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Research Paper

IL-4 Overexpression in Mesenchymal Stem Cells Induced by Chlorzoxazone

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ABSTRACT: *Mesenchymal stem cells (MSCs) secrete various proteins, including growth factors, cytokines, and chemokines, known collectively as the secretome. The MSC-derived secretome has emerged as a promising therapeutic approach in regenerative medicine, particularly for conditions like osteoarthritis. Additionally, the MSC secretome can be formulated into topical preparations, such as creams, for easier application. One key anti-inflammatory cytokine secreted by MSCs is interleukin-4 (IL-4), which protects cartilage during joint inflammation and suppresses pro-inflammatory cytokines like IL-1β. To enhance the therapeutic efficacy of the MSC secretome cream, MSCs were treated with various concentrations of chlorzoxazone (CZ) (0 µM, 5 µM, 10 µM, 20 µM, and 50 µM) to promote IL-4 overexpression. The concentration of IL-4 in the MSC secretome was quantified using ELISA. Results indicated that the most significant increase in IL-4 concentration occurred in the secretome of MSCs cultured with 20 µM CZ.*

KEYWORDS: *Secretome, Mesenchymal stem cells, Interleukin-4, Chlorzoxazone*

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I. INTRODUCTION

Osteoarthritis (OA) affects approximately 250 million people worldwide and is a leading cause of disability, particularly among the elderly [1]. The World Health Organization (WHO) estimates the global prevalence of OA at 9.6% in men and 18% in women over 60. In Indonesia, with a population of 255 million, OA prevalence is 15.5% (about 39 million) in men and 12.7% (approximately 32 million) in women [2]. Although knee replacement surgery can restore function in degenerated knee joints, up to 20% of patients continue to experience pain and complications postoperatively. Consequently, cell-based therapies, such as mesenchymal stem cells (MSCs), have emerged as promising biological alternatives [3].

MSCs are characterized by their high self-renewal capacity and multipotent differentiation potential. MSCs derived from the synovial membrane of OA patients can differentiate into osteocytes, chondrocytes, and adipocytes. These regenerative properties make MSCs strong candidates for stem cell-based therapy in OA [4][5].

MSCs secrete various bioactive proteins into their culture medium, known as conditioned medium-mesenchymal stem cells (CM-MSCs) or the MSC secretome. Protein profiling of the MSC secretome has revealed growth factor-like proteins secreted by MSCs [6]. Numerous studies have highlighted the therapeutic potential of the MSC secretome in health and cosmetic applications, including anti-aging [7], hair regeneration [8], burn treatment [9], and osteoarthritis [10].

The MSC secretome offers several advantages over MSCs, including lower immunogenicity, anti-inflammatory properties, non-tumorigenicity, ease of storage, and improved tissue targeting [11]. Additionally, the secretome can be formulated into topical preparations such as creams, enhancing its practicality and ease of use [7]. These features make MSC secretome-based creams a more effective therapeutic option for OA.

To enhance the regenerative potential of the MSC secretome in OA therapy, MSCs have been treated with insulin-like growth factor 1 (IGF-1) to increase the concentrations of growth factors and cytokines within

the secretome [12]. IGF-1 treatment has elevated levels of several growth factors, including BMP2, FGF18, and TGF-β1 [12]. However, this method has not effectively increased levels of the anti-inflammatory cytokine interleukin-4 (IL-4), which is crucial in OA.

IL-4, an anti-inflammatory cytokine secreted by MSCs, plays a vital role in reducing inflammatory responses and supporting tissue repair. In OA, IL-4 helps protect cartilage during joint inflammation by inhibiting inducible nitric oxide synthase (iNOS) and nitric oxide (NO) and downregulating pro-inflammatory cytokines such as interleukin-1β (IL-1β) [3].

However, MSCs secrete only minimal amounts of IL-4. Given IL-4's importance in OA recovery, strategies to enhance its expression in the MSC secretome are needed. Previous efforts have included genetically modifying MSCs to promote sustained IL-4 secretion, demonstrating therapeutic benefits in OA models [3][13]. Nevertheless, these methods are complex and costly. Therefore, a more efficient approach, such as inducing IL-4 expression with chlorzoxazone (CZ), is required.

II. METHODOLOGY

2.1 Materials and Equipment

The equipment used in this study included an ELISA reader, CO_2 incubator, Class A2 biosafety cabinet (LAF), water bath, centrifuge, and inverted microscope. The materials consisted of adipose-derived mesenchymal stem cells (AD-MSCs) from the INA LAB Stem Cell Collection in Padang; Dulbecco's Modified Eagle Medium (DMEM; Gibco®); fetal bovine serum (FBS; Gibco®); penicillin-streptomycin (PenStrep); a human IL-4 ELISA kit (Feiyue Biotech); and chlorzoxazone (CZ; Sigma-Aldrich).

2.2 Experimental Design

This study employed a laboratory-based experimental method with two stages. The first stage involved inducing IL-4 overexpression in MSCs using chlorzoxazone (CZ) over 24 hours, following the procedure by Deng et al. (2020) [14]. A completely randomized design (CRD) was applied with the following treatment groups:

- K0 = 0 μM CZ (control),
- K1 = 5 μM CZ,
- K2 = 10 μM CZ,
- K3 = 20 μM CZ,
- K4 = 50 μM CZ.

Each treatment was conducted in triplicate.

The second stage involved analyzing IL-4 concentration in the MSC secretome using the enzyme-linked immunosorbent assay (ELISA). Secretomes from MSCs treated with varying CZ concentrations were analyzed using the human IL-4 ELISA kit, following the manufacturer's instructions. All samples were assayed in duplicate.

2.3 Data Analysis

Data were analyzed using IBM SPSS Statistics version 25.0.0. The concentrations of IL-4 in the MSC secretome were assessed using one-way analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$. Duncan's post hoc test was performed for multiple comparisons.

III. RESULTS AND DISCUSSION

In this study, mesenchymal stem cells (MSCs) were isolated from adipose tissue obtained from the stem cell stock at INA-Lab, Padang. The MSCs were treated with various concentrations of chlorzoxazone (CZ) (0 μM, 5 μM, 10 μM, 20 μM, and 50 μM) for 24 hours. The concentration of interleukin-4 (IL-4) in the MSC secretome was analyzed using an enzyme-linked immunosorbent assay (ELISA).

Table 1. IL-4 ELISA Standard Measurement Results

Standard	Concentration (pg/mL)	Well	OD Value
1	0	H1	0,0314
2	3,13	G1	0,1047
3	6,25	F1	0,2059
4	12,5	E1	0,4101
5	25	D1	0.7267

6	50	C1	1,3738
7	100	B1	1,7853
8	200	A1	2,1216

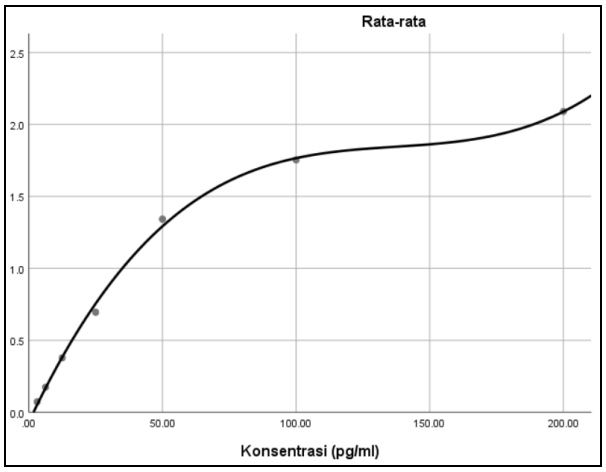

Figure 1. Standard Curve

In this study, the concentration of IL-4 in the MSC secretome induced with Chlorzoxazone (CZ) for 24 hours was analyzed using an ELISA kit from Feiyue Biotech. The standard was lyophilized IL-4, which was reconstituted and diluted according to the manufacturer's protocol. The IL-4 standards were measured based on their optical density (OD) values. The absorbance values obtained indicated a positive correlation with increasing concentration, demonstrating a directly proportional relationship. The results of the IL-4 standard measurements are presented in Table 1.

A standard curve was generated, with the x-axis representing concentration (pg/mL) and the y-axis representing absorbance (OD). A cubic polynomial (non-linear) model was used to construct the curve. The relationship between IL-4 standard concentrations and OD values was assessed using a regression equation. The resulting regression model showed a correlation coefficient (r) of 0.999 ($p = 0.000$; $p < 0.05$), indicating a very strong positive correlation (Figure 1). This regression equation was then used to calculate the IL-4 concentrations in the experimental samples.

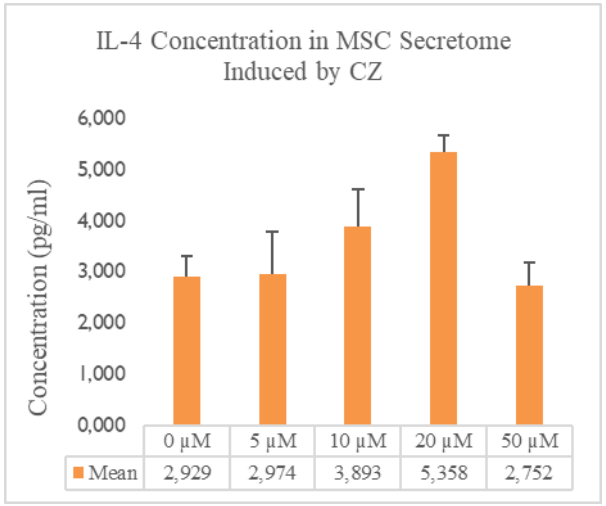

Figure 2. ELISA Results Diagram

ELISA results for IL-4 concentration in MSC secretome following CZ induction are presented in Figure 2. The data show that IL-4 concentration reached its peak at the 20 µM treatment but decreased at the 50 µM concentration. In the 0 µM group, the mean absorbance was 0.0472, corresponding to an IL-4 concentration of 2.929 pg/ml based on the standard curve equation. At 5 µM, the mean absorbance was 0.0490, yielding an IL-4 concentration of 2.974 pg/ml. The 10 µM treatment, with a mean absorbance of 0.0848, resulted in an IL-4

concentration of 3.893 pg/ml. For the 20 µM and 50 µM treatments, mean absorbance values were 0.1420 and 0.0403, corresponding to IL-4 concentrations of 5.358 pg/ml and 2.752 pg/ml, respectively.

ANOVA

Respon

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.210	4	3.552	10.445	.001
Within Groups	3.401	10	.340		
Total	17.611	14			

Figure 3. Statistical analysis

Table 2. Duncan's Post Hoc Test Analysis

CZ Treatment	Mean (pg/ml)
0 µM	2,92867±0,392[ab]
5 µM	2,97433±0,831[ab]
10 µM	3,89300±0,748[b]
20 µM	5,35833±0,327[c]
50 µM	2,75233±0,432[a]

Based on the one-way ANOVA and Duncan's post hoc test (Figure 3 and Table II), the IL-4 concentration in the MSC secretome treated with 20 µM CZ was significantly higher than in other treatment groups ($p < 0.05$; $p = 0.001$). According to Deng et al. (2020), the optimal CZ concentration to enhance the immunomodulatory capacity of MSCs is 10 µM. However, the difference in IL-4 levels between 10 µM and 20 µM CZ was not substantial. The observed increase in immunomodulatory capacity appears to correlate positively with CZ concentration in the culture medium [14].

At 50 µM CZ, IL-4 concentration decreased, possibly due to overstimulation or cytotoxic effects at high doses. MSCs reside in a manipulable microenvironment that can be optimized for therapeutic purposes. However, excessive stimulation may impair MSC function. For example, prolonged hypoxic manipulation can lead to loss of function, and high levels of reactive oxygen species (ROS) can induce oxidative stress, apoptosis, and reduced regenerative capacity [15][16].

A previous study by Marlina et al. (2021) showed that IGF-1–induced MSCs increased secretion of growth factors such as BMP2, FGF18, and TGF-β1, but did not enhance IL-4 levels—an anti-inflammatory cytokine with a key role in osteoarthritis (OA) [12]. In contrast, Deng et al. (2020) reported that chlorzoxazone (CZ) induction could shift pro-inflammatory MSC1 toward the anti-inflammatory MSC2 phenotype, leading to increased IL-4 secretion [14].

Deng et al. (2020) also highlighted the role of FOXO3 in CZ-mediated MSC polarization. FOXO3, previously linked to muscle atrophy, is a relevant target due to CZ's muscle relaxant properties. FOXO3 is typically phosphorylated via the PI3K-AKT and MAPK-ERK pathways. CZ inhibits FOXO3 phosphorylation largely independent of these pathways, thereby promoting the expression of immunomodulatory factors, including IL-4. This mechanism supports CZ's ability to enhance anti-inflammatory cytokine secretion and improve MSC secretome-mediated immunomodulation, which may contribute to OA therapy [14].

IV. CONCLUSION

Different CZ concentrations significantly affected IL-4 levels in the MSC secretome ($p < 0.05$; $p = 0.001$). Treatment with 20 µM CZ resulted in the highest IL-4 concentration, significantly greater than other groups.

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