

## **Fibrotic effect of monoculture, conditioned media and coculture of fibroblast NIH3T3 cells on collagen secretion and mRNA TGF- $\beta$ expressions**

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### **ABSTRACT**

Fibrosis is common profound in diabetes condition, they characterized by accumulation of extracellular matrix in tissue then cause tissue injury. The study aim to investigate effect of monoculture, conditioned media, and coculture fibroblast of NIH3T3 cells (NIH3T3 cells) in LPS-high glucose media (HG-LPS media) conditions on mRNA TGF- $\beta$  and collagen secretion. The fibroblast NIH3T3 were cultured with various culture type there is mono culture NIH3T3 cells, conditioned media from macrophage RAW264.7 cells culture media, and coculture with RAW264.7 cells (ratio 1:1 and 1:2) in normal media (media containing 4500  $\mu$ g/mL of glucose) and HG-LPS media (media containing LPS 0.25  $\mu$ g/mL and D-glucose 5400  $\mu$ g/mL). Our findings shows that the TGF- $\beta$  mRNA expression in RAW 264.7 and NIH 3T3 cell coculture media with a culture ratio of 1:1 (Coculture 1:1) were the highest ( $p < 0.05$ ) compared than monoculture of NIH3T3 cells (cultured in normal media or HG-LPS Media), conditioned media from RAW264.7 cells in normal media (CM-M0 Media), and the RAW 264.7 and NIH 3T3 co-culture cells with 1:2 cultured ratio in HG-LPS Media (Coculture 1:2). On other hand, the the relative ratio of collagen secretion on Coculture 1:1 were the highest compared to CM-M0 media ( $p < 0.05$ ). Our study conclude that RAW 264.7 and NIH 3T3 coculture cells with a (1:1) cultured ratio in LPS and high glucose media (Coculture 1:1) is appropriate for diabetic fibrosis in vitro models.

**Keywords:** fibrosis, coculture, NIH3T3 cells, RAW264.7 cells, TGF- $\beta$  mRNA, collagen

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## INTRODUCTION

Diabetes cause profound long-term effect on multiple organs fibrosis there are skeletal muscle, brain, liver, eyes, kidney, heart, and lungs. Hiperglycemia induce pathological change, including inflammation and fibrosis. Tissue fibrosis initially results from tissue injury caused by dysregulated production of extracellular matrix (Talakatta et al., 2018). This pathological condition may contribute to cellular stress and cellular dysfunction, characterized by prolonged inflammation and fibrosis (Zhang et al., 2018). TGF- $\beta$  is a potent pro-fibrotic agent that plays a role in regulating collagen secretion (Meng et al., 2016).

Fibrosis models are widely used to understand the pathological conditions in diabetes such as renal fibrosis, aortic fibrosis, or idiopathic pulmonary fibrosis (Chung & Lan, 2013; Wang et al., 2020). But, fibrosis in vitro models is still controversial because TGF- $\beta$  and collagen secretion are not optimally expressed (Ploeger et al., 2013). In the present study, we investigated the fibrotic effect of fibroblast NIH3T3 cells in monoculture, conditioned media, and coculture cells in LPS and high glucose media conditions. In this study, TGF- $\beta$  mRNA expression and collagen secretion ratios were evaluated to investigate TGF- $\beta$  and collagen correlation and determine the optimal in vitro models.

## MATERIALS AND METHOD

### Materials

Fetal bovine serum-qualified (Gibco® 26140-079), Dulbecco's modified eagle media (Gibco® 12800-058), Penicillin-streptomycin (Gibco® ), Fungizone (Gibco® ) were purchased from Gibco. Materials used for treatments was D-glucose (Merck®), lipopolysaccharides from Escherichia coli 055:B5 (Sigma L2880). Materials used for identification were Tri-RNA Reagent (Favorgen® FATRR 001), Go Taq Green Master Mix (Promega), FluoroVue™ Nucleic Acid Gel Stain (Smobio® NS1000), ExcelRT cDNA Synthesis Kit (Smobio® RP-1300), and Sirius Red (Sigma® 2610108).

### Methods

#### Ethical approval

This study was approved by The Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Public Health, and Nursing, Gadjah Mada University, Yogyakarta, Indonesia (Ref. KE/FK/0878/EC/2018) approved on 21 August 2018.

#### Cell Culture in vitro model

The RAW 264.7 and NIH 3T3 cells were cultured and maintained in DMEM medium containing 4500 ug/ml of glucose (Gibco® 12800-058), supplemented with 10% fetal bovine serum (FBS, Gibco® 26140-079), and 2% penicillin/streptomycin (Gibco®). The cells were incubated at 37°C in a humidified 5% of CO<sup>2</sup> atmosphere. The fibroblast NIH3T3 cells (1x10<sup>6</sup> cells/well) were cultured overnight in three different culture types there are the NIH3T3 cells monoculture, conditioned media from macrophage, and coculture of NIH3T3 and RAW264.7 cells. The monoculture of NIH3T3 cells cultured in medium LPS 0.25  $\mu$ g/mL and D-glucose 5400  $\mu$ g/mL (HG-LPS), and medium containing 4500 ug/ml of glucose (Normal Media) as a negative control. On other treatment, the NIH3T3 cells cultured in conditioned media were obtained from RAW264.7 cells (1x10<sup>6</sup> cells/well) in normal media (CM-M0 Media) and HG-LPS media (CM-M1 Media) after 24h. While coculture cells were conducted with culture together NIH3T3 and RAW264.7 cells in various number ratios of the cells (Coculture 1:1 and 1:2) in HG-LPS media (Holt et al., 2010).

#### RNA Isolation semi-quantitative reverse transcriptase PCR assay

The total RNA was isolated from the cells of control and treated cells using Tri-RNA reagent, and cDNA was synthesized using ExcelRT™ Reverse Transcription Kit II (Smobio, RP1400) according a previously described protocol (Toni et al., 2018). The RNA (100 $\mu$ g/  $\mu$ L) was reverse transcribed using ExcelRT™ Reverse Transcription Kit II. Thermal cycle conditions were 25°C for

11 min, 42°C for 50 min, and 85°C for 5 min. Complementary DNA (1 µL) was used as template and primers used for PCR analysis mentioned in the [Table 1](#). The TGF-β mRNA expression with GAPDH as housekeeping gene were measured by reverse transcriptase PCR (RT-PCR). The Go Taq Green (Promega, M7122) Master Mix was used and RT-PCR reaction was performed for 35 thermal cycles, following the conditions of the thermal cycle: Denaturation at 95°C for 7 minutes; Annealing at 60°C (GAPDH), 54°C (TGF-β) for 1 minute; and Extension at 72°C for 4 minutes 45 seconds. The expression of the target and housekeeping gene each of experiment was carried out in duplicate. The PCR product were subjected to 2% agarose gel electrophoresis and gel red (FluoroVue™ Nucleic Acid Gel Stain) staining. The gene expression of PCR product in gel electrophoresis was quantified using densitometry analysis using ImageJ software.

**Table 1. Primers used for PCR analysis**

Name <sup>1</sup>	Forward Primer	Reverse Primer
TGF-β	TTCCGCTGCTACTGCAAGTCA	GGGTAGCGATCGAGTGTCCA
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

<sup>1</sup>Primer's names: Transforming Growth Factor-β, Mouse (TGF-β); Glyceraldehyde-3-phosphate dehydrogenase, Mouse (GAPDH)

### Analysis of collagen secretion in culture medium

Collagen contained in the cells culture medium in each well was evaluated using the Sirius Red assay based on the protocol described previously with modification ([Hashim et al., 2011](#)). Collagen was isolated using polyethylene glycol 400 overnight, and dissolved pellet by 0.1 M CH<sub>3</sub>COOH (Acetic acid). Acetic acid as solvent control. A sample (50 uL) and solvent control were added 250 µL Sirius Red solution 1% and mixed at room temperature for 20 min to allow precipitation of collagen (reddish pellet). The excess solution was removed using HCl and NaOH was added to each tube and mixed gently to allow dissolved. The absorbance was measured at 540 nm. The collagen secretion ratio was calculated by comparing the sample and solvent control absorbance.

### Data Analysis

The data of TGF-β mRNA and Collagen secretion ratio's are presented as mean ± standard of deviation (SD). All data were analyzed by One-Way ANOVA of variance test followed by post-hoc tests by tukey for TGF- β mRNA and Dunnett T3 for Collagen secretion ratio's data. A value of p<0.05 was considered significant.

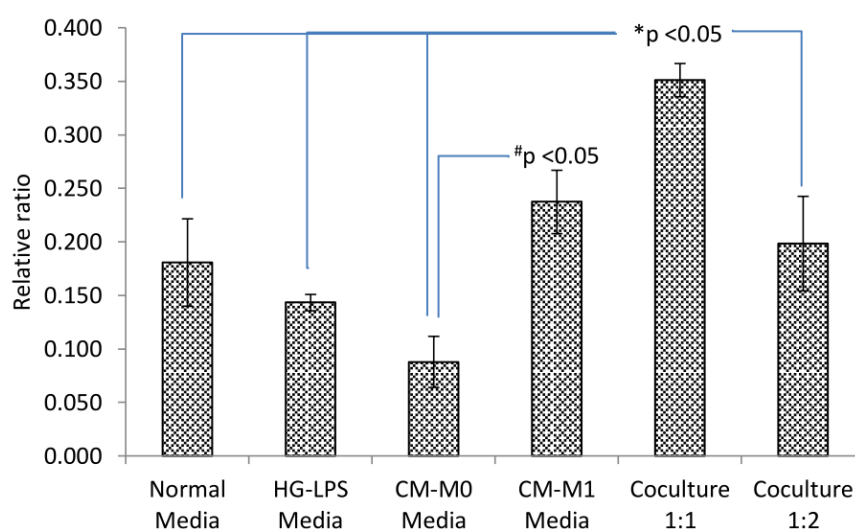
## RESULT AND DISCUSSION

Diabetes is a pathological condition that induces dysregulation of TGF-β in the cells. Tissue fibrosis is initially the result of overexpressing of TGF-β that induces collagen secretion in fibroblast cells ([Chang et al., 2015](#)). Therefore, mRNA TGF-β and collagen secretion were evaluated in this study to obtain the appropriate cell culture type for in vitro model diabetic fibrosis in vitro models.

Hyperglycemia and long-term inflammation can trigger diabetes mellitus. Proinflammatory macrophage produces various types of inflammatory mediators such as cytokines, chemokines, growth factors, etc ([Thomsen & Rosendahl, 2015](#)). The condition of hyperglycemia triggers macrophage cells to secrete proinflammatory cytokines. These conditions induce collagen hypersecretion (fibrosis response) in fibroblast cells ([Ploeger et al., 2013](#)). Direct contact of macrophage and fibroblast co-culture cells induces cell signaling through autocrine and paracrine interactions ([Holt et al., 2010](#)). In this study, coculture cells of RAW 264.7 and NIH 3T3 cells with culture ratio 1:1 show the higher mRNA TGF-β and collagen secretion relative ratio.

### Effect of monoculture, conditioned media, and coculture of NIH 3T3 cells on mRNA TGF- $\beta$ expressions

To understand the effect of culture type on the fibrosis effect of fibroblast cells, TGF- $\beta$  mRNA was evaluated in NIH3T3 cells induced LPS and high glucose. our study shows that NIH3T3 cells treated LPS and high glucose media result TGF- $\beta$  mRNA expression is not significant different, while NIH3T3 cells treated with conditioned media from inflammatory macrophage (M1) show mRNA TGF- $\beta$  is higher than NIH3T3 cells treated with LPS and high glucose (Figure 1). Cocultures were prepared by co-culturing macrophage cells and fibroblast cells on LPS-containing and high-glucose media. The mRNA TGF- $\beta$  of fibroblast cells on coculture of NIH3T3 and RAW264.7 cells (1:1 culture ratio) on LPS-containing and high glucose media is more higher than Normal Media, NIH3T3 cells treated LPS and high glucose media (HG-LPS Media), NIH3T3 cells cultured in conditioned media were obtained from RAW264.7 cells (CM-M0 Media) and Coculture (1:2) cells.

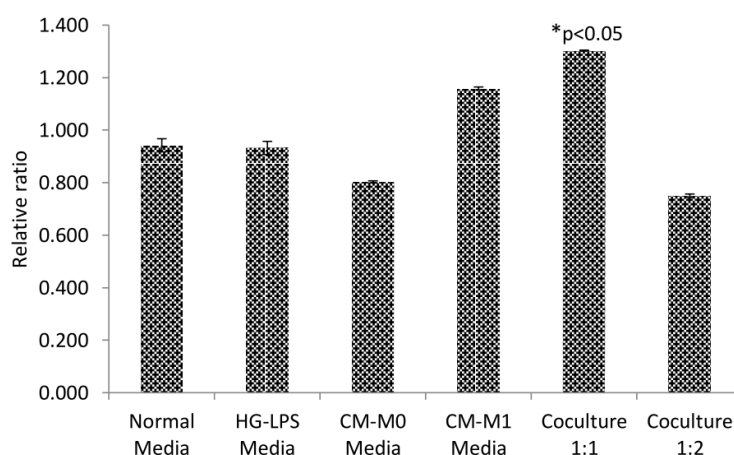


**Figure 1. The TGF- $\beta$  mRNA expression in various culture type of fibroblast cells NIH3T3**

Normal media, NIH 3T3 monoculture cultured in DMEM medium (FBS 10%) contain 4500  $\mu$ g/mL glucose; HG-LPS Media, NIH 3T3 monoculture cultured in DMEM medium (FBS 10%) contain 5400  $\mu$ g/mL glucose and 0.25  $\mu$ g/mL LPS; CM-M0, conditioned media from RAW264.7 cells in normal media; CM-M1, conditioned media from RAW264.7 cells in HG-LPS Media; Coculture (1:1 and 1:2 ratio), the RAW 264.7 and NIH 3T3 co-culture cells with various cultured in HG-LPS Media. \* $p < 0.05$  vs. Normal Media, HG-LPS Media, CM-M0 Media and Coculture (1:2); # $p < 0.05$  vs. CM-M0 Media.

### Effect of monoculture, conditioned media, and coculture of NIH 3T3 cells on collagen secretion

The collagen secretion in culture media of NIH3T3 cells was evaluated to understand of fibrotic effect on various culture type. Our study shows that collagen secretion occurs in all types of NIH3T3 cells cultures. The Collagen secretion ratio on NIH3T3 cells treated in conditioned media from inflammatory macrophage is higher than treated in LPS-containing and high glucose media, but both of them are not significantly different based on statistic analysis (Figure 2). While the coculture of NIH3T3 and RAW264.7 cells (1:1 culture ratio) in LPS-containing and high glucose media show collagen secretion ratios higher than CM-M0 Media culture type and statistically different ( $p < 0.05$ ).



**Figure 2. The Collagen secretion ratio's in various culture type of fibroblast cells NIH3T3**

Normal media, NIH 3T3 monoculture cultured in DMEM medium (FBS 10%) contain 4500  $\mu\text{g/mL}$  glucose; HG-LPS Media, NIH 3T3 monoculture cultured in DMEM medium (FBS 10%) contain 5400  $\mu\text{g/mL}$  glucose and 0.25  $\mu\text{g/mL}$  LPS; CM-M0, conditioned media from RAW264.7 cells in normal media; CM-M1, conditioned media from RAW264.7 cells in HG-LPS Media; Coculture (1:1 and 1:2 ratio), the RAW 264.7 and NIH 3T3 co-culture cells with various cultured in HG-LPS Media. \* $p < 0.05$  vs. CM-M0 Media.

High glucose and LPS medium stimulates elevate of proinflammatory mediators (IL-6, IL-1, IL-4, TGF- $\beta$ , etc) (Naudi et al., 2011). Macrophage is part of the immune system cells, they can communicate with other cells and/or tissue cells through a biological mediator such as cytokines, chemokines, hormones, etc. Meanwhile, tissue cells (fibroblast cells) receive signals through other cells and act according to the signal that has been given. The biological mediator can contribute to proliferation, apoptosis, differentiation, or tissue repair (Duque & Descoteaux, 2014). Our study reveals that mRNA TGF- $\beta$  and collagen secretion on NIH3T3 fibroblast cells induced with LPS and high glucose are not statistically different than NIH3T3 fibroblast cells in normal media. On the other hand, TGF- $\beta$  mRNA expression and collagen secretion on conditioned media from inflammatory macrophages were slightly higher, although they are not statistically different. The previous research explains that the autocrine signaling pathway (conditioned media) is not enough to induce fibrosis than juxtacrine signaling pathways (coculture cells) (Holt et al., 2010).

Transforming Growth Factor- $\beta$ , a potential fibrotic agent that has been reported to play a role in tissue remodeling and fibrosis (Caja et al., 2018). TGF- $\beta$  induce the gene encoding human collagen type 1 alpha 2 (COL1A2) via Smad-dependent pathway (Ikeda et al., 2016). Our study showed that TGF- $\beta$  mRNA expression and the relative ratio of collagen secretion in RAW 264.7 and NIH 3T3 cell coculture media with a culture ratio of 1:1 were the highest compared to other models. Therefore, mRNA TGF- $\beta$  expression correlates with collagen secretion.

## CONCLUSION

The co-culture of RAW 264.7 and NIH 3T3 cells (1:1) in LPS and high glucose media is appropriate for fibrosis in vitro models.

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