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Systemic effects of clinical follicular fluid from polycystic ovary syndrome and non-polycystic ovary syndrome in female mice

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The follicular fluid's composition changes physiologically to meet the demands of specific local phenomena during oogenesis and folliculogenesis. Here, we investigated whether follicular fluid from polycystic ovarian syndrome (PCOS) patients influence the systemic functioning of body compared to the follicular fluid from non-PCOS patients. Follicular fluid was pooled separately from both healthy and PCOS subjects. Tissues and other detritus were separated from the collected fluid by centrifugation. Female Swiss albino mice received 1 and 2 mL/kg of the fluid intraperitoneally, and were observed for 21 days for physiological changes. Variation in body weight was tracked intermittently. Serum was obtained for estimation of oestrogen, progesterone, testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), insulin and glucose on the day the animals were euthanized. Liver, kidney, heart, lung, pancreas, spleen and ovary were examined histologically. Haemoglobin and haematocrit levels were found significantly lowered in animals administered with follicular fluid from PCOS patients. Beginning at day 14, there was a significant increase in total body weight. Blood glucose level increased consistently and almost doubled on day 21 from 101.2±0.86 to 201.0±5.34 mg/dL. Serum concentrations of oestrogen and progesterone reduced significantly and testosterone level got increased in PCOS follicular fluid treated animals compared to non-PCOS follicular fluid. Both the LH/FSH ratio and insulin level rose significantly (P <0.001) increased in PCOS follicular fluid treated animals. The HOMA-IR was likewise statistically significantly increased (P <0.001). However, both the HOMA-Beta and QUICKI scores dropped significantly (P <0.001). A PCOS follicular fluid treatment group of female mice showed clear signs of myocarditis, cardiac atrophy and numerous ovarian cysts. The follicular fluid of PCOS patients showed a localised effect, and the components, through the systemic circulation, posed detrimental consequences systemically suggesting involvement in the pathogenesis of PCOS. Follicular fluid from PCOS patients should be further studied for proteomics and its potential in therapeutic, diagnosis and prognosis.

Keywords: Cystic fluid, Folliculoogenesis, Ovarian fluid, Ovarian cyst, Polycystic ovarian fluid

Women account for 38% of the reasons for infertility due to multiple factors such as hormonal imbalances, genetics, epigenetics, the environment, and certain other conditions. The well-known pathophysiological factor such as polycystic ovarian syndrome (PCOS) is the underlying cause for the infertility in women[1,2]. PCOS has been associated with a rise in hyper-lipidemia, insulin resistance, type II diabetes and hyperadiposity[3]. One of the major criteria for diagnosis of PCOS involves the presence of multiple cysts in the ovary. These cysts are formed from ovarian follicles through folliculogenesis process. Folliculoogenesis is the process through which a primordial follicle becomes an antral follicle and then a preovulatory follicle. When mature, these follicles are able to release an egg that is fertile and ready to be implanted[4]. The first sign that tertiary follicles are being made is the formation of an antrum around granulosa cells. The antrum is filled with follicular fluid, which is the result of plasma transfusion and the secretory activities of granulosa and thecal cells[5,6].

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Abbreviations: AGT, Aberrant glucose tolerance; AMH, Anti Müllerian hormone,; cAMP, Adenosine 3',5'-cyclic monophosphate; EDTA, Ethylene diaminetetra acetate; FF, Follicular fluid; FSH, Follicle stimulating hormone; GnRH, Gonadotropin releasing hormone; IPTG, isopropyl Beta -D-thiogalactopyraanoside.; IGF-I & II, Insulin-like growth factors I & II; IGT, Impaired glucose tolerance; IVF, *In vitro* fertilization; LH, Leutinizing hormone; MCV, Mean corpuscular volume; MCHC, Mean corpuscular haemoglobin concentration; MPV, Mean platelet volume; NGT, Normal glucose tolerance; P-LCR, Platelet larger cell ratio; PCT, Plateletcrit; PCOS, Polycystic ovarian disease; RDW, Red cell distribution width; T2DM, Type II diabetes mellitus

Plasma and their regional ovarian production are the sources of intrafollicular inflammatory cytokines includes interleukins (IL). The follicular fluid protein IL-1 beta is believed to affect oocyte maturation and fertilisation but not embryo development after fertilisation. The follicular fluid of embryos with high implantation potential has been found to contain significant concentrations of interferon-12 (IF-12) and granulocyte-macrophage colony-stimulating factor (GM-CSF)[7,8]. Oocyte formation is ensured by follicle vascularity, intrafollicular oxygen concentration, and mitochondrial activity. Cellular signalling and homeostasis are significantly influenced by reactive oxygen species (ROS). Higher the ROS lower the oocyte quality leading to apoptotic alterations in the follicular environment. Different proteins found in the follicular fluid are taken from serum filtrate and produced from granulosa and thecal cells[9]. The formation of follicular fluid involves the proteoglycans hyaluronic acid and chondroitin sulphate produced by granulosa cells due to an osmotic gradient that allows fluid to flow from blood vessels in the thecal layer. In addition to isopropyl beta -D-thiogalactopyraanoside (IPTG) and versican (Vcan), follicular fluid contains a variety of other proteins. They help keep these molecules in the antrum for longer by producing hyaluronic acid compounds. Further, it contains regulatory compounds used by granulosa cells and oocytes to progress in development[9,10] . Biochemical analysis of follicular fluid composition revealed the oocytes' quality and maturity level. Proteomic analysis reveals that follicular fluid contains proteins belonging to many different functional classes, including insulin-like growth factor, receptors, hormonal factors, anti-apoptotic proteins, and metalloproteinases essential for the growth of follicles and oocytes[11,12] .

Granulosa cells of preantral follicles and tiny antral follicles produce anti-Müllerian hormone (AMH). This hormone's concentration enables the assessment of embryo quality. Adenosine 3',5'-cyclic monophosphate (cAMP), a component of signal transduction mediator that leutinizing hormone (LH) interacts with target cell membrane receptors[13,14]. Additionally, follicular fluid contains polypeptides insulin-like growth factors I and II (IGF-I & II), which encourage tissue proliferation and differentiation. IGF-1 is essential for follicular development up to the gonadotropin-dependent phase[15]. One illustration is Laron's condition, in which regular ovulation can take place despite less IGF-1 levels[16]. The synthesis of IGF receptors in primordial follicles increases fivefold and IGF-1 concentration rises threefold in response to androgens, which also significantly enhance the number of basic follicles[17].

Gonadotropins, which act as growth hormone, influence granulosa cell synthesis of a variety of compounds important for oocyte maturation, such as hyaluronic acid. They enhance oocyte cytoplasmic maturation and regulate meiosis by working in concert with estradiol to release cyclic AMP[18]. Estradiol amounts within follicular fluid of preovulatory follicles usually slightly greater than in the other follicles just prior to and around the time of LH surge, and thereafter they start to decline. The progesterone concentration rapidly rises just before the LH surge in subsequent 25 hours of this surge, the level gradually declines. Ovulation has occurred when the progesterone concentration changes and the capacity to evaluate an oocyte's ability to be fertilised is made easier by the levels of both hormones as well as their reciprocal proportions. The follicular fluid also contains androgens such as androstenedione as well as testosterone are higher during the preovulatory stage, but they are still much lower than those of estradiol[19].

PCOS is an ovarian local pathogenic factor linked to irregular menstruation, high androgen levels, cardiovascular disease, infertility, and a number of other physiological issues, apart from type-2 diabetes, insulin resistance and obesity. Above description explains the follicular fluid and its composition which may have significant effect on local ovarian environment, folliculogenesis, oocytosis, and inter-intracellular signalling process[20]. However, the systemic effect of follicular fluid and its components is not well studied and established. Therefore, in the present study, we observed the systemic, biochemical and histological effect of follicular fluid collected from normal and women diagnosed with PCOS on female mice.

Materials and Methods

Collection of follicular fluid

Follicular fluid was collected from regularly menstruating, healthy women and those with PCOS who were undergoing *in vitro* fertilization (IVF) at Indira IVF Centre (Dehradun, India). To achieve regulated ovarian hyperstimulation, all women underwent a protracted IVF treatment involving a

gonadotropin releasing hormone (GnRH) agonist and recombinant FSH combination. After 34-36 h of administration of human chorionic gonadotropin (hCG) (10,000 IU), follicular fluid was drawn from the follicles during transvaginal ultrasound-guided oocyte aspiration. The samples of follicular fluid used for examination were macroscopically clean and free of flushing medium contamination. To remove cellular debris, the samples were centrifuged at 10,000×g for 30 min at 4°C. Syringe-filtered (0.22 m) supernatants were then kept at 3-4°C in deep freezer for further usage. This work was approved by the University Research Ethics Committee of DIT University, Dehradun and written informed consents were obtained from all the participants[21,22].

Animals and groups

Animal studies were carried out after the approval by Institutional Animals Ethics Committee of DIT University, Dehradun. All animal study protocols were as per the CPCSEA guidelines. Adult female 30 mice aged 60 days, were used in this experiment. The mice were randomly grouped into five groups of six each. The Group I (control) consisted of mice that were given only a standard laboratory meal and water along with normal saline 5 mL/kg i.p; Gr. II and III received normal patient's follicular fluid injected 1 and 2 mL/kg i.p., respectively; Gr. IV and V were given an i.p. injection of PCOS patients follicular fluid 1 and 2 mL/kg, respectively for 21 days.

Blood sampling method and sample handling

The animals were fasted overnight and euthanized with overdose (100 mg/kg i.p.) of ketamine on last day of the experiment after withdrawing 2 mL of blood in vacutainer for biochemical investigation. For histological examination kidney, liver, lung, pancreas, spleen, heart and ovary were removed and stored in 10% neutral buffered formalin[23] until we prepare 5 μM thick slides of each organ.

Biochemical analysis

All biochemical analyses were carried out in pathology lab centre in Dehradun. The biochemical constituents' concentrations in blood plasma were determined in accordance with the pathology laboratory's guidelines[24].

Biochemical parameters

The levels of plasma glucose were measured using diagnostic tools of Span Diagnostic Ltd., Gujurat, India. The plasma level of LH and FSH, an immunoenzymometric test was carried out by employing ERBA Fertikits. Plasma levels of estradiol, progesterone, and testosterone were estimated using the GenXbio kit and microplate reader (MultiskanTM GO, Thermo Fischer scientific)[25].

Estimation of HOMA-IR, HOMA-Beta and QUICKI

Following procedures were used in calculating HOMA-IR, HOMA-Beta, and QUICKI.[26,28]

$$\text{HOMA - IR} = \frac{\text{fasting glucose (mg/dL)} \times \text{insulin (}\mu\text{U/mL)}}{405}$$

$$\text{QUICKI} = \frac{1}{[\log(\text{fasting insulin } \mu\text{U/mL}) + \log(\text{fasting glucose mg/dL})]}$$

$$\text{HOMA - }\beta = \frac{\text{fasting insulin} \times 20}{\text{fasting blood glucose - 3.5}}$$

Haematological estimation methods

Following the advice of physician, blood was collected for hematological investigation in a tube containing dipotassium ethylene diaminetetra acetate (EDTA) to prevent clotting as reported by Dacie & Lewis (2006)[27]. Blood samples were centrifuged for 20 min at 25000 rpm in a centrifuge tube the clear supernatant was separated in a glass tube for biochemical analysis[29]. Total red blood cells, white blood cell count, platelet counts, haemoglobin concentration, mean corpuscular volume, and mean corpuscular haemoglobin concentration (MCHC) were estimated using haematological analyzer (GI-HA3000).

Histopathological examinations

Using the usual procedures, isolated organs were processed, embedded in paraffin, sectioned, deparaffinized, and dehydrated. Examining the microscopic changes allowed researchers to gauge the full impact of the follicular fluid-induced modifications. Hematoxylin and eosin (H & E) was used to stain tissue sections, which were then observed under a microscope for microscopic changes[30].

Statistical analysis

Means and standard error of the mean of the data are reported (SEM). Student t-test was used to determine if differences were significant, and a value of P <0.05 was deemed to indicate statistical significance.

Results

Change in haematological profile

Treatment of mice for 21 days with normal follicular fluid resulted in significant increase in RBC, platelet, haemoglobin, haematocrit, Mean corpuscular

hemoglobin (MCH), and Platelet larger cell ratio (P-LCR). Whereas significant decrease in Mean corpuscular volume (MCV) and Red cell distribution width corpuscular volume (RDW-CV). Similarly treatment with PCOS follicular fluid significantly increased MCV, RDW-CV, Red cell distribution width size distribution (RDW-SD), Mean platelet volume (MPV), plateletcrit (PCT), and P-LCR. But multiple factors such as haemoglobin, haematocrit, MHC, Mean corpuscular haemoglobin concentration (MCHC), and PCT were significantly reduced. (Table 1)

Change in body weight

Chronic treatment of female mice for 21 days with normal and PCOS follicular fluid treatment are presented in Fig. 1. Normal follicular fluid treatment with 2 mL/kg shown significant (P <0.05) improvement in body weight on day 1 (27.20±0.3742) 14 (28.76±0.66) and 21 days (29.72±0.42). Highly significant (P <0.001) gain in body weight of female mice was recorded on day 14 (31.48±0.84) and 21 (34.86±0.67) with 2 mL/kg PCOS FF treatment.

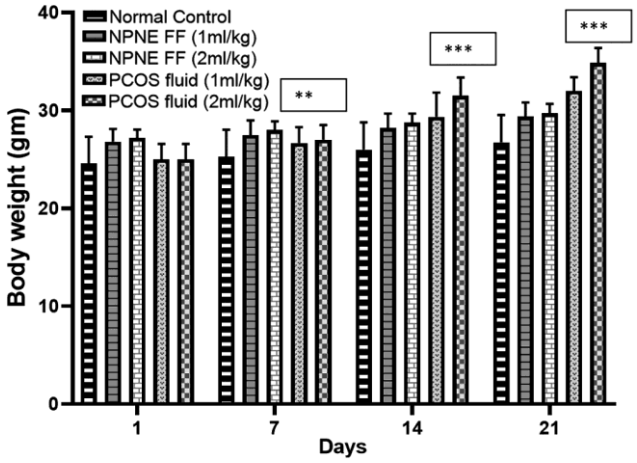

Fig. 1 — Influence of follicular fluid parenteral treatment on change in body weight. [**P <0.01; ***P <0.001]

Whereas very significant (P <0.01) increase in PCOS FF treated female mice was observed at a dose of 1 mL/kg on day 14 (29.34±1.10) and 21 (31.98±0.02) compared to day 1 (25.07±0.70).

Blood glucose level

The blood glucose level highly significantly (P <0.001) increased in female mice on parenteral PCOS follicular fluid treatment to (100±0.99) from (170±0.98). It was found to be dose dependent and duration of treatment. The level of blood glucose almost doubled on day 21 with 2 mL/kg from 101.2±0.86 to 201.0±5.34. As shown in Fig. 2 the hyperglycemic effects were noticed on day 7 (157±2.30) onwards and consistent elevation of blood glucose.

Serum estradiol, progesterone and testosterone

Treatment of female mice with PCOS follicular fluid highly significantly (P <0.001) dose dependently decreased the serum estradiol (24±1.07) and progesterone (1.100±0.100) level and increased testosterone level (16.29±0.37) (Fig. 3). Comparatively progesterone

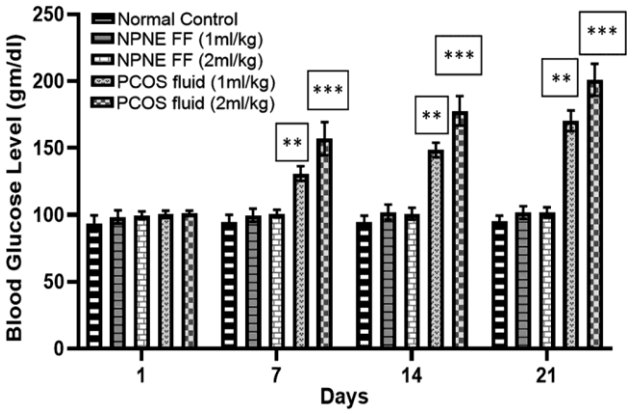

Fig. 2 — Effect of follicular fluid parenteral treatment on change in blood glucose level the blood glucose level highly significantly (P <0.001) increased in female mice on parenteral PCOS follicular fluid treatment. [**P <0.01; ***P <0.001]

Table 1: — Changes in haematological profile of mice due to follicular fluid parenteral administration

Haematological Parameters	Normal control	Normal follicular fluid (1 mL/kg)	Normal follicular fluid (2 mL/kg)	PCOS follicular fluid (1 mL/kg)	PCOS follicular fluid (2 mL/kg)
RBC (million/mm^3)	7.780±0.29	9.620±0.07348***	10.18±0.10***	7.240±0.1030	6.300±0.14
PLT (mcL)	989.0±13.64	1048±13.74***	1075±7.77***	986.4±6.36	965.0±13.04
HGB (g/dL)	13.12±0.17	13.84±0.09**	14.46±0.14***	12.46±0.20*	12.18±0.10*
MCV (μm^3)	43.26±0.25	40.50±0.18***	39.84±0.14***	47.74±0.31***	49.10±0.14***
HCT (million/mm3)	40.16±0.12	40.80±0.26*	41.28±0.12***	39.70±0.20*	39.66±0.19*
MHC (Ig)	14.52±0.17	15.20±0.095**	15.52±0.18**	14.60±0.29	13.36±0.18***
MCHC (g/dL)	33.52±0.16	34.10±0.13*	35.36±0.2731***	30.34±0.19***	29.86±0.33***
RDW-CV (%)	16.40±0.28	15.34±0.19**	16.28±0.1319	20.54±0.19***	21.62±0.1***
RDW-SD (fL)	29.70±0.12	30.30±0.21	31.00±0.27**	34.74±0.28***	35.90±0.24***
PDW (%)	10.54±0.16	10.74±0.11	11.24±0.23*	11.40±0.19**	11.76±0.23**
MPV (fL)	10.60±0.19	10.90±0.19	11.00±0.084*	14.46±0.20***	16.16±0.35***
PCT (ng/mL)	0.62±0.02	0.62±0.03	0.65±0.003	0.73±0.01**	0.70±0.02**

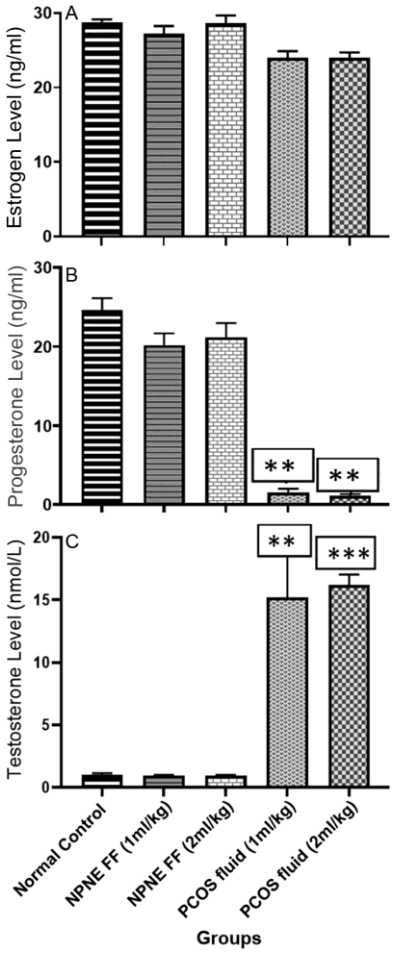

Fig. 3 — Effect of follicular fluid parenteral treatment on serum (A) Estradiol; (B) Progesterone; and (C) Testosterone levels. [Treatment of female mice with PCOS follicular fluid highly significantly (P <0.001) dose dependently decreased the serum estradiol and progesterone level and increased testosterone level serum LH, FSH and ratio. **P <0.01; ***P <0.001]

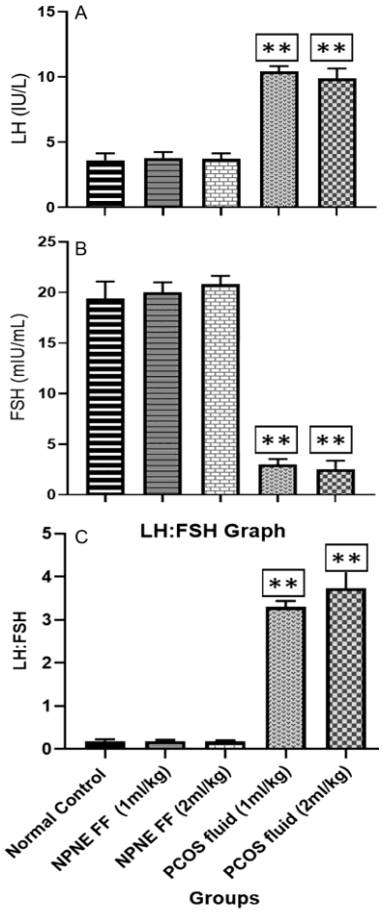

Fig. 4 — Effect of follicular fluid parenteral treatment on change in serum (A) LH; (B) FSH; and (C) LH:FSH ratio. [There was significant (**P <0.01) increase in LH to FSH ratio.]

level reduced dose dependently by normal follicular fluid very significantly (P <0.01) otherwise no significant changes were observed in the level of estradiol and testosterone.

Parenteral PCOS follicular fluid treatment of female mice at both 1 and 2 mL/kg dose highly significantly (P <0.001) increased serum LH level almost to three times of the normal (Fig. 4). Contradictorily, the level of serum FSH reduced from 19.40±0.75 to 3.16±0.1 and 2.7±0.2 at 1 and 2 mL/kg dose of PCOS follicular fluid. Consequently, there was highly significant (P <0.001) increase in LH to FSH ratio.

Insulin sensitivity and resistance

In an attempt to understand the influence of PCOS follicular fluid on insulin activity and profile of female mice they were parenterally administered with 1 and 2 mL/kg dose for 21 days. There was highly significant (P <0.001) dose dependent increase in serum insulin level was observed. Similarly, HOMA-IR was also highly significantly (P <0.001). However, HOMA-Beta and QUICKI were highly significantly (P <0.001) and dose dependently reduced (Fig. 5).

Effect on heart, liver, kidney, lung, pancreas, spleen and ovary

We noticed no substantial changes in histopathology of heart, liver, kidney, lung, pancreas, spleen and ovary of female mice treated with follicular fluid of normal subjects of 1 and 2 mL/kg dose i.p. for 21 days. As presented in Fig. 6, the follicular fluid of PCOS patient at 1 and 2 mL/kg ip dose substantially affected vital and other organs. Pancreas are affected by vascular occlusion and infiltration by inflammatory cells leading to swelling and loss of beta cells. Liver was noted to have central and peripheral vascular occlusion, inflammatory cellular infiltration, necrosis, fatty liver, thickening of

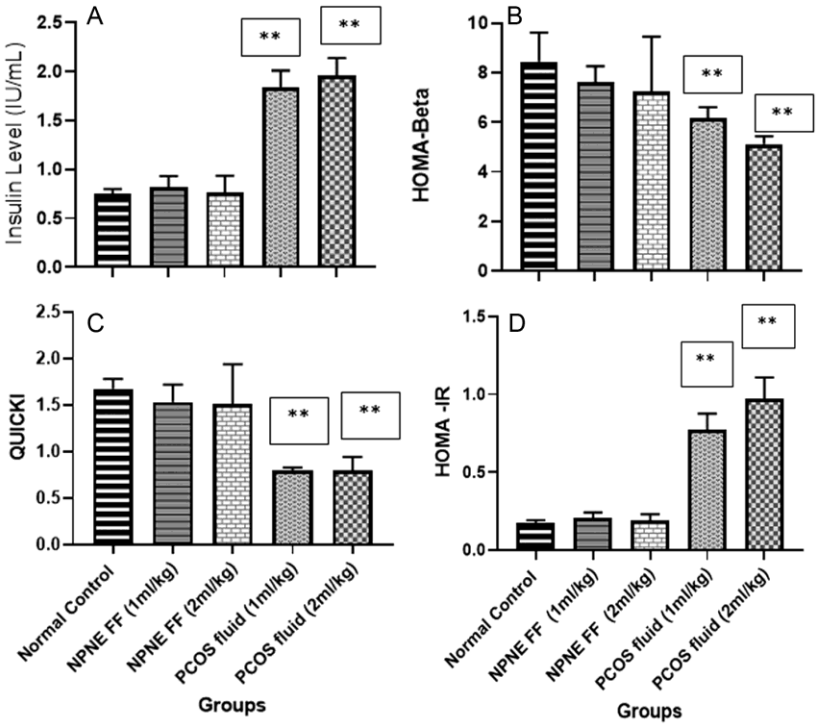

Fig. 5 — Effect of follicular fluid parenteral treatment on insulin sensitivity and resistance. (A) Insulin resistance; (B) HOMA-Beta; (C) Quiki; and (D) HOMA IR. [There was significant (P <0.01) dose dependent increase in serum insulin level was observed. Similarly, HOMA-IR was also significantly (P <0.01). However, HOMA-Beta and QUICKI were highly significantly (P <0.01) and dose dependently reduced]

hepatocytes and collagen deposition. Myocarditis and myocardial hypertrophy were clearly visible in PCOS follicular fluid treated female mice. Abnormality in kidney structure was observed with an extensive fibrosis. The histology of ovary clearly shows that there were multiple cysts in mice. The lungs were extensively infiltrated by inflammatory cells, thickening of alveolar sac, and alveolar membrane.

Discussion

Our results imply that the development of PCOS in mice is attributable to the increased body weight and elevated androgen and LH hormone of groups treated with PCOS follicular fluid. Body weight in the group given normal follicular fluid was found to be much more stable than in the control group.

The metabolic abnormalities in PCOS are linked in large part to insulin resistance and the compensatory hyperinsulinemia that results from it could be due to the presence of insulin like growth factors[31]. However, not all PCOS-affected women are insulin-resistant or experience compensatory hyperinsulinemia[32,33]. Hyperandrogenemia is primarily characterised by an excess of androgens and an elevated LH level, and its clinical manifestations typically emerge during adolescence in women with PCOS[34,35]. Increased serum androgens and luteinizing hormone are associated with obesity and abdominal obesity, which in turn worsens the clinical characteristics of monthly irregularity and infertility[36]. Both women with PCOS and healthy controls experience higher androgen levels when they gain body weight. Thus, the aetiology and pathogenesis of PCOS involve a complicated interplay between obesity, abdominal obesity, insulin resistance, androgen level, and LH level[37,38].

PCOS increases the risk for diabetes and cardio-vascular disease in young women, in addition to being the leading cause of hyperandrogenism and female infertility[39]. The risk of developing impaired glucose tolerance (IGT) or type II diabetes mellitus (T2DM) is also two to five times higher in the PCOS population compared to the general population[40,41]. Similar to previous research, we showed that after 21 days of dosing with PCOS follicular fluid, glucose intolerance is greatly increased[42]. When compared to the control group, we observed a statistically significant decrease in fasting blood glucose levels when using normal follicular fluid, regardless of dosage[43,44]. Similarly,

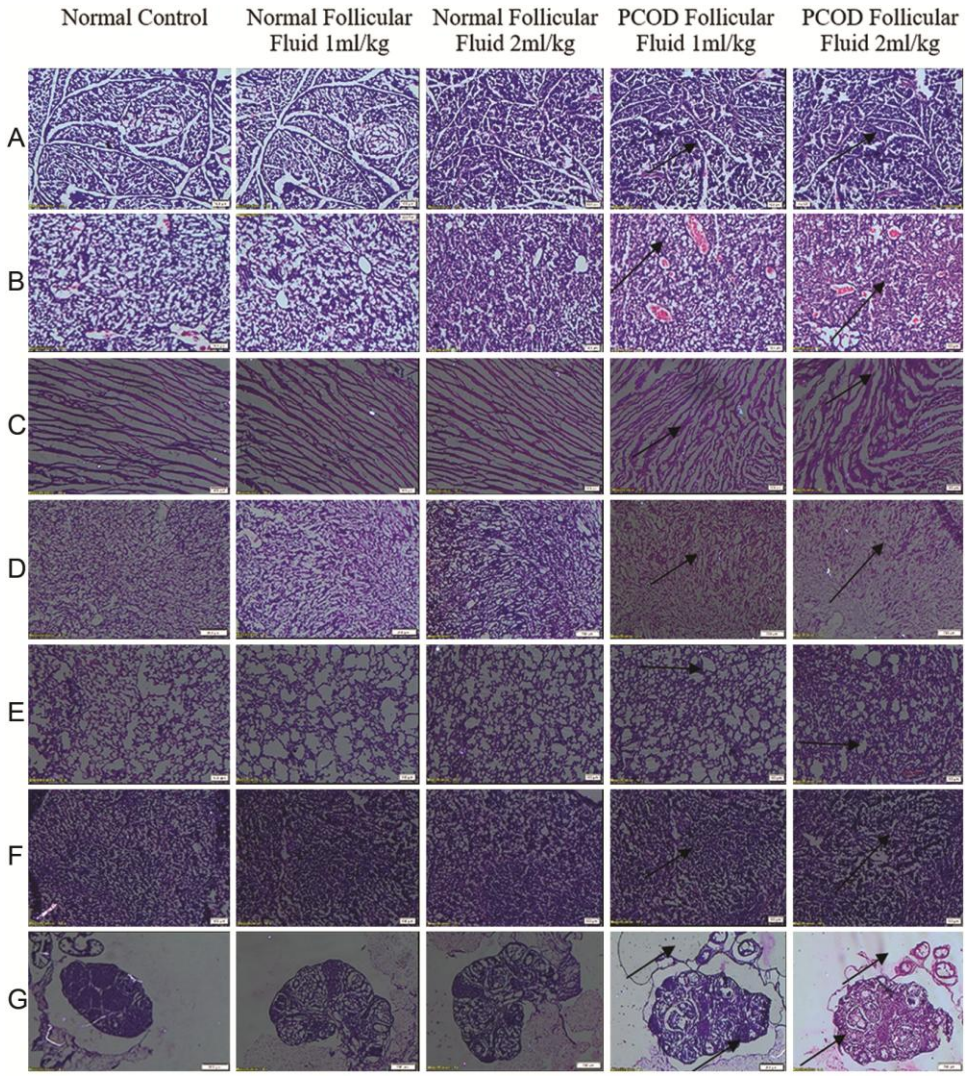

Fig. 6 — Effect of parenteral follicular fluid treatment on histopathology of (A) Pancreas; (B) Liver; (C) Heart; (D) Kidney; (E) Lungs; (F) Spleen; and (G) Ovary

consistent effects on glucose tolerance can be observed in otherwise normal follicular fluids. As a result, normal fluid may function as an insulin sensitizer and affect the selection of PCOS treatment by bringing about the reversal of aberrant glucose tolerance (AGT) to normal glucose tolerance (NGT)[45,46]. Groups of female mice treated with PCOS follicular fluid lead to gain in body weight when given normal follicular fluid was found to be much more stable than in the control group.

Ovulation success is proportional to the amount of estrogen (E) produced by the dominant follicle. Androgen concentrations in follicular fluid (FF) are higher and estradiol (Es) concentrations are lower in PCOS compared to non-PCOS women[47,48]. Regarding our research, both 1 and 2 mL/kg of follicular fluid were able to restore their levels to within the normal range. Treatment with normal follicular fluid also results in a rise in estrogen and a decrease in progesterone. After finishing the research, both estrogen and progesterone levels increase significantly, which may explain the observed decline in the diestrus phase and improvement in estrous cycle regularity.

Over-production of ovarian androgens is facilitated by elevated LH secretion, which in turn is linked to elevated pituitary sensitivity to GnRH, as has been shown in multiple investigations examining women with polycystic ovary disease[49,50]. Our research confirms that PCOS follicular fluid 2 mL/kg

considerably raises LH levels. The LH: FSH ratio increases as a result, but in the normal follicular fluid 1 and 2 mL/kg groups, it drops dramatically. The raise in the ratio is an indication of onset of PCOS[51].

The outcome of the study proves that follicular fluid of PCOS not only has a localized effect but also has a significant systemic effect on various organs and systems of the body leading to PCOS complications. This also proves that PCOS follicular fluid can be used in developing PCOS models in female mice. However, the study should have had more range of doses of the fluid to see variability and extent of systemic and histological effects.

Conclusion

The follicular fluid of PCOS patients, it is determined, not only has a localized effect, but the follicular fluid components absorbed into the systemic circulation and have detrimental consequences and may be involved in important clinical symptoms of PCOS such as highly significant gain in body weight of female mice, glucose intolerance, hyperglycemia, hyperandrogenism, and surge in levels of LH. Whereas the level of estrogen, progesterone, and FSH were reduced consequently there was highly significant increase in LH to FSH ratio. Dose dependent increase in serum insulin level was observed leading to elevation in HOMA-IR, and HOMA-Beta. At the same time there was reduction in QUICKI. Pancreas are affected by vascular occlusion and infiltration by inflammatory cells leading to swelling and loss of beta cells. Liver was noted to have central and peripheral vascular occlusion, inflammatory cellular infiltration, necrosis, fatty liver, thickening of hepatocytes, and collagen deposition. Myocarditis and myocardial hypertrophy were clearly visible in PCOS follicular fluid treated female mice. Abnormality in kidney structure was observed with an extensive fibrosis. The histology of ovary clearly shows that there were multiple cysts in mice. The lungs were extensively infiltrated by inflammatory cells, thickening of alveolar sac, and alveolar membrane. Further, follicular fluid from PCOS patients should be studied for its potential in diagnosis and prognosis. In our laboratory, the study is undergoing to quantitatively estimate the components of follicular fluid using advanced technologies and systems.

Conflict of interest

Authors declare no competing interests.

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