ORIGINAL ARTICLE

The concentration-dependent pro-fibrotic effect of metformin on LPS and high glucose induced fibroblast NIH 3T3 and Macrophage RAW 264.7 Cell co-culture

Rizki Awaluddin[1], Dwi Aris Agung Nugrahaningsih, PhD[2], Eti Nurwening Solikhah, Dr[2]

[1]Master in Biomedical Science Program, Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, [2]Departmen of Pharmacology and Therapy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada

ABSTRACT

Introduction: Diabetes mellitus is known as one of the risk factors for Idiopathic Pulmonary Fibrosis (IPF) development. Recently, metformin, the commonly used antidiabetic medication, is reported to have a therapeutic effect in IPF. However, the benefit of metformin therapy in IPF is still controversial. The study aims to investigate the metformin effect on the fibroblast and macrophage co-culture under lipopolysaccharides (LPS) and high glucose treatment.

Method: The NIH 3T3 and RAW 264.7 co-culture were induced with LPS and high glucose before it was treated with metformin in different concentration. After 24 hours of treatment, the media and the cells were collected for further examination. The collagen expression was measured using Sirius red dye in the media. The IL-6 and TGF β mRNA examination were done using real-time PCR.

Result: Our study showed that NIH 3T3 and RAW 264.7 co-culture treated with metformin has higher collagen expression, but lower IL-6 mRNA expression compares to those on co-culture without treatment.

Conclusion: Metformin increases fibrosis markers in LPS and high glucose-induced NIH 3T3 and RAW 264.7 co-culture despite its ability to improve IL-6 mRNA expression.

KEY WORDS:
Metformin, idiopathic Pulmonary Fibrosis, Fibroblast, Macrophage

INTRODUCTION

Uncontrolled diabetes mellitus (DM) is a well-known risk factor for organ damage such as diabetic nephropathy.[1] peripheral arterial disease,[2] aortic fibrosis,[3] including lung damage.4 A clinical and experimental study by Talakatta et al., showed that uncontrolled DM induces inflammation and fibrosis in the lung via TGF-β1- SMAD7 pathway.[4] Clinical study revealed that idiopathic Pulmonary Fibrosis (IPF) is commonly found in DM patients.[5] Therefore, DM is considered to be one of the risk factors of IPF development.[6]

Metformin, the first-line anti-diabetic medication, has been widely used to control blood glucose concentration in diabetic patients. The mechanism of metformin in controlling blood glucose concentration is by inhibiting liver gluconeogenesis and improving glucose by increased tissue sensitivity towards insulin. However, other than in DM, metformin is also known to have several therapeutics actions such as in cancer,[7] and cardiovascular diseases.[8] Pre-clinical study in bleomycin induced lung fibrosis mice model showed that metformin can reverse established lung fibrosis.[9] Interestingly, post hoc analysis data from phase-3 clinical studies of Pirferidone treatment in IPF patient revealed that metformin does not affect the clinical outcomes in IPF patients.[10]

In this study, we aimed to investigate the effect of metformin in lipopolysaccharide (LPS) and high glucose-induced 3T3-NIH and RAW 264.7 cell co-culture to mimic diabetic and inflammatory condition.

MATERIALS AND METHODS

Direct-coculture cells RAW264.7 macrophages and NIH3T3 fibroblast cells

The study was conducted using direct co-culture of NIH 3T3 and RAW 264.7 cells. Both NIH 3T3 (ATCC® CRL-1658) and RAW264.7 (ATCC® TIB-71) cells were kindly provided by Prof. Tzhou Chi Huang from the Department of Food and Science, National Pingtung University Science and Technology, Taiwan. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) complete media containing 10% FBS and 2% penicillin-streptomycin and incubated in a 5% CO_2 incubator. The co-culture model was conducted by culturing both RAW264.7 macrophage cells and NIH3T3 fibroblast cell in 6 wells micro-plate. The direct co-culture model was incubated for 48 hours in a culture medium containing LPS 0.25ug/mL, D-glucose 5400µg/mL. Monoculture of NIH 3T3 was used for negative control. After 24 hours of incubation, metformin was added to the co-culture at a concentration of 75µg/ml, 150µg/ml and 300µg/ml. Metformin was given for 24 hours. The cells and the media were harvested for further examination.

Extraction and measurement of collagen levels in culture media

Collagen secretion was measured in culture media using Sirius red dye. Collagen was extracted from the media using polyethylene glycol 400 (PEG 400) and dissolved using acetic acid 0.1 M. Sirius red dye was added to stain the collagen. As a positive control, collagen standard (Collagen type-1 from

This article was accepted: 23 January 2020
Corresponding Author: Dwi Aris Agung Nugrahaningsih
Email: dwi.aris.a@ugm.ac.id

Table I: List of Primer used in this study

Gene name	Forward primer	Reverse primer
IL-6	5'-ACTGATGCTGGTGACAACCACG-3	5'- AGCCTCCGACTTGTGAAGTGG-3
TGF-β	5 '-TTCCGCTGCTACTGCAAGTCA-3 '	5'-GGGTAGCGATCGAGTGTCCA-3'
GAPDH	5 '-TGTGTCCGTCGTGGATCTGA-3'	5 '-TTGCTGTTGAAGTCGCAGGAG-3'

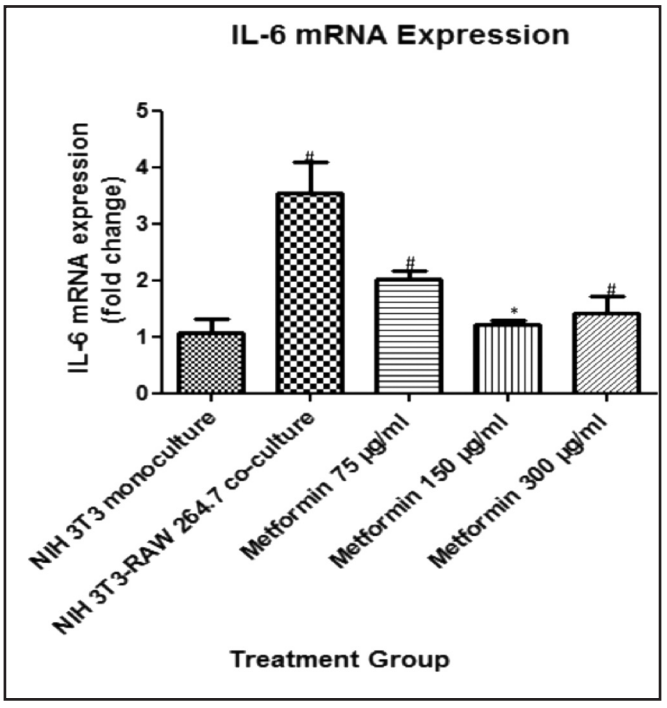

Fig. 1: The expression of IL-6 mRNA on LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture. Data are presented as mean ± SEM. Statistical significance: *P <0.05 vs monoculture; #P <0.05 vs. untreated co-culture of NIH 3T3-RAW 264.7.

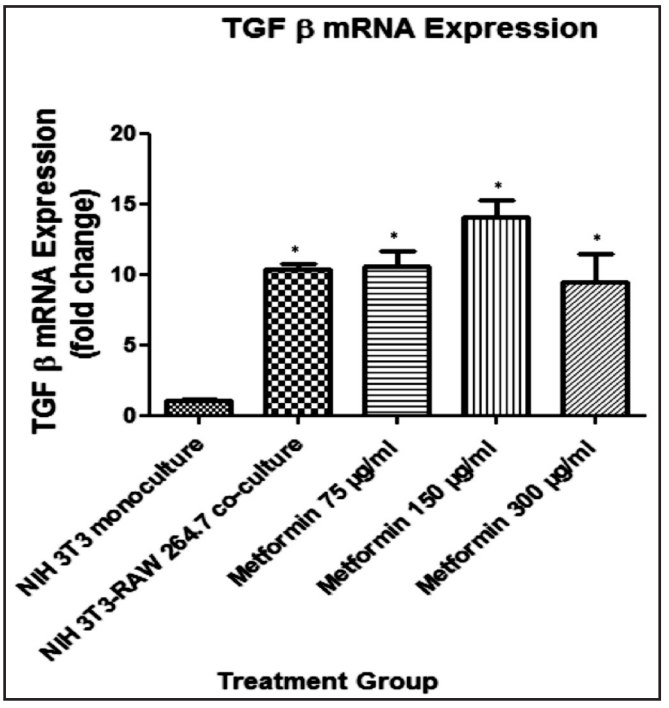

Fig. 2: The expression of TGF β mRNA on LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture. Data are presented as mean ± SEM. Statistical significance: *P <0.05 vs monoculture; #P <0.05 vs. untreated co-culture of NIH 3T3-RAW 264.7.

rat tail, SIGMA) was used. The colour formed after Sirius red addition was measured using ELISA reader at 550nm wavelength. Collagen concentration was calculated by using linear regression of standard collagen data.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated using Tri-RNA reagent (Favorgen, Taiwan). The RNA concentration was measured using Nanovue (GE Healthcare Life Science). The RNA was reverted into cDNA by using a cDNA synthesis kit (SMOBIO®) reagent. The real-time PCR was conducted by using a two-step Real-time PCR kit (SMOBIO®) in a C1000 Thermal cycler CFX96TM Real-Time System (Biorad®). Table I shows the primer used in this study.

The PCR condition was 95°C for 1 minute, 95°C for 30 seconds, annealing 62°C (GAPDH, IL-6); 55.3°C (GAPDH-TGF-β) for 30 seconds. The mRNA expression was calculated using delta-delta Ct formula.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was carried out using One-Way ANOVA followed by a post-hoc test, with a value of $p<0.05$ considered as statistically significant.

RESULTS

Metformin inhibits IL-6 mRNA expression in LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture

To investigate the metformin impact on inflammation, we cocultured NIH 3T3 and RAW 264.7 cells and induced them with LPS and high glucose medium. Our result showed that LPS and high glucose-induced NIH 3T3-RAW 264.7 co-culture showed higher IL-6 mRNA expression compare those on NIH 3T3 monoculture. Metformin treatment at 300µg/mL on NIH 3T3-RAW 264.7 co-culture showed lower expression of mRNA IL-6 compares those on untreated co-culture. Figure 1 shows the expression of IL-6 mRNA on NIH 3T3-RAW 264.7 cell co-culture.

Metformin does not improve TGF mRNA expression in LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture

Metformin treatment at 300µg/mL showed higher TGF β mRNA expression in LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture compare to those on NIH 3T3 mono-culture. However, metformin treatment does not have an impact on TGF β mRNA expression on LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture. Figure 2 shows the expression of TGF β mRNA on NIH 3T3-RAW 264.7 co-culture.

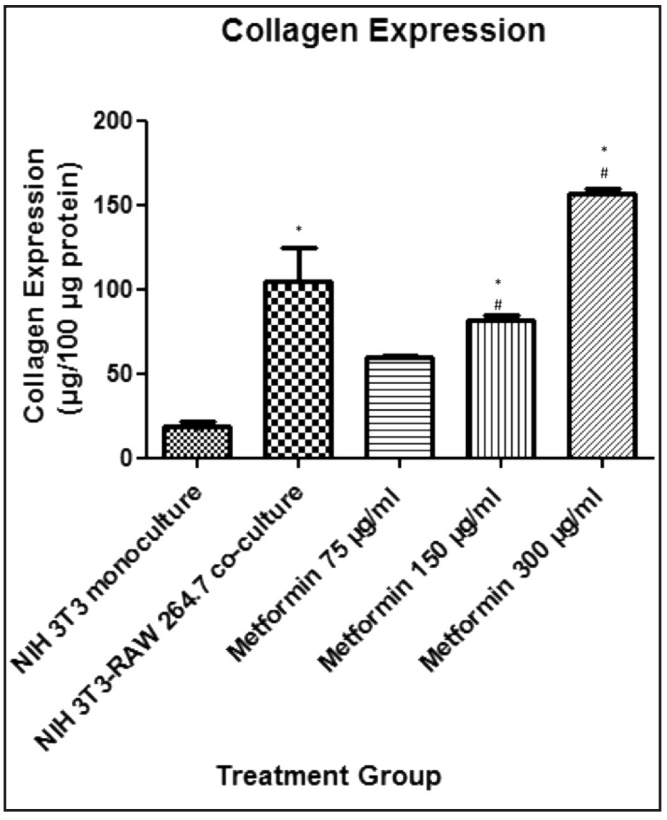

Fig. 3: The expression of collagen secretion on LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture. Data are presented as mean ± SEM. Statistical significance: *P <0.05 vs monoculture; #P <0.05 vs untreated co-culture of NIH 3T3-RAW 264.7.

Metformin increases collagen secretion in LPS and high glucose-induced NIH 3T3-RAW 264.7 cell co-culture

Collagen secretion is markedly higher in LPS-High glucose-induced NIH 3T3-RAW 264.7 co-culture compare to those on NIH 3T3 monoculture. Metformin treatment in the co-culture showed higher collagen secretion compare to those on untreated co-culture. Figure 3 shows the collagen secretion in LPS and high glucose-induced NIH 3T3-RAW 264.7 co-culture.

DISCUSSION

Diabetes mellitus has been known as a risk factor for IPF. A clinical study in the diabetic patient showed reduced lung vital capacity and total lung capacity.[11] Meanwhile, preclinical study in human peritoneal mesothelium cell (HPMC) showed that high glucose treatment-induced epithelial to mesenchymal transition (EMT) through downregulation of hepatocyte growth factor and bone morphogenic protein expression.[12] Study on diabetic alveolar epithelial cell (AEC) induced by high glucose showed that under high glucose treatment the AEC maintains their mesenchymal morphology. Meanwhile, diabetic AEC treated with low glucose showed the transformation of AEC morphology into epithelial morphology.[4]

In our study LPS and high glucose treated fibroblast-macrophage co-culture showed higher expression of IL-6 mRNA, TGF β mRNA and collagen secretion compare to those on fibroblast monoculture. The LPS and high glucose treatment could induce inflammation in the co-culture of NIH 3T3 and RAW 264.7 cells. The LPS and high glucose treatment in the co-culture also induce fibrotic changes in the co-culture shown as the marked increase of TGF β mRNA and collagen secretion in co-culture media. The treatment of metformin in LPS and high glucose-induced NIH 3T3-RAW 264.7 co-culture showed that metformin induces collagen secretion despite the depressed expression of IL-6 mRNA expression in the co-culture. Based on this finding, we suggest that the increase of collagen expression in LPS and high glucose-induced NIH 3T3-RAW 264.7 co-culture is not associated with IL-6.

Metformin is one of the most important DM medication from biguanide family. Metformin improves insulin resistance, decreases hepatic gluconeogenesis, and increase peripheral glucose uptake.[13] Interestingly, preclinical and clinical studies have shown other potential benefits of metformin, not only controlling blood glucose but also preventing complication of DM such as cardiovascular disease.[14-16] The cardio-protective mechanism of metformin is unlikely related to its effects on controlling blood glucose concentration.[17] In addition to hyperglycaemia, inflammation has been known as an important contributing factor to the development of cardiovascular disease in diabetes.[18,19] Previous studies on primary hepatocytes showed that metformin inhibits degradation of NF-κB negative regulator. The study also showed that metformin at 2mmol/L, equal with 258.32 µg/mL, inhibits pro-inflammatory cytokines IL-6, IL-1β, and CXCL1/2.[20] Our result also showed that metformin 300µg /mL can prevent the expression of the inflammatory cytokines, IL-6, in co-culture of NIH 3T3-RAW 264.7. Interestingly, our results showed that metformin at the same concentration that shows anti-inflammatory effect also showed fibrotic effect marked by the higher collagen secretion. Previous *in vitro* studies showed that metformin antifibrotic effects appear on a high concentration of metformin. Metformin showed antifibrotic activity in hypertrophied adipocyte at 5mM, equal with 645.8µg/mL.[21] *In vitro* study on TGF-β induced fibrogenesis in primary lung fibroblast showed that metformin could prevent fibrogenic differentiation of the fibroblast at 5mM.[22] Based on these results, we suggest that the antifibrotic effect of metformin appear in higher concentration than the concentration that shows anti-inflammatory effect. However, it must be noted that in human, the maximum concentration (Cmax) of metformin in the blood after one dose of 500 mg metformin is much lower than the concentration used in in vitro study. Study of different formulation of metformin showed that metformin C max after consumption of 500mg metformin was approximately 1200ng/mL or 1,1µg/mL.[23] Other studies of metformin pharmacokinetic also showed that metformin C max after taking 500mg metformin was 2.1µg/mL.[24] The difference in metformin concentration between pre-clinical and clinical setting might explain the finding in the clinical setting that metformin did not improve clinical symptoms in IPF patient.

CONCLUSION

Metformin can enhance fibrogenic differentiation despite its anti-inflammatory effect. However, to translate this finding in the clinical setting more study regarding metformin concentration needs to be done to find the optimal concentration to be able to prevent fibrosis and inflammation.

ACKNOWLEDGEMENT

This study was funded by Faculty of Medicine Dana Masyarakat Grant 2018-2019.

REFERENCES

1. Shahwan MJ, Gacem SA, Zaidi SK. Prevalence of Diabetic Nephropathy and associated risk factors among type 2 diabetes mellitus patients in Ramallah, Palestine. Diabetes Metab Syndr 2019; 13(2): 1491-6.
2. Aubert CE, Liabeuf S, Amouyal C, Kemel S, Lajat-Kiss F, Lacorte JM, et al. Serum concentration and vascular expression of adiponectin are differentially associated with the diabetic calcifying peripheral arteriopathy. Diabetol Metab Syndr 2019; 11: 32.
3. Hawas AA, Nugrahaningsih DA. Sholikhah EN, Syarifuddin S, Wijayaningsih RA, Ngatidjan N. Anti-inflammatory effect of Centella asiatica extract on prevented aortic intima-media thickening in diabetic rats. Thai Journal of Pharmaceutical Science 2018; 42(2): 1-7.
4. Talakatta G, Sarikhani M, Muhammed J, Dhanya K, Shomashekar BS, Maseh PA, Sundaresan N, Ravindra PV. Diabetes induces fibrotic changes in the lung through the activation of TGF-β signaling pathways. Sci Rep. 2018; 8(1): 11920.
5. Kim YJ, Park JW, Kyung SY, Lee SP, Chung MP, Kim YH, Lee JH, Kim YC, Ryu JS, Lee HL, Park Cs, Uh ST, Lee YC Kim KH, Chun YJ, Park YB, Kim DS, Jegal Y, Lee JH, Park MS, Jeong SH. Clinical characteristics of idiopathic pulmonary fibrosis patients with diabetes mellitus: the national survey in Korea from 2003 to 2007. J Korean Med Sci 2012; 27: 56-60.
6. Enomoto T, Usuki J, Azuma A, Nakagawa T, Kudoh S: Diabetes mellitus may increase risk for idiopathic pulmonary fibrosis. Chest 2003; 123(6): 2007-11.
7. Yu T, et al. Metformin inhibits SUV39H1-mediated migration of prostate cancer cells. Oncogenesis 2017; 6: e324.
8. He J, Huang Z, Zhu H, Qiu W, Fan L, Yi X. Diabetes mellitus in idiopathic pulmonary fibrosis: a systematic review and meta-analysis. Int J Clin Exp Med 2016; 9(9): 27-38.
9. Rangarajan S, Bone NB, Zmijewska AA, Jiang S, Park DW, Bernard K, et al. Metformin reverses established lung fibrosis in a bleomycin model. Net Med 2018; 24(8): 1121-7.
10. Spagnolo P, Kreuter M, Maher TM, Wuyts W, Bonella F, Corte TJ, et al. Metformin does not affect clinically relevant outcomes in patients with idiopathic pulmonary fibrosis. Respiration 2018; 96(4): 14-22.
11. Shah SH, Sonawane P, Nahar P, Vaidya S, Salvi S. Pulmonary function tests in type 2 diabetes mellitus and their association with glycemic control and duration of the disease. Lung India 2013; 30(2): 108-12.
12. Yu MA, Shin KS, Kim JH, Kim YI, Chung SS, Park SH, et al. HGF and BMP-7 ameliorate high glucose-induced epithelial to mesenchymal transition of peritoneal mesothelium. J Am Soc Nephrol 2009; 20(3): 67-81.
13. Fullerton MD, Galic S, Marcinko K, Sikkema S, Pulinilkunnil T, Chen ZP, et al. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nat Med 2013; 19:49-54.
14. Selvin E, Bolen S, Yeh HC, Wiley C, Wilson LM, Marinopoulos SS, et al. Cardiovascular outcomes in trials of oral diabetes medications: a systematic review. Arch Intern Med 2008; 168: 70-80.
15. Furouzandeh F, Salazar G, Patrushev N, Xiong S, Hilenski L, Fei B, et al. Metformin beyond diabetes: pleiotropic benefits of metformin in attenuation of atherosclerosis. J Am Heart Assoc 2014; 3: e001202.
16. Hattori Y, Suzuki K, Hattori S, Kasai K. Metformin inhibits cytokine-induced nuclear factor κb activation via amp-activated protein kinase activation in vascular endothelial cells. Hypertension 2006; 47: 3-8.
17. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet 1998; 352: 54-65.
18. Sasaki H, Asanuma H, Fujita M, Takahama H, Wakeno M, Ito S. Metformin prevents progression of heart failure in dogs: role of AMP-activated protein kinase. Circulation 2009; 119: 68-77.
19. Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006; 444(7121): 860-7.
20. Cameron AR, Morrison VL, Levin D, Mohan M, Forteath C, Beall C, et al. Anti-inflammatory effects of metformin irrespective of diabetes status. Circ Res 2016; 119(5): 52-65.
21. Malekpour-Dehkordi Z, Teimourian S, Nourbakhsh M, Naghiaee Y, Sharifi R, Mohiti-Ardakani J. Metformin reduces fibrosis factors in insulin resistant and hypertrophied adipocyte via integrin/ERK, collagen VI, apoptosis, and necrosis reduction. Life Sci 2019; 233: 116682.
22. Kheirollahi V, Wasnick RM, Biasin V, Vazquez-Armendariz AI, Chu X, Moiseenko A, et al. Metformin induces lipogenic differentiation in myofibroblasts to reverse lung fibrosis. Nat Commun 2019; 10(1): 2987.
23. Montoya-Eguía SL, Garza-Ocañas L, Badillo-Castañeda CT, Tamez-de la O E, Zanatta-Calderón T, Gómez-Meza MV. Comparative pharmacokinetic study among 3 metformin formulations in healthy mexican volunteers: a single-dose, randomized, open-label, 3-period crossover study. Curr Ther Res 2015; 77: 18-23.
24. McCreight LJ, Stage TB, Connelly P, Lonergan M, Nielsen F, Prehn C, et al. Pharmacokinetics of metformin in patients with gastrointestinal intolerance. Diabetes Obes Metab 2018; 20(7): 1593-60.