Tropical Journal of Pharmaceutical Research December 2022; 21 (12): 2611-2617
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i12.15

Original Research Article

Effect of berberine on serum hormonal levels and endometrial gene and protein signaling in a rat model of polycystic ovary syndrome

Jun Guo[1,2]*, Huiling Cao[2]
[1]Ganzhou Hospital of Guangdong Provincial People's Hospital (Ganzhou Municipal Hospital), [2]Department of Obstetrics and Gynecology, Maternal and Child Health Hospital, Zhanggong District, Ganzhou 341000, Jiangxi Province, China

For correspondence:* ***Email:* *guojun7624@126.com*

Sent for review: 10 September 2022 *Revised accepted: 27 November 2022*

Abstract

Purpose: *To study the effect of berberine on serum hormonal levels, endometrial gene, and protein signaling in a rat model of polycystic ovary syndrome (PCOS).*
Methods: *Eighty-seven healthy female Sprague Dawley (SD) rats were assigned to control group, PCOS model group, and berberine group, each with 29 rats. The rats were assigned to three groups: control, model, and berberine groups, each with 29 rats. A rat model of PCOS was established via the administration of letrozole (1 mg/kg) to rats in the model group continuously for 21 days through gavage. Rats in berberine group were also given letrozole at the same dose for the same duration through gavage, while rats in control group received physiological saline in place of berberine. Estrus cycle, serum hormonal levels, ovarian tissue morphology, estrogen receptor α (ERα) levels, and mRNA expression levels of protein kinase B subtype 3 (Akt-3β) were evaluated. RAS-C3 botulinum toxin substrate 1 (Rac1), human chromosome 10 deleted phosphatase (PTEN) and mouse sarcoma-like virus oncogene (KRAS) were also determined using appropriate procedures.*
Results: *Berberine treatment resulted in a significant decrease in the mean body weight of PCOS rats, relative to model rats ($p < 0.05$). Estrus interval in model rats was significantly prolonged. Levels of testosterone and estradiol, and mRNA expression levels of ERα, Akt3, Racl, PTEN, and KRAS were significantly increased, while progesterone level was significantly decreased, relative to control ($p < 0.05$). Estrus interval in the berberine group was shortened and estrus cycle gradually returned to normal, while the levels of testosterone and estradiol, and mRNA levels of ERα were gradually restored, relative to untreated PCOS rats ($p < 0.05$). Moreover, mRNA levels of Akt3, RAC1, PTEN, and KRAS were significantly decreased, while progesterone level was significantly increased ($p < 0.05$).*
Conclusion: *Berberine appreciably restores heat cycle and ovary morphology in PCOS rats, and regulates serum hormonal levels, and endometrium-related gene and protein signaling.*

Keywords: *Berberine, Polycystic ovary syndrome, Endometrium-related genes, Protein, Signaling*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a high-incidence disease in women of childbearing age. It manifests as reproductive, metabolic, endocrine, and other abnormalities, with insulin resistance, persistent ovarian anovulation, and elevated androgen as its main clinical

characteristics [1]. In recent years, the incidence of PCOS has gradually increased as a result of changes in lifestyle. However, the pathogenesis of PCOS has not yet been clearly elucidated. Some scholars believe that PCOS might be closely associated with disorders in glucose and lipid metabolisms, as well as abnormal hormonal levels, all of which are interrelated, thereby forming a vicious circle [2]. At present, oral contraceptives, reduction of insulin resistance, and induction of ovulation are used in the clinical treatment of PCOS. These treatments have produced some curative effects, but they are associated with adverse and toxic side effects. Traditional Chinese medicine has continued to attract attention because of its high safety, low toxic side effects, and better curative effects. Berberine is a common antipyretic and anti-infective drug in clinics. It has been found that berberine lowers blood glucose, blood lipids, and insulin resistance. However, the effect of berberine on PCOS, and the mechanism involved, have not been investigated [3]. In this study, the effect of berberine on PCOS, and its influence on various PCOS indices were investigated in a rat model of the disease.

EXPERIMENTAL

Animals

Eighty-seven (87) healthy female Sprague Dawley (SD) rats weighing 215 ± 15 g were used in this study. The rats were obtained from Harbin Pharmaceutical Group Biological Vaccine Co. Ltd. (production license SCXK black, 2017-002, license number: SYXK (black) 2016-002). All rats were kept in the laboratory in an environment with a mean temperature of 23 ± 2 ℃, a humidity of 50 ± 15 %, and a 12-h light/12-h dark photoperiod. This study received approval from the Animal Ethics Authority of Ganzhou Municipal Hospital (approval no. GMH2022093) in line with the 1985 revision of NIH guidelines. publication no. 85-23 [4].

Major instruments and reagents

The main equipment and reagents used in this study, and their sources (in brackets) were: cryogenic high-speed centrifuge (Hebei Huizai Technology Co. Ltd, model: A1331053); real-time fluorescence quantitative PCR instrument (Xi 'an Tianlong Technology Co. Ltd, model: TL988); paraffin slicer (Beijing Shengke Xinde Technology Co. Ltd, model: RM2255); -80 ℃ ultra-low temperature refrigerator (Zhejiang Jisheng Low-temperature Equipment Co. Ltd, model: DW-86W300); electron microscope (Beijing Jingkelida Technology Co. Ltd, model: EM208s); electronic balance (Shandong Xin Coal Mine Mountain Equipment Group Co. Ltd., model: SZ-139E); Human villi Gonadotropin Detection Kit (Zhejiang Dongfang Gene Biological Products Co. Ltd); letrozole (Jiangsu Hengrui Pharmaceutical Co. Ltd, batch number: 20171001, specification: 2.5 mg x 10s); and berberine (Guangdong South China Pharmaceutical Group Co. Ltd, batch number: 44020757, specification: 0.1 g x 100s).

Grouping and treatments

The rats were assigned to three groups: control, model, and berberine groups, each with 29 rats. A rat model of PCOS was established via the administration of letrozole (1 mg/kg) to rats in the model group continuously for 21 days through gavage. Rats in berberine group were also given letrozole at the same dose for the same duration through gavage, while rats in control group received physiological saline in place of berberine. Successful establishment of PCOS was indicated by the appearance of sparse ovulation or anovulation, hyperandrogenemia, and/or ultrasonographic appearance of polycystic ovary. In addition to letrozole, rats in the berberine group were given letrozole (0.1 g/kg per day) for 21 days.

Determination of parameters/indices

Body weight changes

Variations in the weights in rats after the establishment of the PCOS model were compared amongst the groups. The estrus cycle was monitored in rats in each group.

Days in the estrus cycle

Continuous examination of vaginal smears was carried out from the beginning of drug therapy: two sex cycles were examined, one for 5 days. Cotton swabs were soaked in sterile saline and inserted into the vaginal wall, and gently turned clockwise for one week. Thereafter, the cotton swabs were taken out and smeared on slides. After drying in natural air, the smears were fixed in alcohol and examined under the microscope to determine changes in the number of days in the estrus period of the rats in each group, based on the proportions of leucocytes, cornified epithelial cells, and nucleated epithelial cells in the vaginal secretion.

Changes in serum hormonal levels

At the end of treatment (on day 22), 1 mL of orbital venous blood was taken from each rat and

centrifuged. The supernatant was used for the determination of testosterone levels in each group with ELISA, while estradiol and progesterone levels were determined using an electrochemical luminescence immunoassay. In the latter case, 50 µL of the sample was incubated with 90 µL of substrate 37 ℃ for 20 min. The reaction was stopped by the addition of 50 µL of terminating solution, and changes in electrochemical luminescence were monitored at 450 nm using an electrochemical luminescence detection kit. The assay was carried out in triplicate.

Histological examination of ovaries

The rats were sacrificed and their ovaries were excised. Morphological alterations in ovarian tissues were determined using H & E stain. Paraffinized sections were prepared, dewaxed with xylene, hydrated in ethanol, and rinsed and dried in phosphate buffer. Then, the sections were first stained with hematoxylin and rinsed in phosphate buffer, prior to staining with 0.5 % eosin solution for 5 min. Thereafter, the stained sections were dehydrated in a gradient of alcohol for 30 sec, soaked twice in xylene for 5 min, sealed with neutral gum, and examined under a light microscope.

Expression levels of ERα

Levels of ERα were determined using immunohistochemistry. Tissue specimens were fixed in formalin solution, and paraffinized sections were routinely prepared. The sections were then heated in an oven at 60 ℃ for about 60 min, followed by dewaxing in xylene, dehydration in anhydrous ethanol, and rinsing thrice in phosphate buffer, each rinse for 5 min. Then, the slides were put into a pressure cooker, and citrate buffer solution was added for antigen retrieval, followed by rinsing in tap water to cool the slides to room temperature. This was followed by rinsing thrice in phosphate buffer. Then, the slides were incubated with diluted ER antibody (200 µL) overnight at 4 ℃, prior to sequential incubation with biotin-labeled secondary antibody at room temperature for 60 min, washing with phosphate buffer solution, development with DAB chromogenic solution, re-staining with hematoxylin, washing, dehydration, clearing, and sealing. Finally, the slides were examined under a light microscope. Four fields were randomly selected for the determination of the expression of positive cells.

The expressions were scored as follows: 0 point was scored when the proportion of positive cells was less than 5 %, 1 point was scored when the proportion was more than 5 % but less than 25 %, 2 points were scored when the proportion was more than 25 % but less than 50 %, while 3 points were scored when the proportion was more than 50 %. The intensity of staining was scored viz: 0 point for no stain, 1 point for light yellow stain, 2 points for brownish yellow stain, and 3 points for tan. In addition to scores for the two results, a total score of 1 or less than 1 was taken as negative, while a total score of 2 points or more was taken as positive.

Real-time fluorescence quantitative polymerase chain reaction (qPCR)

The mRNA expressions of T-PCRAKT3, RAC1, PTEN, and KRAS of rats in the three groups were assayed with real-time fluorescence quantitative PCR. The endometrial tissues were homogenized, and statically centrifuged, and the phase layer was mixed with an equal volume of isopropanol. Then, the mRNA was reverse-transcribed to cDNA using a reverse transcriptome kit, followed by RT-PCR using a real-time fluorescence quantitative PCR instrument. Relative mRNA expressions were calculated using the $2^{-\triangle\triangle Ct}$ method. The primers used are as indicated in Table 1.

Table 1: Primers used in PCR

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
AKT3	TGGCACCAGAGG TATTAG AAG A	TATCAAGAG CCTGA AAG CAA
RAC1	ATGCAGGCATCA AGTATGTGGTG	TTACAACAGCA GGCATTTTCTCT TCC
PTEN	GCGAGCT-GTTTTTCCACCTC T	TGATGTCGCGG TACA CACAT
Kras	CAAGAGCGCCTT GACGATACA	TGATGCGC GTCTCGGAAAT
β-actin RNA	CAACCGT-GAAAAGATGACC CA	AATGCCAG GGTACGACAGA

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The SPSS ver. 20.0 software was used for statistical analysis. Two-group comparison of measurement data was done with an independent sample *t*-test, while a multiple-group comparison was done with a single-factor multivariate analysis. Values of $p < 0.05$ indicated that differences were statistically significant.

RESULTS

Changes in rat weights

Prior to the establishment of the PCOS model, body weights were comparable in all groups ($p > 0.05$). However, after the establishment of the PCOS model, the mean weight of model rats was significantly boosted, relative to control rats, but there was no marked disparity in mean rat weight between the berberine group and the model group. After the treatment, rats in the untreated PCOS group (model) were significantly heavier than control rats ($p < 0.05$). Compared with model rats, there was a significant decrease in the mean weight of rats in the berberine group ($p < 0.05$). These results are presented in Table 2.

Table 3: Changes in estrous periods of the rat groups after treatment (n = 29)

Group	Number of estrous days (days)
Control	2.81±0.47
Model	6.42±1.25[a]
Berberine	2.88±0.33[ab]
F	195.92
P-value	<0.001

[a,b]$P < 0.05$: [a]vs control; [b]vs model

Effect of berberine on levels of testosterone, estradiol, and progesterone

Plasma levels of testosterone and estradiol in model rats were significantly boosted, but progesterone concentrations were significantly decreased, relative to the control ($p < 0.05$). However, berberine significantly decreased the level of testosterone and estradiol, while it significantly increased the level of progesterone, relative to model rats. These data are presented in Table 4.

Influence of berberine on ovarian tissue

Figure 1 shows that in the control group, there were protrusions of several luteal tissues from the surface of the ovary showing follicles at different stages of development. In the model group, there were deleterious follicular lesions, with decreases in the number of granulocyte layers, and significant increases in the corpus luteum. However, berberine significantly reduced the follicular lesions in PCOS rats and increased the number of granular cell layers.

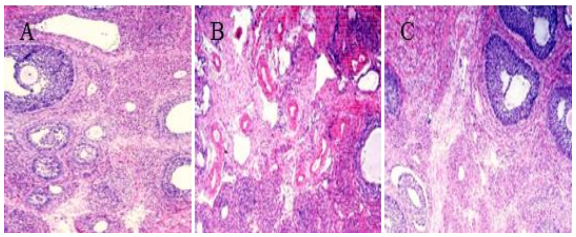

Figure 1: Morphological changes in ovarian tissues of rats. A: control group; B: model group; FC: berberine group

Effect of berberine on expression levels of ERα

Compared with the control group, the level of Erα-positive expression in the model group was significantly increased ($p < 0.05$). However, berberine significantly decreased the level of Erα positive expression, when compared with the model group ($p < 0.05$; Table 5).

Table 2: Weight changes in rats in the 3 groups (n = 29)

Group	Before PCOS model	After PCOS model	After treatment
Control	183.81±19.20	197.48±21.85	355.41±21.37
Model	176.94±19.62[a]	221.28±23.62[a]	402.28±16.52[a]
Berberine	172.41±14.76[ab]	229.88±16.98[ab]	382.02±11.62[ab]
F	2.95	18.51	54.30
P-value	0.058	<0.001	<0.001

Data are presented as mean ± SD. [a,b]$P < 0.05$: [a]vs control; [b]vs model

Table 4: Effect of berberine on levels of testosterone, estradiol, and progesterone (n = 29)

Group	Testosterone (pg/mL)	Estradiol (pmol/L)	Progesterone (nmol/L)
Control	207.05±68.09	117.88±10.99	51.78±6.15
Model	340.34±62.42[a]	308.77±38.63[a]	46.98±8.77[a]
Berberine	246.15±13.64[ab]	163.20±15.88[ab]	62.66±7.77[ab]
F	46.84	463.97	32.07
P-value	<0.001	<0.001	<0.001

[a,b]$P < 0.05$: [a]vs control; [b]vs model

Table 5: Effect of berberine on ERα expression levels (n = 15)

Group	Erα expression	
	Positive	**Negative**
Control	5(17.24)	24(82.76)
Model	27(93.10)	2(6.90)
Berberine	10(34.48)	19(65.52)
χ^2	36.733	
P-value	<0.001	

Results are expressed as mean ± SD. [a]$P < 0.05$, vs control; [b]$p < 0.05$, vs model

Effect of berberine on mRNA expression levels of AKT3, RAC1, PTEN, and Kras

The mRNA expression levels of AKT3, RAC1, PTEN, and Kras in the endometrial tissues of rats in the model rats were significantly up-regulated, relative to control rats ($p < 0.05$). However, berberine significantly decreased the mRNA expression levels of AKT3, RAC1, PTEN, and Kras in the endometrial tissues of rats, relative to model rats. These data are presented in Table 6.

DISCUSSION

Polycystic ovary syndrome (PCOS) is a pathophysiological and clinical polymorphic syndrome caused by multiple factors. Its main characteristics are persistent anovulation, excess androgen, and insulin resistance. The pathogenesis of PCOS is still unclear, although it is believed that it may be due to multiple factors such as genetics, psychology, environmental factors, and chronic inflammation [5]. At present, the clinical treatment of PCOS is aimed at the reduction of androgen levels, adjustment of the menstrual cycle and enhancement of ovulation, mitigation of insulin resistance, and use of drugs such as metformin and thiazolidinediones (TZDS). Although TZDS is frequently used in clinics, its gastrointestinal side effects and adverse impact on weight may limit its clinical application. In recent years, the advantages of traditional Chinese medicine in the treatment of PCOS have become increasingly appreciated due to its fewer adverse side effects and higher efficacy, when compared to orthodox drugs. Ancient Chinese medicine did not describe the disease as polycystic ovary syndrome (PCOS). Based on its clinical symptoms and signs, it was classified as “amenorrhea”, “leakage of fluids”, and “lump”, with *kidney shortage* regarded as the root cause of the disease [6]. Berberine is an isoquinoline alkaloid. It is an important component of *Coptis chinensis* and it exerts important pharmacological effects such as antibacterial, anti-inflammatory, *heat-clearing* and detoxification properties [7]. The purpose of this study was to investigate the effect of berberine on the treatment of PCOS, and the mechanism involved.

Insulin resistance and hyperinsulinemia are considered key factors in the etiology of hyperandrogenemia and loss of ovulation. Studies have found that hyperinsulinemia secondary to insulin resistance induces hyperandrogenemia, while insulin resistance aggravates hyperandrogenemia or even non-ovulation, thereby forming the pathological basis of abnormal glucose metabolism and reproductive dysfunction in patients with PCOS [8]. Berberine is an effective insulin sensitizer that reduces insulin resistance in follicular membrane cells and mitigates metabolic and endocrine disorders in patients [9]. The results of this study showed that berberine improved the estrous cycle of rats and restored the morphology of ovaries to appreciable extents.

Estrogen receptor (ER), a member of the nuclear receptor superfamily, is a hormone-regulated transcription factor consisting of two subtypes: α type and β type, with ERα being the dominant receptor of the uterus. Estrogen exerts its role through ERα [10]. In this study, it was found that the sex hormones of PCOS rats were obviously disordered, which affected the normal expression of ER. In PCOS, estrogen stimulation of the endometrium leads to significant increases in the ratio of ER and progesterone receptors, as well as increases in the mitotic potential of endometrial cells and DNA replication errors, resulting in the occurrence of endometrial lesions [11].

Table 6: Effect of berberine on mRNA expression levels of AKT3, RAC1, PTEN, and Kras in endometrial tissues of rats (n = 29)

Group	AKT3 mRNA	RAC1 mRNA	PTEN mRNA	Krasn mRNA
Control	1.01±0.01	1.02±0.01	1.01±0.01	1.00±0.01
Model	1.52±0.22[a]	21.10±9.30[a]	14.47±0.32[a]	82.92±8.88[a]
Berberine	1.08±0.03[ab]	1.15±0.14[ab]	6.38±1.41[ab]	12.44±1.86[ab]
F	134.61	134.29	1910.51	2080.24
P-value	<0.001	<0.001	<0.001	<0.001

[a,b]$P < 0.05$, [a]vs control; [b]vs model

Phosphatidyl inositol 3 kinase/protein kinase B (PI3K/Akt-3β) signal pathway is an important signaling route of PCOS which is influenced by growth factors, insulin, and signal compounds involved in activation and regulation of glucose metabolism, and regulation of apoptosis, all of which affect the occurrence of tumors [12]. The Akt-3β is the downstream substrate of the PI3K signaling pathway, and its activation is crucial for growth regulation. Studies have found significantly increased expression levels of Akt-3β in endometrial cancer cells, which indicates that it may be an important target of uterus-related tumor genes [13].

The RAC1 belongs to the RHO group, and it has important regulatory functions in vascular endothelial cell migration, cell proliferation and differentiation, and activation of the PI3K signaling pathway [14]. It is known that PTEN is an important tumor suppressor gene that inhibits cell proliferation and differentiation and blocks downstream signal transduction. In addition, PTEN inhibits the phosphorylation of Akt. It has been reported that the PTEN level which has an important relationship with the insulin signaling pathway, may inhibit the occurrence of tumors by suppressing the PI3K/Akt signaling pathway [15]. It may be an important target gene for endometrial cancer. *Kras* is a proto-oncogene. Normally, the combination of Kras with GTP or GDP keeps cell growth in a relatively stable state, but when *Kras* is mutated, the cells undergo malignant transformation and may become cancer cells [16].

In this study, it was found that the mRNA expression levels of Akt-3β, RAC1, PTEN, and Kras in the endometrial tissues of PCOS rats were significantly increased, but these increases were reduced by berberine. Therefore, berberine mitigated PCOS and blocked its carcinogenesis potential.

CONCLUSION

Berberine significantly restores the estrus cycle and ovarian morphology of rats with PCOS. Moreover, berberine exerts a regulatory effect on serum hormonal levels and endometrium-related genes and protein signals. Therefore, this compound has potentials has potentials for use in the management of polycystic ovarian syndrome.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jun Guo designed the study, supervised the data collection, and analyzed the data. Huiling Cao interpreted the data and prepared the manuscript for publication. Huiling Cao supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

REFERENCES

1. *Meier RK. Polycystic ovary syndrome. Nurs Clin North Am 2018; 53(3): 407-420.*
2. *Carmina E, Napoli N, Longo RA, Rini GB, Lobo RA. Metabolic syndrome in polycystic ovary syndrome (PCOS): lower prevalence in southern Italy than in the USA and the influence of criteria for the diagnosis of PCOS. Eur J Endocrinol 2006; 154(1): 141-145.*
3. *Mishra S, Maurya VK, Kumar S, Ankita, Kaur A, Saxena SK. Clinical management and therapeutic strategies for the thyroid-associated Ophthalmopathy: Current and future perspectives. Curr Eye Res 2020; 45(11): 1325-1341.*

4. World Health Organization. Principles of laboratory animal care. WHO Chron 1985; 39: 51-56.
5. Macut D, Bjekić-Macut J, Rahelić D, Doknić M. Insulin and the polycystic ovary syndrome. Diabetes Res Clin Pract 2017; 130: 163-170.
6. Barthelmess EK, Naz RK. Polycystic ovary syndrome: current status and future perspective. Front Biosci (Elite Ed) 2014; 6(1): 104-119.
7. Yin J, Xing H, Ye J. Efficacy of berberine in patients with type 2 diabetes mellitus. Metabolism 2008; 57(5): 712-717.
8. Matsuzaki T, Munkhzaya M, Iwasa T, Tungalagsuvd A, Yano K, Mayila Y, Yanagihara R, Tokui T, Kato T, Kuwahara A, Matsui S, Irahara M. Relationship between serum anti-Mullerian hormone and clinical parameters in polycystic ovary syndrome. Endocr J 2017; 64(5): 531-541.
9. Su L, Xianmei HAN, Xiao C. Effect of berberine on insulin resistance in rats with polycystic ovary syndrome. Shaanxi Trad Chin Med 2017; 38(1): 132-134.
10. Zhang N, Liu X, Zhuang L, Liu X, Zhao H, Shan Y, Liu Z, Li F, Wang Y, Fang J. Berberine decreases insulin resistance in a PCOS rats by improving GLUT4: Dual regulation of the PI3K/AKT and MAPK pathways. Regul Toxicol Pharmacol 2020; 110: 104544.
11. Fujiwara S, Terai Y, Kawaguchi H, Takai M, Yoo S, Tanaka Y, Tanaka T, Tsunetoh S, Sasaki H, Kanemura M, et al. GPR30 regulates the EGFR-Akt cascade and predicts lower survival in patients with ovarian cancer. J Ovarian Res 2012; 5(1): 35.
12. Zhao H, Zhou D, Chen Y, Liu D, Chu S, Zhang S. Beneficial effects of Heqi san on rat model of polycystic ovary syndrome through the PI3K/AKT pathway. Daru 2017; 25(1): 21.
13. Nepstad I, Hatfield KJ, Grønningsæter IS, Reikvam H. The PI3K-Akt-mTOR signaling pathway in human acute myeloid leukemia (AML) cells. Int J Mol Sci 2020; 21(8): 2907.
14. Ubba V, Soni UK, Chadchan S, Maurya VK, Kumar V, Maurya R, Chaturvedi H, Singh R, Dwivedi A, Jha RK. RHOG-DOCK1-RAC1 signaling axis is perturbed in DHEA-induced polycystic ovary in rat model. Reprod Sci 2017; 24(5): 738-752.
15. Chu MT, Fenelon A, Rodriguez J, Zota AR, Adamkiewicz G. Development of a multidimensional housing and environmental quality index (HEQI): application to the American Housing Survey. Environ Health 2022; 21(1): 56.
16. Alhejji AE, Alsultan MA, Alshareet MA, Alkhamis FA, Aljaafary MA, Alshaqaqiq AI, Nabouli MR, Ibrahim Ali S. Knowledge, attitudes, and practices regarding diabetic retinopathy among primary health care physicians in Al-Hasa, Saudi Arabia. J Prev Med Hyg 2020; 61(1): E85-E91.