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Effect of α-cyperone-containing serum on H_2O_2-induced oxidative stress of ovarian granulosa cell apoptosis in rats

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Abstract

Objective: The paper investigated the effect and mechanism of α-Cyperone-containing serum on H_2O_2-induced oxidative stress in ovarian granulosa cell apoptosis in rats. Methods: Female SD rats of 21-25 days old were obtained. Mechanical separation and trypsin digestion method were used to collect rat ovarian granulosa cells. H_2O_2 of 50 μM, 100 μM, 200 μM, 500 μM, and 1000 μM were applied to intervene in ovarian granulosa cells. CCK-8 method was employed to screen appropriate drug concentration and establish rat ovarian granulosa cell oxidative stress model. 5%, 10%, and 20% drug-containing serum intervention were performed, CCK-8 method was used to screen serum concentration and intervention time. 200 nm, 400 nm, and 800 nm JNK signaling pathway inhibitor SP600125 were used to intervene with oxidatively stressed ovarian granulosa cells, and CCK-8 method was applied to screen the appropriate concentration. Ovarian granulosa cells in the logarithmic growth phase were randomly divided into the blank group, the H_2O_2 intervention oxidative stress model group (the model group), the drug-containing serum group, and the JNK pathway inhibitor group. After intervention, the laser confocal microscope was used to observe the expressions of intracellular ROS, and the average optical density of each group was compared. The laser confocal microscope was used to observe TUNEL staining, and the apoptosis rate of each group was compared. Western blot was used to detect the expressions of p-JNK, Bax, and caspase-3 proteins. Results: 200 μM H_2O_2 was used to induce ovarian granulosa cell model of oxidative stress. The optimal concentration of drug-containing serum was 10%, and the intervention time was 24 h. The intervention concentration of JNK signal pathway inhibitor SP600125 was 800 nm. Compared with the blank group, the average optical density of intracellular ROS and apoptosis rate increased in the model group, the drug treatment group, and the JNK pathway inhibitor group. Compared with the model group and the JNK pathway inhibitor group, the intracellular ROS expression and apoptosis rate of the drug group decreased. The Western blot expressions of p-JNK, Bax, and caspase-3 proteins in the model group were higher than that of the blank group, the drug administration group, and the JNK pathway inhibitor group. Conclusion: The serum containing α-Cyperone may inhibit the ROS-JNK signaling pathway and reduce the H_2O_2-induced oxidative stress ovarian granulosa cell apoptosis.

Keywords: α-Cyperone; ROS-JNK signaling pathway; H_2O_2; rat ovarian granulosa cells; apoptosis.

Practical Application: α - cyperone on apoptosis of ovarian granulosa cells

1 Introduction

Endometriosis is a common and frequently-occurring disease in women of childbearing age. 50% of patients with endometriosis have infertility (Lew, 2019; Thézénas et al., 2020). Mature follicles and smooth ovulation are necessary conditions for pregnancy. Previous studies have shown that patients with endometriosis have impaired ovarian function, dysplasia of follicles, a large amount of atresia of follicles, low number of eggs obtained during IVF, poor quality of follicles, poor quality of embryos, low pregnancy rate, and high abortion rate (Lin et al., 2020). Ovarian granulosa cells play an important role in the development of follicles. The morphology and number of granulosa cells are markers of follicular development and pregnancy outcome. Previous studies believed that ovarian granulosa cell apoptosis is an important cause of follicular atresia. In recent years, the relationship between ovarian granulosa cell autophagy and follicular atresia has received increasing attention. Apoptosis and autophagy work together to regulate follicular atresia and affect follicular development, and both are regulated by the ROS-JNK signaling pathway. The ectopic endometrium, as a "foreign body", produces a large amount of ROS after long-term stimulation, which induces oxidative stress. Excessive ROS activates the JNK signaling pathway (Da Broi et al., 2018; Zhao et al., 2020; Li et al., 2020).

α-Cyperone is an effective ingredient extracted from the traditional Chinese medicine Cyperus Rotundus. It relaxes intestinal smooth muscle, relieves bronchial smooth muscle spasm, increases bile flow and solid content in bile, inhibits uterine and estrogen-like effects, and promotes the completely keratinization of vaginal epithelium cells. Whether α-Cyperone can reduce H_2O_2-induced oxidative stress ovarian granulosa cell apoptosis has not been studied before, so this study has a certain degree of innovation and can lay the foundation for future clinical research.

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2 Methods

2.1 Preparation of medicated serum

SD rats were adaptively fed for 5 days and randomly divided into a blank group of 30 rats and an α-Cyperone group of 30 rats. The blank group received 2 mL/day distilled water intravenous injection, and the α-Cyperone group received 20 mg/kg continuous intravenous injection for 5 days. 1 h after the last administration, 3.5 mL/kg of 10% chloral hydrate was applied for intraperitoneal anesthesia. Blood was obtained from the abdominal aorta and let stand for 2 h, then centrifuged at 3000 rpm for 15 min. The supernatant was water-bathed under 56 °C for 30 min to deactivate, then filtered and sterilized with 0.22 μm filter membrane. The supernatant was dispensed into 2 mL cryovials, stored at -20 °C for later use.

2.2 CCK-8 method for measuring various indicators

Experiment 1: The cells were obtained. The prepared 50 μM, 100 μM, 200 μM, 500 μM, and 1000 μM H_2O_2 solution was added 100 μL/well to the culture plate, which was then incubated for 2 h.

Experiment 2: The cells were obtained. 5%, 10%, and 20% drug-containing serum, 5%, 10%, and 20% blank serum were added 100 μL/well to the culture plate respectively. The cell culture plate of the non-H_2O_2 intervention group was placed in 37 °C cell incubator with 5% CO_2 for 24 h, 48 h, and 72 h respectively. The H_2O_2 intervention group cell culture plates were incubated for 24 h, 48 h, and 72 h respectively, followed by 2 h incubation with 200 μM H_2O_2.

Experiment 3: The cells were obtained. 10% blank serum containing the JNK signaling pathway inhibitor SP600125 200 nm, 400 nm, and 800 nm was added 100 μL/well respectively to the culture plate. The cells were incubated for 2 h.

The above-mentioned experimental cell culture solution was pumped and discarded respectively. 10% CCK-8 solution was added to each well. The cells were incubated for 2 h, and the absorbance OD value was measured at 450 nm with a microplate reader.

2.3 Detection of ROS in cells

The cells were divided into four groups: the non-intervention group (blank group), the model group, the drug-containing serum group, and the JNK inhibitor group. Respectively, the model group and the administration group were added with complete medium, 10% normal rat serum, 10% drug-containing serum, and 10% normal rat serum containing 800 nm SP600125. In the JNK inhibitor group, 200 μM H_2O_2 solution was added to make the final concentration of DCFH-DA 10 μM and the diluted DCFH-DA was added to the 12-well plate. The 488 nm excitation wavelength and 525 nm emission wavelength were used for observation and photographing. The blue fluorescence spot under the microscope is the nucleus, and the green fluorescence spot in the nucleus denotes the positive expression of ROS.

2.4 TUNEL cell apoptosis detection

The cells were grouped as in the ROS detection experiment. The cells were fixed with 4% paraformaldehyde solution, added with 50 μL/well TUNEL detection solution to prevent from evaporating, and then incubated at 37 °C in darkness for 60 min. On a clean glass slide, a little DAPI mounting tablet was dropped. The cell slide was taken out and mounted in darkness. A laser confocal microscope with 488 nm excitation wavelength and 525 nm emission wavelength was used for observation and photographing. Under the microscope, the TUNEL positive signal is in green fluorescence, located in the nucleus of granular cells, which is in round or oval shape, while the blue fluorescence spot is the nucleus.

2.5 Western blot detection of p-JNK, Bax, caspase-3 protein expressions in ovarian granulosa cells

The cells were grouped as in the ROS detection experiment. Protein extraction, quantification, sample protein preparation, and Western blot were performed successively. After scanning the film, TanonImage software GelImageSystem (ver.4.00) was used to calculate the gray value of different groups.

3 Result

3.1 H_2O_2 induced oxidative stress model of rat ovarian granulosa cells

Cell inhibition rates induced by 5 concentrations of H_2O_2 in rat ovarian granulosa cells were compared. There was no significant difference in cell inhibition rates between the 50 μM and 100 μM groups. While in the 200 μM, 50 μM, and 1000 μM groups, the cell inhibition rates were all greater than that in the 50 μM and 100 μM groups, and the difference was statistically significant. In the H_2O_2-induced rat ovarian granulosa cell oxidative stress model, the H_2O_2 concentration is positively correlated with cell inhibition rate, in a dose-dependent relationship. The H_2O_2 concentration at 200 μM was chosen to establish an oxidative stress model, as shown in Figure 1.

The 24 h, 48 h, 72 h cell survival rate of 5%, 10%, 20% blank serum and drug-containing serum intervened in oxidative stress ovarian granulosa cells were compared. There was no

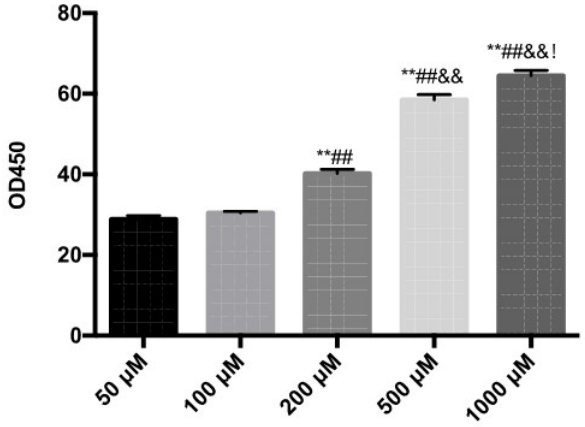

Figure 1. The inhibition rates of ovarian granulosa cells induced by H2O2 at different concentrations. ** p<0.01 vs 50 μM; ## p<0.01 vs 100 μM; && p<0.01 vs 200 μM; ! p<0.05 vs 500 μM.

statistically significant difference in 5% serum at 24 h, 48 h, or 72 h. Compared with 10% and 20%, the cell survival rate after the intervention of drug-containing serum was higher than that of the blank serum group at all the 3 time points. Therefore, it is shown that 10% and 20% drug-containing serum can promote the proliferation and survival of oxidative stress granular cells at 24 h, 48 h, and 72 h. 10% drug-containing serum was chosen. After the cells were cultured for 48 hours, different serum interventions were performed. After 48 hours of intervention, the cells had entered the plateau phase and the survival rate was affected. Therefore, the appropriate intervention time point was 24 h, as shown in Figure 2.

After application of 200 nm, 400 nm, and 800 nm SP600125 intervention induced oxidative stress ovarian granulosa cells. Cell survival rate was compared. It was found that the survival rate of ovarian granulosa cells in 800 nm SP600125 intervention was higher than that in the 200 nm and 400 nm cells, and the difference was statistically significant. The concentration of 800 nm was selected to intervene in ovarian granulosa cells with H_2O_2-induced oxidative stress, as shown in Figure 3.

3.2 Intracellular ROS expression

Compared with the blank group, the average optical density of intracellular ROS in the model group, the administration group, and the JNK inhibitor group all increased, and the intracellular ROS in the administration group was lower than that in the model group and the JNK inhibitor group, as shown in Figure 4.

3.3 TUNEL apoptosis rate

Compared with the blank group, the apoptosis rate of the model group, the administration group, and the JNK inhibitor group increased, and the difference was statistically significant. Compared with the model group, the apoptosis rate of the administration group and the JNK inhibitor group decreased, also with a statistically significant difference. See Figure 5.

3.4 Western blot detecting apoptosis-related protein expressions

The expressions of p-JNK, Bax, and caspase-3 proteins in ovarian granulosa cells in the H_2O_2-induced oxidative stress model group were higher than those in the blank group, the administration group, and the JNK inhibitor group, as shown in Figure 6.

4 Discussion

ROS is a product of oxidative stress, which not only participates in normal physiological and biochemical metabolism, but also plays an important role in maintaining redox homeostasis. Oxidative stress is considered to be the initiating factor of apoptosis (Nagata, 2018; Saleem et al., 2020), and the JNK signaling pathway mediated by ROS is involved in the process of apoptosis, which can cause obvious cytotoxicity and hinder

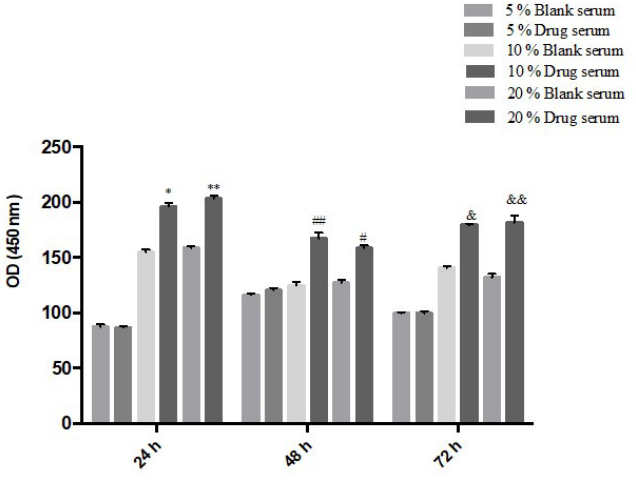

Figure 2. The survival rates of ovarian granulosa cells with different concentrations and different time points of serum intervention in oxidative stress. ** p<0.01 vs 24h Blank serum; * p<0.05 vs 24h Blank serum; ## p<0.01 vs 48h Blank serum; ## p<0.05 vs 48h Blank serum; && p<0.01 vs 72h Blank serum; & p<0.01 vs 72h Blank serum.

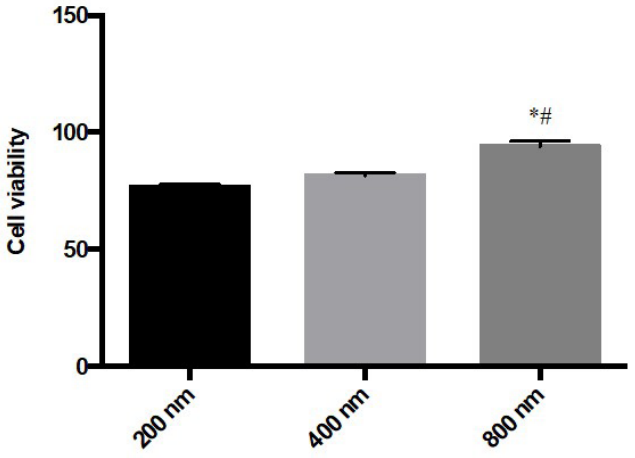

Figure 3. The survival rates of H_2O_2-induced ovarian granulosa cells intervened with different concentrations of SP600125. * p<0.05 vs 200μm; # p<0.05 vs 400μm.

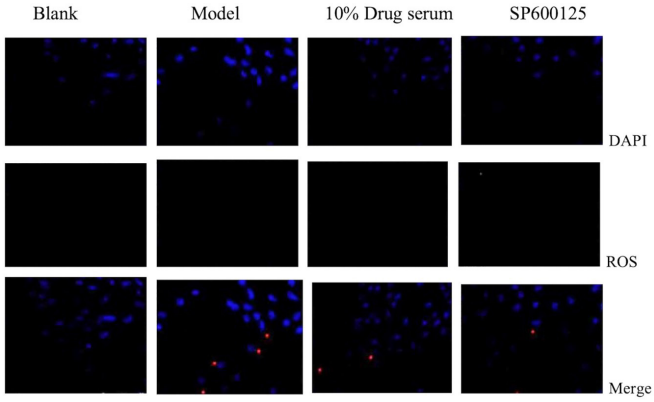

Figure 4. Intracellular ROS expression in each group under confocal laser microscope.

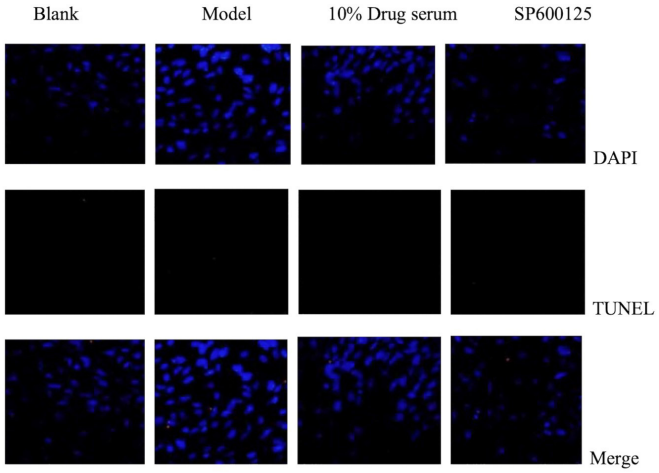

Figure 5. The TUNEL expression in each group under confocal laser microscope.

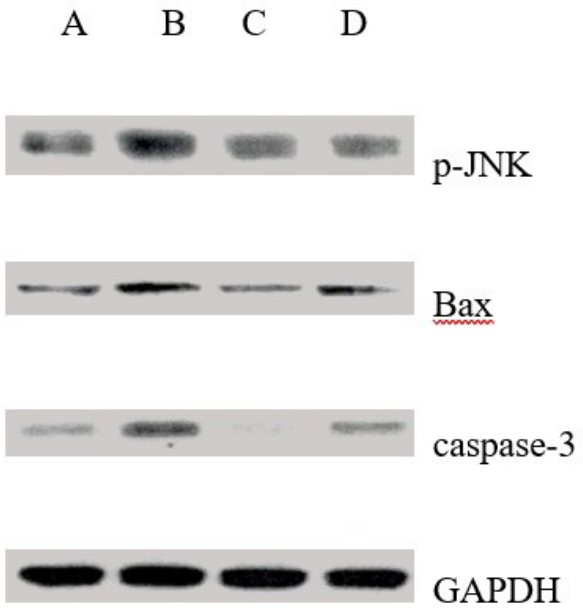

Figure 6. Western blot results of apoptosis-related protein expressions. A: Blank; B: Model; C: 10% Drug serum; D: SP600125.

cell survival. Excessive ROS causes phosphorylation of JNK, promotes the activation of downstream transcription factor c-Jun, and regulates the expression of downstream pro-apoptotic and anti-apoptotic genes. The anti-apoptotic Bcl-2 gene is almost completely inhibited, while the pro-apoptotic gene Bax increases. The downstream apoptosis-related caspase-3 protein activates and induces apoptosis (Kwak et al., 2020; Wang et al., 2020a). When ROS decreases, the JNK signaling pathway will be blocked, the expression of c-Jun will decrease, the expression of Bcl-2 will increase, the expression of Bax protein will decrease, and the activity of caspase-3 will be inhibited (Wang et al., 2020b; Ren et al., 2020), thus reducing the occurrence of apoptosis. Mitochondria are one of the most important ways to produce a large number of ROS in cells, and they are extremely sensitive to oxidative stress. A large amount of ROS is produced in ovarian granulosa cells induced by H_2O_2, and causes severe damage to mitochondria. However, after the administration of medicated serum, the level of intracellular ROS decreases (Hwang et al., 2019).

In the experiment, H2O2 successfully induced an oxidative stress model of ovarian granulosa cells, which were intervened with medicated serum and JNK signaling pathway inhibitor SP600125. The results showed that ROS in the oxidative stress model group increased significantly and the rate of granulosa cell apoptosis increased. After the administration of medicated serum, intracellular ROS and apoptosis rate decreased significantly, and the ultrastructural damage of ovarian granulosa cells was less than that in the oxidative stress model group. In the JNK inhibitor group, the intracellular ROS expression did not decrease significantly, but the apoptosis rate decreased significantly. According to the Western blot results, the expressions of p-JNK, Bax, and caspase-3 proteins in the model group increased, while the expressions of p-JNK, Bax, and caspase-3 proteins in the administration group and the JNK inhibitor group were lower than that in the model group. This indicated that H2O2 may activate the ROS-JNK signaling pathway and induce the apoptosis of ovarian granulosa cells by inducing excessive ROS production in cells. The application of drugs with anti-oxidative stress effects can reduce the production of ROS, reduce the expression of p-JNK protein, inactivate the ROS-JNK signaling pathway, and reduce the apoptosis rate of granulosa cells. As an inhibitor of the JNK signaling pathway, SP600125 had no significant effect on the intracellular ROS production after drug intervention, but it did reduce the expression of P-JNK protein and the apoptosis rate of granulosa cells. Therefore, it is considered that SP600125 can inhibit the JNK signaling pathway to inactivate ROS-JNK signaling pathway, thus reducing granular cell apoptosis. In the H2O2-induced oxidative stress model, H2O2 induces ovarian granulosa cell apoptosis through the ROS-JNK signaling pathway (Costa et al., 2010).

5 Conclusion

In conclusion, H_2O_2 stimulated ovarian granulosa cells, activated the ROS-JNK signaling pathway and induced ovarian granulosa cell apoptosis by producing a large amount of ROS. After the intervention of medicated serum with the cells, the production of intracellular ROS was reduced and the ROS-JNK signaling pathway was blocked, and ovarian granulosa cell apoptosis was reduced. SP600125 intervention in cells has no effect on intracellular ROS, but by inhibiting JNK signaling pathway, ovarian granulosa cell apoptosis was reduced, thus clarifying that the oxidative stress induced ovarian granulosa cell apoptosis through ROS-JNK signaling pathway, and α-Cyperone.

References

Costa, P F P., Ferraz, M. B. M., Ros-Polski, V., Quast, E., Collares Queiroz, F. P., & Steel, C. J. (2010). Functional extruded snacks with lycopene and soy protein. *Food Science and Technology (Campinas)*, 30(1), 143-151. http://dx.doi.org/10.1590/S0101-20612010005000017.

Da Broi, M. G., Jordao, A. A., Jr., Ferriani, R. A., & Navarro, P. A. (2018). Oocyte oxidative DNA damage may be. involved in minimal/mild endometriosis-related infertility. *Molecular Reproduction and Development*, 85(2), 128-136. http://dx.doi.org/10.1002/mrd.22943. PMid:29247565.

Hwang, J. S., Lee, S. B., Choi, M.-J., Kim, J. T., & Seo, H. G. (2019). Anti-adipogenic effect of a turmeric extract-loaded nanoemulsion in 3T3-L1 preadipocytes and high fat diet-fed mice. *Food Sci. Technol*, 39(Suppl. 2), 439-447. http://dx.doi.org/10.1590/fst.20718.

Kwak, A. W., Choi, J. S., Liu, K., Lee, M. H., Jeon, Y. J., Cho, S. S., Yoon, G., Oh, H. N., Chae, J. I., & Shim, J. H. (2020). Licochalcone C induces cell cycle G1 arrest and apoptosis. in human esophageal squamous carcinoma cells by activation of the ROS/MAPK signaling pathway. *Journal of Chemotherapy (Florence, Italy)*, 32(3), 132-143. http://dx.doi.org/10.1080/1120009X.2020.1721175. PMid:32009586.

Lew, R. (2019). Natural history of ovarian function including assessment of ovarian reserve. and premature ovarian failure. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 55, 2-13. http://dx.doi.org/10.1016/j.bpobgyn.2018.05.005. PMid:30420162.

Li, B., Zhou, P., Xu, K., Chen, T., Jiao, J., Wei, H., Yang, X., Xu, W., Wan, W., & Xiao, J. (2020). Metformin induces cell cycle arrest, apoptosis and autophagy. through ROS/JNK signaling pathway in human osteosarcoma. *International Journal of Biological Sciences*, 16(1), 74-84. http://dx.doi.org/10.7150/ijbs.33787. PMid:31892847.

Lin, X., Dai, Y. D., Tong, X. M., Xu, W., Huang, Q., Jin, X., Li, C., Zhou, F., Zhou, H., Lin, X., Huang, D., & Zhang, S. (2020). Excessive oxidative stress in cumulus granulosa cells. induced cell senescence contributes to endometriosis-associated infertility. *Redox Biology*, 30, 1-32. http://dx.doi.org/10.1016/j.redox.2020.101431. PMid:31972508.

Nagata, S. (2018). Apoptosis and clearance of apoptotic cells. *Annual Review of Immunology*, 36(1), 489-517. http://dx.doi.org/10.1146/annurev-immunol-042617-053010. PMid:29400998.

Ren X, Wang S, Zhang C. Selenium ameliorates cadmium-induced mouse Leydig TM3 cell apoptosis via inhibiting the ROS/JNK/c-jun signaling pathway. Ecotoxicology Environment Safety, 2020(192), 110266. http://dx.doi.org/10.1016/j.ecoenv.2020.110266.

Saleem, M. Z., Nisar, M. A., Alshwmi, M., Din, S. R. U., Gamallat, Y., Khan, M., & Ma, T. (2020). Brevilin A inhibits STAT3 signaling and induces ROS-Dependent apoptosis, mitochondrial stress and endoplasmic reticulum stress in MCF-7 breast cancer cells. *OncoTargets and Therapy*, 13, 435-450. http://dx.doi.org/10.2147/OTT.S228702. PMid:32021288.

Thézénas, M. L., De Leo, B., Laux-Biehlmann, A., Bafligil, C., Elger, B., Tapmeier, T., Morten, K., Rahmioglu, N., Dakin, S. G., Charles, P., Martinez, F. E., Steers, G., Fischer, O. M., Mueller, J., Hess-Stumpp, H., Steinmeyer, A., Manek, S., Zondervan, K. T., Kennedy, S., Becker, C. M., Shang, C., Zollner, T. M., Kessler, B. M., & Oppermann, U. (2020). Amine oxidase 3 is a novel. proinflammatory marker of oxidative stress in peritoneal endometriosis lesions. *Scientific Reports*, 10(1), 1495. http://dx.doi.org/10.1038/s41598-020-58362-3. PMid:32001775.

Wang, A., Jiang, H., Liu, Y., Chen, J., Zhou, X., Zhao, C., Chen, X., & Lin, M. (2020a). Rhein induces liver cancer cells apoptosis via activating. ROS-dependent JNK/Jun/caspase-3 signaling pathway. *Journal of Cancer*, 11(2), 500-507. http://dx.doi.org/10.7150/jca.30381. PMid:31897245.

Wang, T., Liu, X., & He, C. (2020b). Glucocorticoid-induced autophagy and apoptosis in bone. *Apoptosis*, 25(3-4), 157. http://dx.doi.org/10.1007/s10495-020-01599-0. PMid:32157482.

Zhao, R. H., Liu, Y., Lu, D., Wu, Y., Wang, X. Y., Li, W. L., Zeng, C., Meng, Q. W., Lian, F. M., Zhou, J., Shi, Y., Sun, W. W., Han, Q., Tang, Y., & Shi, G. (2020). Chinese medicine sequential therapy improves pregnancy. outcomes after surgery for endometriosis-associated infertility: a multicenter randomized double-blind placebo parallel controlled clinical trial. *Chinese Journal of Integrative Medicine*, 26(2), 92-99. http://dx.doi.org/10.1007/s11655-019-3208-2. PMid:31997236.