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The Effect of Secretome Hypoxia Mesenchymal Stem Cells (SH-MSCs) in Reducing P65 and TNF-A Gene Expression in Polycystic Ovary Syndrome

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ABSTRACT: Polycystic Ovary Syndrome (PCOS) is an endocrine and metabolic syndrome characterized by a chronic inflammation and an increase of androgen production in ovarian theca cells.[1] The expression of the p65 gene and TNF-α was found to be intracellular inflammation markers in PCOS patients. Previous studies have indicated that the *Secretome of hypoxia mesenchymal stem cells* (SH-MSCs) has anti-inflammatory properties and is widely reported to be effective in treating various diseases. An experimental study by using the *Post Test Only Control Group Design* method. This study involved 24 female wistar rats divided into 4 groups. Group K1 consisted of healthy rats, K2 PCOS rats without any treatment, K3 PCOS rats treated with 200µL dose of SH-MSCs, and K4 PCOS rats treated with 400µL dose of SH-MSCs. On the 33rd day, the rats underwent an examination of the p65 and TNF-α gene expression. The result of Shapiro-Wilk test and Levene test indicated that the data were normally distributed and homogenous. In One-Way ANOVA test, groups K III and K IV of PCOS rats showed significantly lower changes in the expression of the p65 and TNF-α genes compared to the control group ($p<0.05$). The result of Kruskal-Wallis test and Mann-Whitney test showed significant differences in TNF-α gene expression among the groups ($p<0.05$). While, the post hoc Tamhane test showed significant differences in p65 gene expression among the groups ($p<0.05$). SH-MSCs given at a dose of 200 µL/kgBW and 400 µL/kgBW had an effect in reducing the expression of the p65 gene and TNF-α gene on female wistar rats of PCOS model.

KEYWORDS: PCOS, P65, SH-MSCs, TNF-α, Gene Expression

I. INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is an endocrine and metabolic syndrome characterized by a chronic inflammation and an increase of androgen production in ovarian theca cells (Chugh et al, 2021). A chronic inflammation plays a central role in the etiopathology of PCOS. The expression of the p65 and TNF-α genes, intracellular inflammation markers, was found in women with PCOS (Dokuzeylul et al, 2022 ; Garg et al, 2023). Until now, the standard therapies of PCOS used have not been effective and require a long-term use, leading to mild to severe side effects (Abdalla et al, 2020).

Hormonal therapy, as a first-line treatment, has side effects on metabolism, digestion, and can even be teratogenic (Bulsara et al, 2021). Preclinical and clinical studies have shown that SH-MSCs contains various anti-inflammatory cytokines or immunomodulatory significantly higher than *secretome normoxia MSCs* (SN-MSCs), such as *transforming growth factor* (TGF-β), *TNFα-stimulated gene* 6 (TSG6), *prostaglandin E2* (PGE2), IL-10, and *insulin-like growth factor* 1 (IGF-1), and lower levels of proinflammatory cytokines like IL-6 and IL-8 (Xue et al, 2022 ; Putra et al, 2021). A chronic inflammation plays a central role in the inflammatory symptoms of PCOS. Increased levels of inflammatory markers such as *C-Reactive Protein* (CRP), *Tumor Necrosis Factor-Alfa* (TNF-α), *interleukin* (IL)-6, IL-18, *Monocyte Chemoattractant Protein-1* (MCP-1), and *Acute-Phase Serum Amyloid A* (APSAA) have been found in the serum levels and follicular fluid of women with PCOS. Previous research reported that the increased levels of NF-κB p65, an intracellular inflammation marker, have been demonstrated in women with PCOS (Dokuzeylul et al, 2022).

Based on clinical, biochemical, and molecular studies, there is an evidence that TNF-α contributes fivefold to the etiopathogenesis of PCOS (Garg et al, 2023). TNF-α induces an inflammatory response as a normal physiological function for ovulation and affects follicular atresia, adiposity, insulin resistance, ovarian apoptosis, increased ovarian steroid secretion, anovulation, and hyperandrogenism (Physiology et al, 2013). Numerous studies have reported that SH-MSCs are capable of

suppressing the secretion of pro-inflammatory cytokines in various pathological diseases (Sazli et al, 2023 ; Zhao et al, 2022). However, the administration of SH-MSCs on the expression of the p65 and TNF-α genes in the rats of PCOS model has been relatively understudied.

II. MATERIAL AND METHODS

The research was conducted in the laboratory of Stem Cell and Cancer Research in Semarang from July to August 2023. This research has gone through an ethical review test from the Ethics Committee of Medical Faculty of Sultan Agung University (No.341/VIII/2023/Bioethics commission). The sample was prepared by mixing SH-MSC with 0,9% Nacl to achieve a concentration of 250 µL/g. SH-MSCs at doses of 200 mg and 400 mg each contained SH-MSCs with 200 µL (K3) and 400 µL (K4) dose of secretome, respectively. The Isolation Procedure of *Mesenchymal Stem Cell* from *Umbilical Cord* was conducted inside biosafety cabinet class 2, using sterile equipment, and performed with high sterility techniques. Subsequently, the Hypoxia TFF process was conducted.

This research used 24 white female strain wistar rats. The rats were divided into 4 groups that have met the inclusion criteria. Group K1 were healthy rats, K2 were PCOS rats without treatment, K3 were PCOS rats treated with a 200µL dose of SH-MSCs, and K4 were PCOS rats treated with a 400µL dose of SH-MSCs. On day 23, sample validation was conducted by observing the cytological appearance of the epithelium, ovarian follicles, and blood sugar levels. On day 33, samples were examined to observe the expression of the p65 and TNF-α genes. The analysis of p65 and TNF-α used *Real Time-Polymerase Chain Reaction* (RT-PCR). The PCR products were then analyzed using qRT-PCR illumine. The increase of gene expression was analyzed in the ratio of increase against housekeeping gene by using EcoStudy software.

The data were presented descriptively using a ratio data scale. The data were then tested for normality using the Shapiro Wilk test and homogeneity testing using the Levene test. The distribution of data on cholesterol, IL-6, and MDA levels obtained normal and homogeneous results, so One Way Annova test was continued ($p<0.05$) then followed with the post hoc *LSD* test. The Kruskal Wallis test and Man Whitney test were carried out to see the expression of the TNF-α gene between groups, and the *post Hoc Tamhane* test was carried out to see the expression of the p65 gene between groups.

III. RESULT

MSCs was isolated in *Stem Cell and Cancer Research* (SCCR) laboratory of Medical Faculty of Sultan Agung Islamic University, Semarang by using umbilical cord of 21-day-old pregnant rat. Furthermore, the result of isolation was cultured on plastic flask. The result of MCS showed an image of adherent cells at the flask's bottom with morphology resembling spindle-like cells. The research finding showed that MSCs was capable of differentiating into osteocyte and adipocyte, which can be detected through calcium deposit and the presence of red-stained fat in *Alizarin Red* and *oil Red dye* staining in both osteogenic and adipogenic cultures, respectively. Validation was performed on the PCOS rat model, and it was confirmed that DHEA induced ovary, as evidenced by the formation of *cystic follicles* in figure 1.

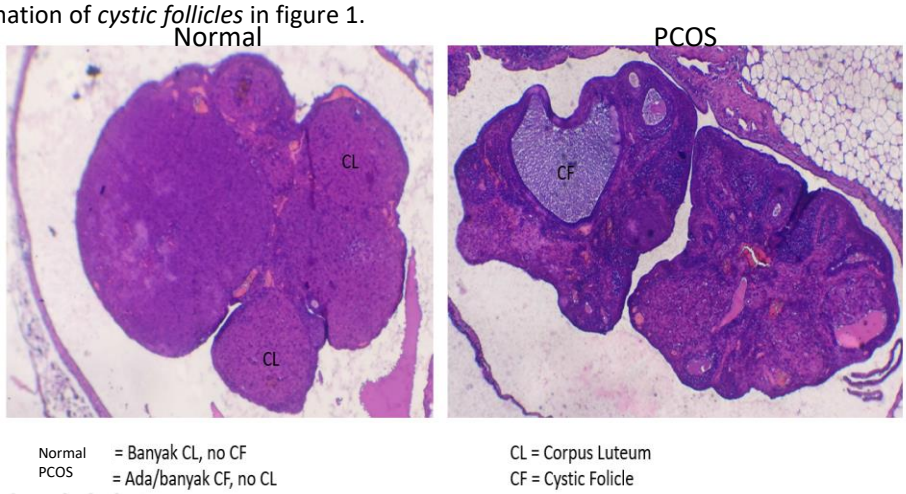

Figure 1. Comparison of HE painting results of healthy mice with PCOS mice

Table 1. Comparison of testosterone levels in healthy mice and PCOS mice

Group	Testosteron Mean (pg/mL)	Stdev (pg/mL)
Healthy	46.67	20.82
PCOS	80	26.46

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SH-MSCs was able to significantly reduce p65 and TNF-α levels in PCOS rats in a dose-dependent manner. Based on the research results, it was showed that the levels of p65 and TNF-α in the K3 group were the highest, followed by the K4 group. In this research, the average expression of the TNF-α gene in the control group was found to be the highest, with an average gene expression value of 2.2006 times, followed by the SH-MSC treatment group of 200 µl/kg with an average value of 0.9019 times. The lowest average expression of the TNF-α gene was observed in the 400 µl/kg treatment group with an average gene expression value of 0.7192 times, as shown in Figure 2. SH-MSC was able to significantly reduce TNF-α levels in male Wistar rats with a PCOS-like model, depending on the dosage.

In this research, it was found that the average p65 levels to be the highest in the control group with an average value of 2.2734, followed by the average p65 levels in the SH-MSC treatment group of 200 µl/kg with an average value of 0.7264. Furthermore, the average p65 levels in the SH-MSC treatment group of 400 µl/kg were 0.5576. It was found that there was significant difference between K2 and K3 (0.009), as well as between K2 and K4 (0.008), indicating meaningful differences as seen in Figure 3. However, there was no significant difference between K3 and K4 (0.674) based on the Post Hoc Tamhane test results. The analysis results also showed that the administration of SH-MSCs at 200µl and 400µl significantly reduced the p65 expression in female Wistar PCOS rats.

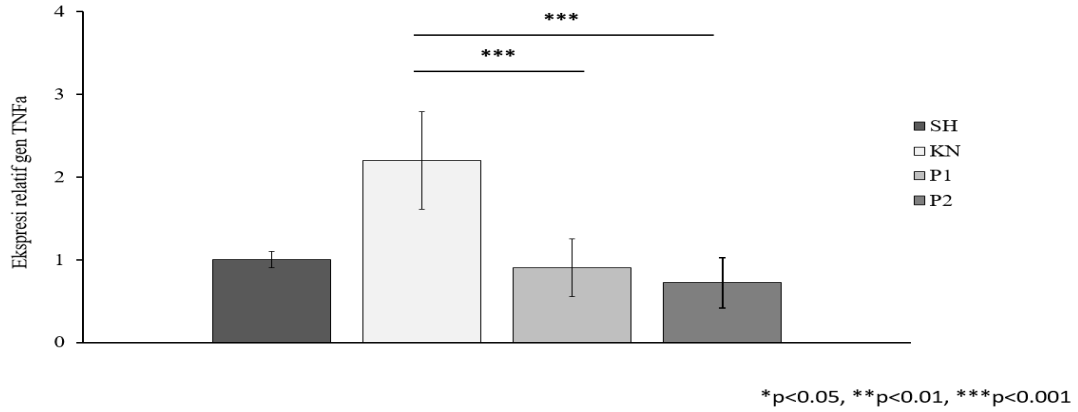

Figure 2. TNF-α gene expression in all study groups

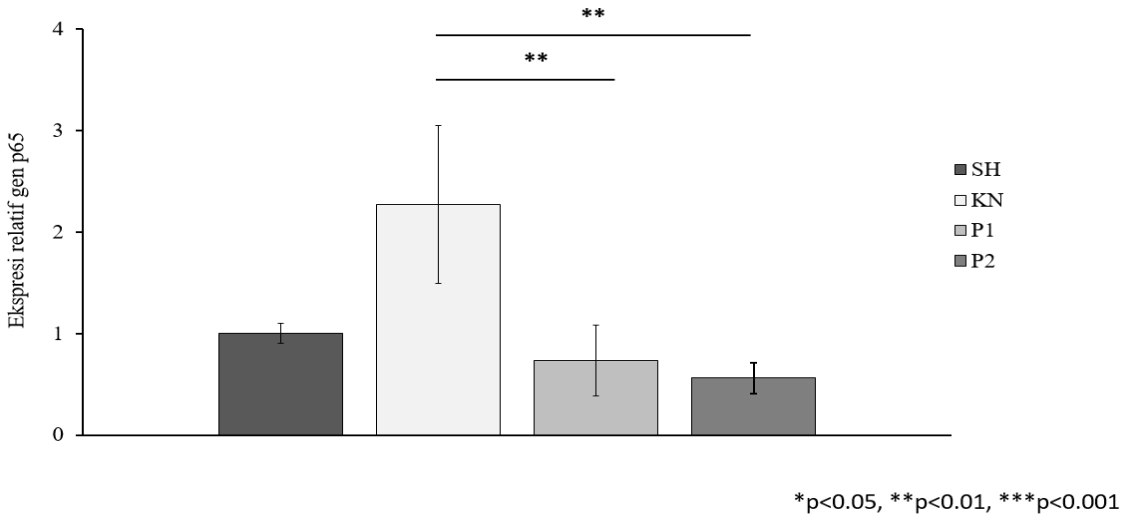

Figure 3. P65 gene expression in all study groups

IV. DISCUSSION

A chronic inflammation plays a central role of PCOS symptoms. It has been found that the serum levels and follicular fluid inflammatory markers such as CRP, TNF-α, IL-6, IL-18, MCP-1, and APSAA are increased in women with PCOS. Previous studies have reported that an increase in NF-κB p65 levels, an intracellular inflammation marker have been demonstrated in women with PCOS (Dokuzeylul et al, 2022). In this research, it was found that the TNF-α levels in the K4 group (SH-MSCs 400µl) significantly decreased compared to the group of rats (p=0.000).

These results indicated that the administration of a 400µl dose of SH-MSCs can reduce the inflammatory process. This may be attributed to the influence of IL-10 contained within SH-MSCs, which acts as an anti-inflammatory agent by inhibiting the NF-κB pathway through the mechanism of IL-10 STAT3 *Pathways* (Wilson et al, 2013 ; Muhl et al, 2019). This research also demonstrated that the TNF-α levels in both the K4 group (SH-MSCs 400µl) and the K3 group (administration of SH-MSCs 200µl) decreased compared to the group of PCOS rats (p=0.000).

This research aligned with the previous researches that used MSCs, where there was an increase in the number of corpus luteum after being given MSCs. In the context of MSC mechanism in wound areas, it is believed that they contribute through cell differentiation and promotion of vascularization by releasing PDGF (Baju et al, 2011). The increase of *cystic follicles* and testosterone hormone are caused by inflammation in the ovary. Secretome can mediate paracrine mechanisms within stem cells. Based on the research findings, there was a suppression of granulosa cell apoptosis.

This research showed that the expression of TNF-α and p65 in both the K4 group (SH-MSCs 400µl) and the K3 group (administration of SH-MSCs 200µl) decreased compared to the control group (p=0.000). The decrease in TNF-α and p65 levels can be associated with the presence of IL-10 in the SH-MSC secretome. IL-10 is believed to have the ability to induce a polarization shift from M1 to M2, which has anti-inflammatory properties through the IL-10 STAT3 pathway (Saraiva et al, 2010).

Cytokine IL-10 produced by SH-MSCs triggers STAT3 phosphorylation and forms homodimers that move to nucleus to activate gene expression that is responsive to STAT3, including SOCS3. SOCS3 also plays a role in inhibiting NF-κB translocation to the nucleus, resulting in the suppression of pro-inflammatory gene expression. Further comparisons are needed between various types of treatments, such as standard drugs used to treat PCOS, and therapy using SH-MSCs. The testosterone levels in ovarian tissue samples were measured by using the ELISA method. The absorbance values obtained were 46.67 pg/mL in healthy rats and 80 pg/mL in PCOS rats (Table 1). The increase of high testosterone levels indicated that PCOS symptoms have developed in the animal model. The GDP values in healthy rats on day 23 showed 94.67 mg/dL, while in PCOS rats, it was 107.67%.

V. CONCLUSIONS

SH-MSCs given at a dose of 200 µL/kgBW and 400 µL/kgBW had an effect in reducing the expression of the p65 gene and TNF-α gene on female wistar rats of PCOS model.

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