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### The Effect of Activated Growth Factor (AGF) from Platelets on Alpha-SMA Levels in Osteoarthritis: Invivo Study

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#### ABSTRACT

**Background:** The most prevalent joint condition and leading contributor to disability is osteoarthritis (OA), resulting in a significant socio-economic impact. It occurs due to an imbalance of pro-inflammatory and anti-inflammatory mediators, influencing anabolic and catabolic activities. This study aims to determine the effect of activated growth factor (AGF) on alpha-SMA levels. **Methods:** This research is an in vivo post-test-only control group experimental study conducted at the Eureka Research Laboratory. Thirty male Wistar rats were divided into five treatment groups: normal control group, negative control group, AGF I group (TGF- $\beta$  100 pg/mL), AGF II group (TGF- $\beta$  1000 pg/mL), and AGF III group (TGF- $\beta$  10,000 pg/mL). AGF was obtained from rat blood intravenously and centrifuged at a predetermined speed. Growth factor activation was performed by adding 10% CaCl<sub>2</sub>, and then TGF- $\beta$  levels were measured. All groups of rats were acclimatized for 7 days. After that, osteoarthritis was induced with intra-articular injection of monoiodoacetate (MIA) 4.8 mg/60 $\mu$ L in all groups of rats except the normal control group. Next, rats were given treatment according to the group for 21 days. On the 21st day, rats were euthanized, and alpha-SMA levels were measured using the sandwich method ELISA kit. **Results:** Sequentially, the mean Alpha-SMA levels for each group in pg/mL were: 91.495  $\pm$  2.36; 10.682  $\pm$  1.09; 30.502  $\pm$  2.00; 52.892  $\pm$  1.29; and 76.180  $\pm$  2.65. In the AGF group, there was an increase in alpha-SMA levels directly proportional to the dose of TGF- $\beta$  injected. **Conclusion:** AGF has an effect on increasing alpha-SMA levels in the joints of rats with a model of osteoarthritis.

#### 1. Introduction

A joint condition known as osteoarthritis (OA) is associated with pain, function loss, and a lower quality of life (QoL).<sup>1-3</sup> About 300 million individuals globally are thought to suffer from osteoarthritis, which is an endemic illness. OA.<sup>2</sup> According to estimates, OA costs society \$303 billion in medical expenses annually.<sup>2</sup>

Anabolic and catabolic activity or pro- and anti-inflammatory mediator imbalances determine the pathophysiology of osteoarthritis.<sup>3-5</sup> The palliative nature of all current osteoarthritis treatments has not been able to stop cartilage degeneration, inflammation, or the disease's development.<sup>6,7</sup>

Nonsteroidal anti-inflammatory drugs (NSAIDs), which have the potential to cause canal perforation, are commonly used to treat osteoarthritis (OA).

It is necessary to conduct research on more gentle OA management that can prevent the degradation of tissue, for example, by utilizing a growth factor from platelets. Platelets include many bioactive proteins and growth factors that are involved in hemostasis or vascular endothelial development.<sup>8,9</sup> According to research done in 2017 by Moussa et al., platelets have anabolic effects on cartilage.<sup>6</sup> One of the platelet's anti-inflammatory GF effects is a result of TGF- $\beta$  that is accompanied by its receptor.

TGF- $\beta$  binds to its receptor, which is one of the reasons platelet-derived GF has anti-inflammatory properties. The transcriptional activation of the fibrotic gene program and the production of  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin) are facilitated by nuclear translocation and SMAD4 signaling, which are the results of TGFBR1-mediated SMAD2/3 phosphorylation in the canonical TGF- $\beta$  pathway.<sup>10</sup>

When compared to normal fibroblasts, cells expressing  $\alpha$ -SMA will produce collagen with more activity and secrete significant amounts of extracellular matrix proteins, which will aid in the reparative response.<sup>11-13</sup> To explore the potential of growth factors (GF) in treating OA, this research was conducted to test the efficacy of activated growth factor from platelets on alpha-SMA levels in Wistar rats, a model of osteoarthritis.

## 2. Methods

### Preparation of activated growth factor

Activated growth factor (AGF) production involves the initial step of extracting intravenous blood from research subjects. Three milliliters of intravenous blood were gathered and deposited into a tube with 0.01% ACD. Following this, centrifugation was carried out at a rate of 3000 rpm for a duration of 20 minutes. Platelet isolation followed, and the platelet growth factor activation process utilized both mechanical and biological techniques. Subsequent centrifugation was conducted at a speed of 5000 for 15 minutes, separating the supernatant and pellet. The supernatant was then examined for TGF-beta levels using the ELISA method, following the ELISA TGF-beta examination procedure provided by the manufacturer.

Platelet activation was initiated by adding 10% CaCl<sub>2</sub>. The addition of CaCl<sub>2</sub> induced progressive growth factor release, starting from 15 minutes and increasing up to 24 hours.

### Preparation and treatment of test animals

This study is an experimental in vivo post-test-only control group and was held at Eureka Research Laboratory, Palembang, Indonesia. The research

samples consist of rats aged 8-10 weeks with a body weight ranging from 200 to 250 grams, and they will be distributed among five treatment groups. The minimum required sample size, determined using the Federer formula, is 25 rats. An additional mouse was included in each group to prevent dropouts, resulting in a total required sample of 30 rats. The Medical and Health Research Ethics Committee of the Universitas Sriwijaya Faculty of Medicine has approved this study, with protocol number 300-2023.

Ad libitum standard food and water were given to the rats, and they were kept in a 12-hour light/dark cycle. The cage environment was regulated at a temperature of approximately 20–25°C and a relative humidity of 50–60%. Rats underwent a one-week acclimatization period before the commencement of the experiment. Following acclimatization, the rats were randomly assigned to the five treatment groups (n = 6 rats per group): normal control (not induced by osteoarthritis, no treatment), negative control (MIA + saline 60uL/7 days for 21 days), AGF I (MIA + TGF-beta 100 pg/mL/7 days for 21 days), AGF II (MIA + TGF-beta 1000 pg/mL/7 days for 21 days), and AGF III (MIA + TGF-beta 10000 pg/mL/7 days for 21 days). Osteoarthritis induction was performed in each group except the normal group. Anesthesia was administered using Biopenthyl® at a dose of 0.1 mL/10 gr BW intraperitoneally, followed by intraarticular injection of monoiodoacetic acid (MIA) at a dose of 4.8 mg/60  $\mu$ L.

After 21 days, euthanasia was conducted using a toxic dose of 1 ml chloroform per test animal. Joint samples were collected and stored at -20°C before measurements. Subsequently, the tissue was homogenized under cold conditions (4°C), and the joint was centrifuged at 3000 rpm for 20 minutes. Alpha-SMA measurements were performed using the sandwich ELISA method following the manufacturer's instructions.

### Statistical analysis

The objective of this study is to examine and compare the mean alpha-SMA levels among all treatment groups. The research data, expressed as

mean  $\pm$  standard deviation for each group, underwent evaluations for data homogeneity using the Levene test and data normality using the Shapiro-Wilk test. The choice of the Shapiro-Wilk test was based on the research sample size being less than 50. If the results of the normality test were statistically significant ( $p < 0.05$ ), a one-way ANOVA hypothesis test was carried

out, followed by the post hoc Bonferroni test.

### 3. Results

The three activated growth factor (AGF) groups had higher mean  $\alpha$ -SMA levels than the negative control group.  $\alpha$ -SMA levels increased along with the concentration of TGF- $\beta$ .

Table 1. Effect of AGF on  $\alpha$ -SMA levels.

No.	Group	Mean $\alpha$ -SMA Level (pg/mL) $\pm$ Standard Deviation
1.	Normal control	91,495 $\pm$ 2,36 <sup>†</sup>
2.	Negative control	10,682 $\pm$ 1,09
3.	AGF I	30,502 $\pm$ 2,00 <sup>†</sup>
4.	AGF II	52,892 $\pm$ 1,29 <sup>†</sup>
5.	AGF III	76,180 $\pm$ 2,65 <sup>†</sup>

†:  $p < 0.05$  when compared to the negative control (MIA). MIA; monoiodoacetic acid, AGF; activated growth factor, TGF- $\beta$ ; Transforming growth factor- $\beta$ .

### 4. Discussion

In this study,  $\alpha$ -SMA levels increased in proportion to the amount of TGF- $\beta$  administered. This occurs as a result of TGF- $\beta$ 's anabolic function in the joints, which balances the catabolic components and promotes joint improvement in osteoarthritis-affected joints.

In osteoarthritis, transforming growth factor-beta (TGF- $\beta$ ) regulates the production of extracellular matrix components by myofibroblasts that express alpha-SMA.<sup>14,15</sup> Anabolic and catabolic processes are out of balance in osteoarthritis due to altered metabolic activity and tissue homeostasis. Catabolic activity is the main cause of osteoarthritis, essentially leading to tissue deterioration.<sup>5</sup> The two cytokines most well-known for inducing and sustaining inflammatory reactions, pain, and inflammation are TNF- $\alpha$  and IL-1 $\beta$ .<sup>16,17</sup>

Molecular debris accumulates in the joints as a result of proinflammatory cytokines and associated pathways. Synovial inflammation is brought on by molecules released into the synovial cavity that are produced from deteriorated articular cartilage. In response to this process, synoviocytes in the synovial membrane release growth factors and proinflammatory cytokines, which stimulate

angiogenesis and cause articular chondrocytes to change phenotypically, thereby drawing immune cells. Further inflammatory cytokines and proteolytic enzymes produced by dedifferentiated articular chondrocytes cause cartilage breakdown and synovial inflammation.<sup>18</sup>

Articular cartilage is unable to mend itself in tissue undergoing osteoarthritis, leading to chondrocyte dedifferentiation.<sup>19</sup> Proliferative chondrocytes undergo drastic changes in cytoskeletal structure, metabolism, and cell shape as they develop into a fibroblastic phenotype. This ultimately results in a higher synthesis. type I collagen, which in turn creates shards of fibronectin, forms the repair.<sup>18,20,21</sup> In order to rebuild and remodel the damaged matrix, fibroblasts in the synovial lining often proliferate more quickly than normal fibroblasts by generating more collagen—the primary building block of the extracellular matrix in ligaments—and reconstructing the matrix than do regular fibroblasts. A contractile phenotype, which is shown by the creation of microfilament bundles and the de novo generation of alpha-smooth muscle actin ( $\alpha$ -SMA), is also necessary for fibroblasts.<sup>12</sup>

The canonical signaling of TGF- $\beta$  will result in increased  $\alpha$ -SMA expression, TGF- $\beta$  stimulation, and cell proliferation. TGF- $\beta$  will attach itself to type I

(TGFBR1/Transforming Growth Factor Beta Receptor 1) and type II (TGFBR1) heterodimeric transmembrane receptors. For both canonical and noncanonical signaling, use TGFBR2/Transforming Growth Factor Beta Receptor 2. The classical TGF- $\beta$  pathway entails nuclear translocation for the activation of  $\alpha$ -SMA transcription after TGFBR1-mediated SMAD2/3 phosphorylation.<sup>10</sup>

Other kinases, including extracellular signal-regulated kinase, MAPK, NF- $\kappa$ B, and JNK, are signaled by the TGF- $\beta$  non-canonical route. Collagen, fibronectin, and proteoglycans are among the ECM components whose secretion is encouraged by the TGF- $\beta$  pathway. In addition to the synthesis of extracellular matrix (ECM) proteins, including type II collagen and aggrecan, the protective impact of TGF- $\beta$  on cartilage matrix turnover also relies on the prevention of ECM protein degradation via enhanced synthesis of protease inhibitors such as TIMP.<sup>22</sup> Alternatively, TGF- $\beta$  modulates cellular responses by activating non-Smad pathways. The phosphatidylinositol-3-kinase (PI3K/AKT) pathway, Rho-like GTPase, and MAPK are examples of these non-Smad pathways. The JNK, p38 MAPK, and PI3K/AKT signaling pathways are among the non-Smad signaling pathways that contribute to TGF- $\beta$ -induced collagen I production.<sup>23</sup>

Smad2 and Smad3 are ligand-responsive proteins in relation to the TGF- $\beta$  pathway. TGF- $\beta$  attaches itself to the TGF- $\beta$  type II receptor (T $\beta$ RII), which then phosphorylates and activates the type I receptor (T $\beta$ RI) through active type II receptor kinase. For a limited period, active T $\beta$ RI phosphorylates its carboxy terminus and binds to Smad2 and Smad3. These receptor-activated Smads go into the nucleus, where target genes are transcriptionally controlled after being phosphorylated and forming heteromeric complexes with Smad4. The process of TGF-induced transcriptional activation can be facilitated by binding regions of Smad3/Smad4 that contain CAGA boxes or by Smad proteins collaborating with other trans-acting factors that affect the production of  $\alpha$ -SMA. 10 Smad-Binding Elements (SBE) or the CAGA box are regulated

by SMAD3-dependent pathways. SMAD3 binds to the promoters of COL1A2, COL3A1, COL5A1, COL6A1, and COL6A3.<sup>23,24</sup>

Furthermore, through regulating the deposition and turnover of matrix-degrading proteolytic enzymes as serine proteinases and MMPs, as well as by suppressing the production of these enzymes, TGF- $\beta$  contributes significantly to the regulation of ECM homeostasis. Furthermore, TGF- $\beta$  stimulates the synthesis of TIMPs and PAI-1, two proteinase inhibitors. Smads have a key role in mediating a variety of TGF- $\beta$  family biological functions, including the production of ECM genes such as MMP-13, collagen types I and VII, aggrecan, PAI-1, and collagen types IV. Furthermore, Smads take part in TGF- $\beta$ -induced downregulation of the human MMP1 promoter activity.<sup>25</sup>

The 2013 study by Leivonen et al. found that TGF- $\beta$ -stimulated fibroblasts activated the p38 MAPK, ERK1/2, and Smad3 pathways. It takes the activation of all three mechanisms for TGF- $\beta$  to induce TIMP-3 expression. Smad3 mediates TGF- $\beta$ -induced stimulation of TIMP-3 expression by forming an association with Smad4.<sup>25</sup> TGF- $\beta$ -induced elevation of TIMP-3 expression is mediated by the ERK1/2 and p38 MAPK pathways in conjunction with Smad3. Disintegrins and metalloproteinases, two membrane-associated ADAMs, as well as ADAMTS matrix-associated thrombospondin, are all strongly inhibited by the extracellular matrix protein TIMP-3. TIMP-3 selectively inhibits the aggrecan-degrading enzymes ADAMTS4 and ADAMTS5, as well as ADAM 12 and ADAM 17.<sup>26</sup> Reducing ADAM and MMP synthesis will break the vicious cycle of producing pro-inflammatory cytokines like TNF- $\alpha$  and IL-1 $\beta$ , which will slow down the advancement of osteoarthritis.

Therefore, based on the review that has been conducted, it is concluded that the anti-inflammatory effect of TGF- $\beta$ , which in turn increases the antianabolic effect and the  $\alpha$ -SMA transcription signaling process, is what caused the increase in  $\alpha$ -SMA levels in the group that received activated growth factor (AGF). Moreover, there is a rise in the synthesis

of TIMP3, collagen, and fibronectin, which prevents the manufacture of proteases and other ADAMTs and slows the advancement of osteoarthritis.

## 5. Conclusion

In osteoarthritis-induced albino rats, activated growth factor at concentrations of 100 pg/mL, 1000 pg/mL, and 10,000 pg/mL statistically significantly increases  $\alpha$ -SMA levels.

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