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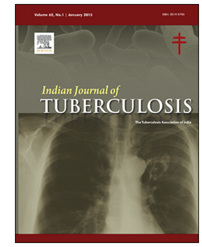

Original article

Metformin induced autophagy in diabetes mellitus – Tuberculosis co-infection patients: A case study

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ABSTRACT

Metformin (MET) is a potential combination drug to elevate anti-TB efficacy. However, the clinical effect, especially smear reversion, during metformin applied with anti-tuberculosis and insulin in patients with type 2 DM newly TB co-infection were remain unknown.

An observational clinical study was done in DM newly TB co-infection outpatients at Surabaya Paru Hospital. This study evaluated MET therapy, at least 2 months, accompanying with insulin and anti-TB regimens and compared to comparison group. The smear, microtubule-associated Protein1 Light Chain 3B (MAP1LC3B) level, as the presentation of autophagy, Superoxide Dismutase (SOD) level, Interferon (IFN)-γ and Interleukin (IL)-10 levels were evaluated twice.

From 42 participants in this study, 22 participants of observation group that received additional MET therapy, 100% had sputum smear reversion after 2-months intensive phase of anti-TB therapy. Whereas 25% of 20 participants of comparison group did not undergo reversion inserts sputum smear.

As conclusion, MET has the potential of being an additive combination therapy to enhance the bactericidal effect of anti-TB on DM-TB coinfection patients. Metformin enhances the effects of anti-TB and insulin therapy in increasing the smear reversion by increasing autophagy.

1. Introduction

Tuberculosis (TB) nowadays is one of "global health emergency" diseases.[1] The increasing evidence in TB patients associated with the rising number of patients at risk for TB, i.e. patients with immunocompromised condition such patients with HIV, diabetes mellitus (DM), cancer and autoimmune diseases. The TB infection at risk in DM patients increased 2.39 times with the risk of failure of

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anti-TB therapy increased 1.69 times with the smear reversion ± 60%.[2,3]

First-line anti-TB regiments, rifampicin, isoniazid (INH), ethambutol, pyrazinamide, use as anti-TB, thus, those etiology therapy aimed for curing the patients, preventing death, preventing recurrence, cutting off the transmission chains and preventing pathogen resistance by eradication *Mycobacterium tuberculosis*.[4–6] The effectiveness of anti-TB are also influenced by the host immune response due to the interaction of anti-TB. Adjunctive therapy with immunomodulators that enhance TB immunity (host-directed therapy, HDT) could shorten treatment durations and improve TB outcomes.[7–9] The identification of new host-directed therapies aimed at improving the clinical outcome of TB patients is a priority for TB management and WHO, including some drugs that work on immune regulation. Currently, several studies have been conducted to determine the function of some immunomodulatory agents, including corticosteroids, TNF blockers, thalidomide, and non-steroidal anti-inflammatory (NSAIDs) as adjunct of OAT therapy.[8,9]

One of HDT mechanism is autophagy due to its ability in inhibiting the TB infection process.[8,9] The process of activating autophagy from formation to maturation and then fusion with lysosomes for phagolysosome or autophagy processes requires many activators and protein (ATGs), one of the proteins representing phagolysosome or autophagy is MAP1LC3B/ATG8.

Metformin hydrochloride (MET), biguanide the oral anti diabetic agent, recently, by a comprehensive in silico study, MET known has possibilities of utilizing as a combination drug with existing antibiotics for TB therapy[10] and by an extensive in vitro study, MET was reported controlling the growth of drug-resistant *M. tuberculosis* strains via production of mitochondrial reactive oxygen species and facilitates phagosome-lysosome fusion.[11] Thus, MET is known as one of highly potential HDT due to target autophagy by AMPK activation or known as mTOR inhibitor.[11,12]

Moreover, MET is not metabolized by P450 enzymes,[4,13,14] thus it has no interaction with rifampicin that could decrease the therapy efficacy. However, interaction MET and rifampicin increases the expression of organic cation transporter (OCT1) and hepatic uptake of metformin, leading to an enhanced glucose-lowering.[4,5,14] MET is also expected enhanced isoniazid (INH) efficacy due to SOD activity.[15] INH a pro-drug, its activation is requiring an interaction with Kat-G produced by *M. tuberculosis*.[4,14] Kat-G activation also produces oxidative stress – reactive oxygen species (ROS), namely H_2O_2 and alkyl hydroperoxides. ROS is neutralized by an antioxidant, superoxide dismutase (SOD).[15,16] It is suspected that SOD contributes to the INH-induced bactericidal effects.

However, the mechanism of immune response changes due to the effects of metformin therapy to enhance the effect of anti-TB was not yet clear. This study, due to clarification aims, we measured the change levels of MAP1LC3B, SOD and IFN-γ levels before and after this observation period and as clinical result, we also evaluated the smear reversion in DM-TB coinfection patients.

2. Materials and methods

2.1. Study design

This involved type 2 DM-TB coinfection patients of observational clinical study and simple random allocation technique with two groups, MET group and non MET group. The MET group was the group receiving metformin therapy 1000–1500 mg along with insulin and anti-TB during the intensive periods, while non MET group received insulin and anti-TB therapy. Patients were carried out at outpatient ward of Surabaya Paru Hospital, with criterias: (1) patient DM with new case of TB co-infection, whom were given insulin and TB treatment regimens; (2) positive AFB in sputum smear; (3) patient's age was 25–60 years old; (4) has normal liver function and renal function; (5) not in hypoxia condition, presenting by peripheral oxygen saturation level must be higher than 92%.

The levels of MAP1LC3B, SOD and IFN-γ was measured before and after this observation period and as clinical result, we also evaluated the smear reversion in DM-TB coinfection patients in both of groups.

2.2. Diagnosis and management therapy

The diagnosis of TB was established by (1) clinical symptoms and signs of TB, such: chronic productive cough, unintentional weight loss; (2) positive sputum smear of acid-fast bacilli (AFB) by microscopic Ziehl-Neelsen-stained sputum slides; and (3) chest radiographs with suggestive features of TB. Diagnosis of DM was established by fasting and 2 h after meal blood glucose. HbA1c was measured after 2 months MET therapy, as evaluation.

Patients diagnosed with TB were registered and treated with anti-TB regimens for a period of 6 months in accordance to WHO guidelines.[17,18] Management therapy for achieving good glycaemic control was insulin therapy. These following drugs were used: MET (Metformin®), insulin (Humulin®), rifampicin (RIF), isoniazid (INH), pyrazinamide (PYR), ethambutol (ETH). MET were given 1000 – 1500 mg in divided daily dose for at least two months or during intensive phase of anti-TB.

2.3. AFB smears

Sputum smears were stained by the Ziehl-Nielsen technique and examined by light microscopy for AFB. Sputum were collected two times: (1) before treatment in order to diagnose and (2) after intensive phase of anti-TB treatment in order to evaluate the treatment.

2.4. Cells culture and ELISA

2.4.1. Cells

PBMC was obtained from patients' whole blood and 1×10^6 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Supernatant were harvested after 72 h and prepare for ELISA methods in order to measure the levels of MAP1LC3B, SOD and IFN-γ.

2.4.2. *ELISA*

MAP1LC3B (MyBioSource MBS760738); SOD (Biovision K335-100); and IFN-γ (RnD DIF50) were used as measurement kits.

3. Result and discussion

3.1. *Characteristic of patients*

This study's ethical clearance was approved by ethical committee of Surabaya Paru Hospital with no. 09.01/KERS/102.6/2016. During this study period, there were 476 cases of new TB infection and 156 cases (~30%) of that were type 2 DM newly TB co-infection. 42 patients were eligible participated in this observational studies. All the basic conditions in both groups were homogenous ($p > 0.05$) (as seen in Table 1).

In order to prevent MET associated lacto acidosis (MALA) during MET therapy in this study, all patients has been determined at the precondition criterias as seen in Section 2.1. Moreover, there was no incidence of lactic acidosis event during this observation period.[19]

3.2. *Acid fast bacilli smears*

Sputum for AFB smears were collected two times: (1) before treatment; and (2) after intensive phase of anti-TB therapy in order to evaluate the treatment (Table 2).

Data in Table 2 shows that prior to intensive phase of anti-TB therapy none of subjects were having negative AFB smear in both groups. The highest number of AFB count (+3) in MET group was 40.9% and in non MET group was 35%. After 2 months MET therapy accompanying with insulin and anti-TB regimens, 100% of patient of MET group were AFB smear reversion (negative AFB smears result), while only 75% of non MET group had AFB smear reversion. Using the Fisher's exact test, results of different test $p = 0.046$ ($p < 0.005$), which means there is a significantly difference of AFB smears reversion between the MET group and the Non MET group.

3.3. *Level of MAP1LC3B, SOD, IFN-γ*

3.3.1. *Autophagy*

Autophagy is a fundamental process of cell biology intimately involved in the interaction between *M. tuberculosis* and the phagocytes, including macrophages, dendritic cells (DC) and neutrophils. *M. tuberculosis* has capacity to evade the autophagy of mononuclear phagocytes (MPs) and leverage the intracellular environment as a replication niche.[20,21] Combined with hyperglycemia state and high free radicals of oxygen or nitrogen, infected MPs of DM patients are faced with a pathogen surviving in phagosomes that fail to incorporate the molecular machinery and also fail to fuse with lysosomes to expose bacilli, then, resulting the failure of anti-TB therapy [22,23]

Autophagy in TB infection is a combined response of innate and adaptive host immune systems that are essential for the process of *M. tuberculosis* elimination. Microtubule-associated protein 1 light chain 3 (MAP1LC3B/ATG8) is ubiquitine-like modifier that represents autophago-lysosome fusion or better known as autophagy,[24] thus in this study MAP1LC3B was used to represent autophagy activity.

MET is known as mTOR inhibitor, by the activation of AMPK [9,12,25] Recent researches of MET that has been done in silico, in vitro and animal's in vivo, expressed that MET is a potential adjunct for anti-TB therapy.[10,12,25]

Table 3 shows that MAP1LC3B level before treatment between MET group and non MET group were alike ($p > 0.005$), thus it shows that patients in both groups, before treatment, were in similar stage of MAP1LC3B. The differences before and after observation period was significant in MET group ($p < 0.005$) while in non MET group was not. Moreover, compared to non MET group, the change of MAP1LC3B after MET therapy during intensive period of anti-TB and insulin was also significant differences. Thus, we concluded that MET improves autophagy and it results 100% AFB smear reversion rate in MET group (as seen in Table 2).

Table 1 – Characteristic of type 2 DM-TB coinfection during observation period of study.

Parameters	MET group	Non MET group	*p* (difference)
Ages (years old)	44.59 ± 8.64	48.40 ± 8.17	0.863
HbA1c (g/dL)	8.82 ± 1.91	9.52 ± 2.02	0.379
Oxygen saturation (SpO_2) (%)	98.06 ± 0.73	97.47 ± 0.83	0.308
BUN (mg/dL)	0.95 ± 0.16	0.93 ± 0.13	0.980
Creatinine serum (U/L)	23.92 ± 11.92	27.3 ± 12.01	0.103
SGOT (U/L)	17.63 ± 6.16	14.44 ± 6.48	0.354
SGPT (U/L)	19.22 ± 8.73	16.09 ± 7.56	0.509

Table 2 – Acid fast bacilli smears result of type 2 DM-TB patients before and after observation period.

AFB result	MET group (N %)		Non MET group (N %)	
	Before	After	Before	After
Negative	0 (0%)	22 (100%)	0 (0%)	15 (75%)
Scanty/+1	9 (40.9%)	0 (0%)	6 (30%)	5 (25%)
+2	4 (18.2%)	0 (0%)	7 (35%)	0 (0%)
+3	9 (40.9%)	0 (0%)	7 (35%)	0 (0%)
Total	22 (100%)	22 (100%)	20 (100%)	20 (100%)

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Table 3 – Microtubule-associated Protein1 Light Chain 3B (MAP1LC3B) level of type 2 DM-TB patient before and after observation period.

MAP1LC3B level (pg/mL)	MET group	Non MET group	Between groups differences
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
Before	1247.7 ± 551.5	1571.6 ± 477.7	0.062
After	1859.4 ± 47.3	1343.6 ± 607.9	0.004
Before and after differences	$p = 0.000$	$p = 0.830$	

Table 4 – Superoxide dismutase (SOD) level of type 2 DM-TB patient before and after observation period.

SOD's level (U/mL)	MET group	Non MET group	Between groups differences
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
Before	47.63 ± 25.76	86.23 ± 18.63	0.427
After	57.33 ± 27.34	61.73 ± 22.57	0.138
Before and after differences	$p = 0.000$	$p = 0.343$	

Table 5 – Interferon (IFN)-γ level of type 2 DM-TB patient before and after observation period.

IFN-γ level (pg/mL)	MET group	Non MET group	Between groups differences
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
Before	1116.4 ± 650.2	1430.9 ± 680	0.072
After	1857.4 ± 48.77	1313.7 ± 617.6	0.004
Before and after differences	$p = 0.000$	$p = 0.899$	

3.3.2. *SOD*

SOD contributes to the INH's mycobactericid effect and inducing autophagy.[4,26]

Table 4 shows that SOD level before treatment between MET group and non MET group were alike ($p > 0.005$), thus it shows that patients in both groups, before treatment, were in similar stage of SOD. The differences before and after observation period was significant in MET group ($p < 0.005$) while in non MET group was not. Moreover, compared to non MET group, determination SOD change as the effect of MET therapy during intensive period of anti-TB was not significant differences in MET group.

Based on those results, we concluded that MET, 1.000–1.500 mg/day, was significantly increased SOD activities which it is enhanced *M. tuberculosis* eliminating process. However, the different of SOD level after observation period in both groups were not significant changes. In our knowledge, SOD activity is generated by the body and adjusted to the existing oxidant levels,[15] thus, this result phenomenon is due to SOD activity triggered not only by MET induced AMPK but also by other factors such oxidative stress, hyperglycemia associated ROS and *M. tuberculosis* elimination process.[11]

In this study, elevated SOD levels were a synergistic effect of MET and anti-TB therapy. Furthermore, high blood SOD levels in the MET group showed that the oxidative stress in the MET group was also high. The increased oxidative stress in this study was the result of respiratory macrophage cell burst of *M. tuberculosis* phagocytosis process in the blood.[11,27] The increase of SOD in this study is assumed as high intracellular killing effect of macrophage cell on *M. tuberculosis* and is proven by AFB smear reversion rate in MET group (100% as seen in Table 2).

3.3.3. *IFN-γ*

IFN-γ is activated by Th1 to maintain IL-12 receptor and provide protection against TB infection.[28] Increased of IFN-γ in chronic TB infection is a cellular immune response. Currently, IFN-γ release assay (IGRA) is used as one of the tools of diagnosis of latent TB infection and IFN-γ elevation is one of the indicators of successful treatment in active TB infection.[29,30] Moreover, IFN-γ plays important key in autophagy.[21,30]

Table 6 – Interaction between metformin, level of SOD, MAP1LC3B, IFN-γ, and AFB smears reversion.

No.	Variable	Beta (β)	*p*
1.	Metformin → IFN-γ	0.621	0.000[a]
2.	Metformin → SOD	0.681	0.000[a]
3.	Metformin → MAP1LC3B	0.636	0.000[a]
4.	Metformin → AFB reversion	0.386	0.012[a]
5.	IFN-γ → MAP1LC3B	0.875	0.000[a]
6.	SOD → MAP1LC3B	0.441	0.004[a]
7.	IFN-γ → AFB reversion	0.295	0.015[a]
8.	SOD → AFB reversion	0.267	0.122
9.	MAP1LC3B → AFB reversion	0.303	0.021[a]

Beta (β): regression coefficient, shows the relationship between independent variables to dependent variable (increase or decrease, negative value means inhibit).
p: probability significance value, is stated significant; significant when *p* value < 0.05.
→: affect to.
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Table 5 shows that IFN-γ level before treatment between MET group and non MET group were alike ($p > 0.005$), thus it shows that patients in both groups, before treatment, were in similar stage of IFN-γ. The differences before and after observation period was significant in MET group ($p < 0.005$) while in non MET group was not. Moreover, compared to non MET group, IFN-γ elevation as the effect of MET therapy during intensive period of anti-TB was also significant differences. Thus, we concluded that MET improves autophagy and it results 100% AFB smear reversion rate in MET group (as seen in Table 2).

3.4. Interaction between metformin, level of SOD, MAP1LC3B, IFN-γ, and AFB smears reversion

Using regression category, we concluded that AFB smear reversion in this study was influenced by MET through autophagy activity and IFN-γ, while SOD did not affect the AFB smear reversion directly, but through the autophagy (as seen in Table 6). Several studies related to the autophagy in TB infection, suggest that SOD and IFN-γ play important role in autophagy[21,25,31,32] and the results of this study support those information.

4. Conclusion

Research and development of new host-directed therapies aimed at improving clinical outcomes of TB patients, are now beginning to be widely practiced in TB therapy strategies, especially using immunomodulatory drugs. The high number of resistance and failure of therapy in TB infection is one of the reasons for the implementation of the activity. Glucocorticoid and IL-12 drugs are ''old'' drugs used in new TB treatment strategies.[8,9,33]

Some previous studies suggest that MET is an immune-modulator that inhibits cancer cell proliferation stimulator, insulin like growth factor (IGF) and activation of PI3K-AKT. The mechanism of inhibition that is the reason Metformin is used for the prevention and adjuvant therapy for cancer, including breast cancer, ovarian cancer, colorectal cancer and prostate cancer.[34,35] Thus, several comprehensive studies in silico, in vitro and animal in vivo also suggest that MET may increase the efficacy of anti-TB treatment,[12,25] however, no human studies have supported it. MET has additive effects on anti-TB due to: (1) MET may increase the expression of organic cation transporter (OCT)-1 rifampicin were instrumental M. tuberculosis inhibition of transcription[10]; (2) MET, based on the results of this study and some previous research, could increase the SOD to prevent resistance to isoniazid.[9,11,16]

This study provides data on patient clinical changes, the AFB smear reversion in DM-TB coinfection patients (Table 2) so that it can be concluded that MET uses as a insulin combination and has additional effect in enhance anti-TB efficacy. Moreover, MET, based on the results of this study and some previous studies, may be concluded as autophagy inducer or mTOR inhibitor. Nevertheless, the mechanism of action of metformin at the molecular level involving phagosomes and lysosomes remains unknown yet in detail and requires further study. In the even of MET associated autophagy through the phagosome, it may increase the efficacy of pyrazinamide induced intracellular phagocytosis of *M. tuberculosis*,[8,36] that need further study.

As preliminary study, this result need to be continued in subsequent studies to develop more effective TB treatment strategies and the development of new adjuvant therapy that works as an immune-modulator by emphasizing the improvement of SOD, autophagy and have less minimal gastrointestinal side effects and the likelihood of more minimal lacto-acidosis. As conclusion, MET has the potential of being an additive combination therapy to enhance the bactericidal effect of anti-TB on DM-TB coinfection patients. Metformin enhances the effects of anti-TB and insulin therapy in increasing the AFB smear reversion by increasing autophagy. MET was also relatively safe for DM-TB coinfection patients due to its result in not elevated lactate levels.[19]

Conflicts of interest

The authors have none to declare.

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