ovarian Research. 11(1): 26.

Karatekea, A., Dokuyucub, R., Doganc, H., Ozgurd, T., Tasd, Z.A., Tutukc, O., Agturkc, G., Tumerc, C. (2018). Investigation of therapeutic effects of erdosteine on polycystic ovary syndrome in a rat model. Medical Principles and Practice. 27: 515-522.

Lan, Z.J. *et al.*, (2017). Selective deletion of Pten in theca-interstitial cells leads to androgen excess and ovarian dysfunction in mice. Mol. Cell. Endocrinol. 444: 26-37.

Lee, Mong-Fong and Lin, Chun-Yen and Chiao, C.C. and Lu, C.C. (2016). Reproductive behavior and embryonic development of the pharaoh cuttlefish, *Sepia pharaonis* (*Cephalopoda: sepiidae*). Zoological Studies. 55: e 41.10.6620/ZS. 2016. 55-41.

Leonardo Augusto Lombardi *et al.,* (2014). Immunohistochemical evaluation of proliferation, apoptosis and steroidogenic enzymes in the ovary of rats with polycystic ovary. Revista Associacio Medica Brasileira. 60(4): 349-356.

Manneras, L., Cajander, S., Holmang, A., Seleskovic, Z., Lystig, T., Lonn, M., Stener-Victorin, E. (2007). A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology. 148(8): 3781-91.

Misugi, T., Ozaki, K., El Beltagy, K., Tokuyama, O., Honda, K.I. and Ishiko, O. (2016). Insulin-lowering agents inhibit synthesis of testosterone in ovaries of DHEA-induced PCOS rats. Gynecologic and Obstetric Investigation. 61(4): 208-215

Mroczko, B. and Mêdras, M. (2007). Some problems with testosterone determination. Endocrinol Pol. 58: 440-5.

Nair, J.R., Pillai, D., Joseph, S.M., Gomathi, P., Senan, P.V. and Sherief, P.M. (2011). Cephalopod research and bioactive substances. Indian Journal of Geo-marine Sciences. 40(1): 13-27.

Pastor, C.L., Griffin-Korf, M.L., Aloi, J.A., Evans, W.S., Marshall, J.C. (1998). Polycystic ovary syndrome: Evidence for reduced sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by estradiol and progesterone. J. Clin. Endocrinol. Metab. 3(2): 582-90.

Rodgers, R.J., Suturina, L., Lizneva, D., Davies, M.J., Hummitzsch, K., Irving-Rodgers, H.F. and Robertson, S.A. (2019). Is polycystic ovary syndrome a 20th Century phenomenon? Medical Hypotheses. 124: 31-34.

Saleh, H., Soliman, A.M., Mohamed, A.S. and Marie, M.A.S. (2015). Antioxidant effect of sepia ink extract on extrahepatic cholestasis induced by bile duct ligation in rats. Biomedical and Environmental Sciences. 28(8): 582-594.

Samarh, S.N., Khalaf, N.A. and Hajhamad, M.M. (2017). Evidence based medical use of aloe vera extracts, short review of literature. International Journal of Research in Medical Sciences. 5(10): 4198.

Senan, V.P. (2015). Antibacterial activity of methanolic extract of the ink of cuttlefish, *Sepia pharaonis* against pathogenic bacterial strains. International Journal of Pharmaceutical Science and Research. 6(4): 1705.

Sheehan, M.T. (2004). Polycystic ovarian syndrome: Diagnosis and management. Clinical Medicine Research. 2: 13-27.

Song, S. and Tan, Y. (2019). Expression of FKBP52 in the ovaries of PCOS rats. International Journal of Molecular Medicine. 43(2): 868-78.

Sundaram, S. (2014). Fishery and Biology of *Sepia pharaonis* Ehrenberg, 1831 off Mumbai, Northwest Coast of India. J. Mar. Biol. Ass. India. 56(2): 43-47.

Sushma, P.R., Nazia, B., Sumith, M., Bakshi. V., (2016). Beneficial effect of Curcumin in Letrozole induced polycystic ovary syndrome. Asian Pacific Journal of Reproduction. 5(2): 116-122.

The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. (2004). Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 19: 41-7.

Wei, D., Yu, Y., Sun, M., Shi, Y., Sun, Y., Deng, X., Li, J., Wang, Z., Zhao, S., Zhang, H., Legro, R.S. (2018). The effect of supraphysiological estradiol on pregnancy outcomes differs between women with PCOS and ovulatory women. The Journal of Clinical Endocrinology and Metabolism. 103(7): 2735-2742.