# THE METFORMIN AND HYPERBARIC OXYGEN THERAPY ON THE NUCLEAR FACTOR KAPPA β (NF-κβ) AND FIBROBLAST GROWTH FACTOR (FGF) EXPRESSION IN THE WOUND OF HYPERGLYCEMIC RATS

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## ABSTRAK

*Kondisi hiperglikemia pada diabetes mellitus (DM) menimbulkan banyak komplikasi seperti hambatan penyembuhan luka akibat penurunan vaskularisasi dan tingginya level stres oksidatif yang menghambat pembentukan kolagen dan fibroblast, sehingga penderita DM lebih mudah menderita gangren. Kombinasi terapi metformin dan oksigen hiperbarik (OHB) memiliki efek sinergis menimbulkan hipoglikemia dan meningkatkan produksi eNOS. Tujuan penelitian ini adalah untuk meneliti efek terapi kombinasi metformin dan OHB untuk penyembuhan luka pada tikus hiperglikemia yang diinokulasi Pseudomonas spp pada lukanya ditinjau dari ekspresi nuclear factor kappa B (NF-© ) dan fibroblast growth factor (FGF). Penelitian ini dilakukan pada 50 ekor tikus Wistar (Rattus norvegicus) jantan yang terbagi menjadi dua kelompok kontrol dan tiga kelompok perlakuan. Tikus dibuat hiperglikemia dengan diinduksi aloksan, diberi luka di punggung kanan dan diinokulasi subkutan dengan Pseudomonas sp. Hasil pemeriksaan glukosa darah, aktivitas fibroblast dan ekspesi NF-© dianalisis menggunakan uji Kolmogorof Smirnov. Hasil penelitian ini menunjukkan penurunan yang signifikan pada ekspresi NF-© dan peningkatan ekspresi FGF pada kelompok yang diterapi dengan kombinasi metformin-OHB (p <0.05) dibandingkan terhadap kelompok kontrol sehingga mengindikasikan penyembuhan luka yang lebih baik. Simpulan, terapi kombinasi metformin dan oksigen hiperbarik dapat menurunkan ekspresi NF-© dan kadar glukosa darah serta meningkatkan ekspresi FGF pada luka tikus Wistar jantan hiperglikemia yang diinokulasi Pseudomonas spp.* ***(FMI 2015;51:7-12)***

***Kata kunci:*** *metformin, hyperbaric oxygen terapy, hyperglycemia, FGF, NF-©*

## ABSTRACT

*Complication in diabetes mellitus may occur due to the decline of vasculature resistance and the increased level of oxidative stress matters that lead to the blockage of fibroblast and collagen formation. This, will eventually prolong the wound healing in diabetes mellitus patient, and is recognized as gangrene. This study, therefore, was conducted to elaborate the outcome of metformin and hyperbaric oxygen (HBO) therapy in accelerating the wound healing of hyperglycemic rats which wound was Pseudomonas spp-inoculated by identifying the nuclear factor kappa B (NF-© ) and fibroblast growth factor (FGF) expression. In this study, 50 male Wistarrats were used. The rats were grouped into 5 groups, consisted of 2 control groups and 3 treatment groups. The treatment groups were administered by aloxan, wounded at the right posterior area, and subcutaneously Pseudomonas spp-inoculated. Some groups were treated with metformin or combination of metformin- HBO therapy. Collected data was analyzed by Kolmogorof Smirnov to evaluate the significant difference of NF-© and FGF expression among groups. It was observed that the expression of NF-© and blood glucose were decreased, however the expression of FGF were increased in the group of rats that treated by the combination of metformin and oxygen hyperbaric (p <0.05). In conclusion, combination of metformin and HBO therapy significantly decreased the expression of NF-© and increased the FGF expression in the wounded areas of male hiperglicemic rats that Pseudomas spp inoculated. Thus, implicated an acceleration of the wound healing process.* ***(FMI 2015;51:7-12)***

***Keywords:*** *metformin, hyperbaric oxygen therapy, hyperglycemia, FGF, NF-©*

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## INTRODUCTION

The incidence of Diabetes mellitus (DM) is increasing in the developing countries. It was estimated in 2025, approximately 7.5 million people will be suffered from DM and mortality due to DM will be 3.5 million per year (American Diabetic Association 2008). In Indonesia, about 5 million people suffered from DM associated retinopathy, neuropathy, nephropathy, macrovascular and microvascular complications (Evans et al 2003, Supari 2005). DM therapy develops rapidly alongside the increased number of diabetes mellitus patient in the last decade. Thus, choice of therapy to cope with hyperglycemia as well as reducing the complication is vital in DM suffered patient.

Subcutaneous infection is another complication of DM (Hooper 2008, Wautier & Guillausseau 2001). The infection may cause the formation of long term wound. The process of wound healing is complex and link the vascular response, cellular activities and formation of chemicals, known as pro inflammation mediators around the wound area. It was known that awound healing process is involving highly regulated responsesof specialized cell types, such as fibroblast growth factors (FGF). The FGF is playing a crucial role in the wound healing process, thus lack of FGF may impair the process (Wautier & Guillausseau 2001).

Hyperglycemia induces several alterations that lead to molecular damage of endothelial cells and increased oxidative stress (Supari 2005). The integrity of endothelial is important for its function as the transductor among cells and as the barrier between the blood and interstitial. Moreover, in hyperglycemia, the biochemistry pathway involved in the formation of Advanced Glication End (AGE), which is known as oxidated carbohydrates, fats, and proteins, is activated (Ojima et al 2005). Further, the complex AGE-RAGE will stimulate the NF-κβ, a transcription factor against infection and inflammation, which is activated during hyperglycemia. The stimulation of NF-κβ also being induces by the oxidative stress and free radicals substances that are produces during hyperglycemia. The infection which is occurred during hyperglycemia may increase the formation of Reactive Oxygen Species (ROS), thus it may stimulate the NF-κβ activ-ation.When NF-κβ is activated, TNF and IL1, cytokines, growth factor and adhesion molecules (Alikhani et al 2005), are produced as a response of inflammation (Morrow et al 2003). Therefore, it is believed that activation of NF-κβ is slowing the wound healing process, due to the worsening infection and inflammation condition (Davis et al 2006).

One of the hyperglycemia therapy, metformin, is known as the first line therapy of hyperglycemia particularly type 2 DM (Richard et al 1995), due to its ability to improve the sensitivity of insulin receptor via the Insulin Receptor Substrate (IRS) by activating cyclic Adenomonophosphat dependent Protein Kinase (c-AMPK). Moreover, metformin has also been identified of its vasodilatation effect by increasing endothelial nitric oxide synthase (eNOS) production (Davis et al 2006). As the vasodilator, metformin may reduce hypoxia that occurred during infection.

Another choice of therapy, which is able to relieve gangrene in type 2 DM patient, is known as hyperbaric oxygen therapy (HBO) (Momose et al 2002). Hiperbaric oxygen therapy is conducted by administering pure oxygen at the pressure level more than 1 absolute atmosfer (ATA). Previously, it was reported that HBO suppressed the pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ (Graves et al 2006). Moreover, HBO also reduced the activity of fibroblast on, perglycemic rat periodontal ligament that were induced by bacterias (Graves et al 2006). Interestingly, other studies identified that combination of metformin with HBO worked synergistically to generate eNOS production and perform hypoglycemic effect (Dewi et al 2010), thus, resulting better treatment outcome. Based on previous studies, we elaborated the ability of metformin and HBO combination therapy when administered to the hyperglycemia rats that were wounded and Pseudomonas spp-infected. It was hypothesized that the combination therapy will hasten the wound healing process by reducing NF-κβ and stimulating FGF expression, as well as stimulating the number of collagen production.

## MATERIALS AND METHODS

The ethical clearance of this study was approved by Komisi Etik Penelitian Fakultas Kedokteran Hewan Universitas Airlangga, Animal Care And Use Committee (ACUC), no. 206-KE on June 25$^{th}$ 2012. In this study, healthy male Wistar rats (Rattus norvegicus) were used. The rats were approximately 2-3 months of age and 150-200 mg of weight. They were considered as healthy by observing the mobility and body weight during the adaptation period in Animal Laboratory, Faculty of Pharmacy, Widya Mandala Catholic University Surabaya. Rats were caged in room temperature with adequate supply of food and water twice a day. They were then divided into 8 groups, namely: K0 (negative control groups-the groups with no treatment), K1 (the group that induced by aloxan), KP1A (the group that induced by aloxan and *Pseudomonas spp.* and was terminated on the 3rd day after bacterial induction), KP1B (the group that induced by aloxan and *Pseudomonas spp.* and was terminated on the 5th day after bacterial induction), KP2A (the group that induced by aloxan, *Pseudomonas spp.*, treated by metformin, and terminated on the 3rd day after bacterial induction), KP2B (the group that induced by aloxan, *Pseudomonas spp.*, treated by metformin and terminated on the 5th day after bacterial induction), KP3A (the group that induced by aloxan, *Pseudomonas spp.*, treated by metformin-HBO therapy and terminated on the 3rd day after bacterial induction), and KP3B (the group that induced by aloxan, *Pseudomonas spp.*, treated by metformin-HBO therapy, and was terminated at the 5th day after bacterial induction).

**Aloxan Administration**

In order to generate hyperglycemic condition, aloxan monohidrate 8% in NaCl 0.9% w/v was administered intraperitoneally (350 mg/kg BW, with the given volume 0.4375 ml/kg BW) to the rats of all groups, except K0 (Setiawan & Monica 2006). Aloxan was prepared an hour before administration to avoid degradation of its components. It was administered to the rats subsequently after the blood glucose test, which should approximately be 80-95 mg/dl. The blood glucose level again tested in the next 48 hours. The rats were given their usual diet and fasted 10 hours before the blood glucose test. The rats with the increased glucose level approximately between 200-400 mg/dl were included in the next steps. The glucose level was tested by the Accucheck Advantage using vena lateralis blood taken from the rats tail.

**Wound Initiation and Subcutaneus Bacterial Inoculation**

*Pseudomonas aeruginosa* (PA) was obtained from diabetes mellitus patient. It was taken from the agar medium and growth in BHI (Brain Heart Infusion) anaerobic medium until the concentration needed was reached. PA was infected subcutaneously to the rats 10 (Ojima et al 2005) CFU of concentration. Subsequently after the hyperglycemia condition was established, rats were wounded around 1 cm in the back right area. PA was subsequently inoculated subcutaneously and followed by giving treatment of Metformin and HBO therapy.

**Metformin and HBO Therapy**

Metformin HCl 0,225% in 4ml/200g BW was given orally three times a day 45 mg/kg BW of dose. Metformin treatment were given for 3 and 5 days. HBO therapy 2.4 ATA was given using animal chamber for 3 and 5 days which was divided into three parts a day, each part took 30 minutes and 5 minutes for a break in between, this procedure was known to give optimum therapy with the lowest side effect (Guritno 2006).

**Fibroblast Growth Factor and NF-© Expression**

Blood was taken from vena lateralis of the rats tail. The blood was dripped into the Advantage®test strip. Glucose level was measured automatically with advantage meter. The thickness of blood vessel wall was performed by measuring the central artery by histopathology method, whereas FGF and NF-κβ expression was done by immuno-histochemistry technique.

**Animal Termination**

Wound healing process is strongly influenced by vascular response, cellular activity and the released of pro-inflammatory mediators in surrounding wound area. The vessels were expected to be dilated and thus improved tissue oxygenation, accelerated the movement of pro-inflammatory mediators and fibroblasts, which then formed fibrins to trigger the formation of collagen (Niinikoshi 2006). The numbers of fibroblast were increased in the 3rd day and showed plateau in the 5th day. Regarding those mentioned findings, the rats in this study were terminated in the 3rd and 5th day after being wounded. The rats were euthanized by placing each of them in a chamber containing ether for 3-5 minutes. Subsequently, the rats were dissected and the wounded areas were taken to conduct the immunohistochemistry analysis of NF-κβ and FGF expression.

**Data analysis**

The data was evaluated to measure the difference between the level of blood glucose, FGF and NF-κβ expression. The normality of data was done by Kolmogorof Smirnov test. In order to identify the homogeneity variation between groups, Levene's test was performed.

**RESULTS**

**Blood Glucose Test Before and After Aloxan Induction**

The blood glucose level before and after aloxan administration, and after Metformin-HBO treatment was analyzed. The blood glucose level showed significant different when compared between groups before and after aloxan induction ($p = 0.001$), as seen on Figure 1.

**NF-© Expression**

The Hematoxylin Eosin (HE) staining was performed followed by immunohistochemical staining on the wounded skin. Subsequently, the activation of NF-κβ and FGF was counted. The expression of NF-κβ was observed by calculating the amount of fibroblast cell nucleus on the subcutaneous tissue of all groups. Comparing group which was given combination therapy of Metformin and HBO to normal group, using paired t-test for means of each group. NF-κβ was significantly decreased (as seen on Figure 2 and 3).

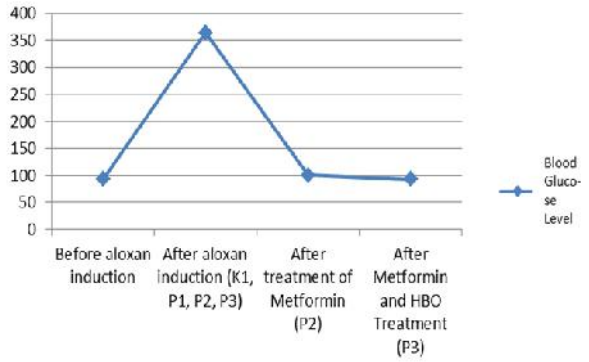


Figure 1. Blood glucose test before and after aloxan induction; after metformin-HBO treatment

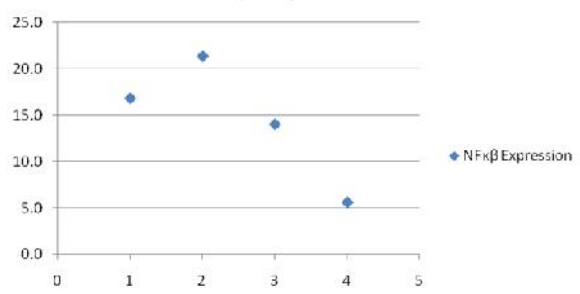


Figure 2. NFκβ expression of Normal Group and Treatment Group
*1 = Normal Group (K0), 2 = Treatment Group-1 (P1), 3 = Treatment Group-2 (P2), 4 = Treatment Group-3 (P3)

This study observed a decreased activation of NF-κβ in the P3 group which was treated with combination therapy of metformin-OHB ($p < 0.05$) when compared to the normal group (K0).

**FGF expression**

FGF expression data showed that combination of metformin-OHB significantly increased the activation of FGF ($p < 0.05$) between K0 and P3, using paired t-test for means ($\dot{x}$) of each group for comparation. However, when comparing the groups which were not treated (K1 and P1) to the group given combination of metformin-OHB ATA (P3), we can see no significant difference ($p > 0.005$). There was also no significant different of FGF activation between P2 and P3 ($p > 0.05$), as seen in figure 4 and 5.

## DISCUSSION

**Hastened Process of Wound Healing**

In this study, we found difficulty when conducted the hastened process of wound healing. This study could only conduct qualitative measurement of healing process by 1) observed the wound condition, and 2) observed the presence of inflammations, like tumorformation, rednessin around the wound. The results showed that the wound were closed on the second day and there was no of inflammation sign in the P3. In the P2, the wound also were closed on the third day and there is no sign of inflammation. While in the P1and K1wounds were not close until the fifth day, however, no signs of inflammation were observed.

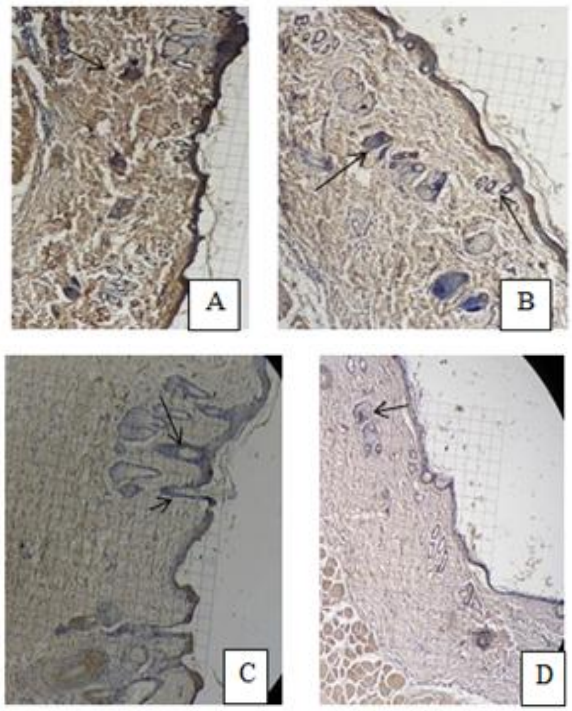


Figure 3. NFκβ expression on immunohistochemistry. A = Normal Group (K0), B = Treatment Group -1 (P1), C = Treatment Group -2 (P2), D = Treatment Group -3 (P3) using binocular microscope with 400 times enlargement. *arrows point to NFκβ

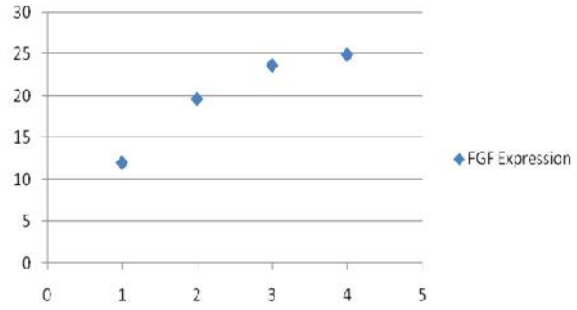


Figure 4. FGF Expression of Normal Group and Treatment Group
*1 = Normal Group (K0), 2 = Treatment Group-1 (P1), 3 = Treatment Group-2 (P2), 4 = Treatment Group-3 (P3)

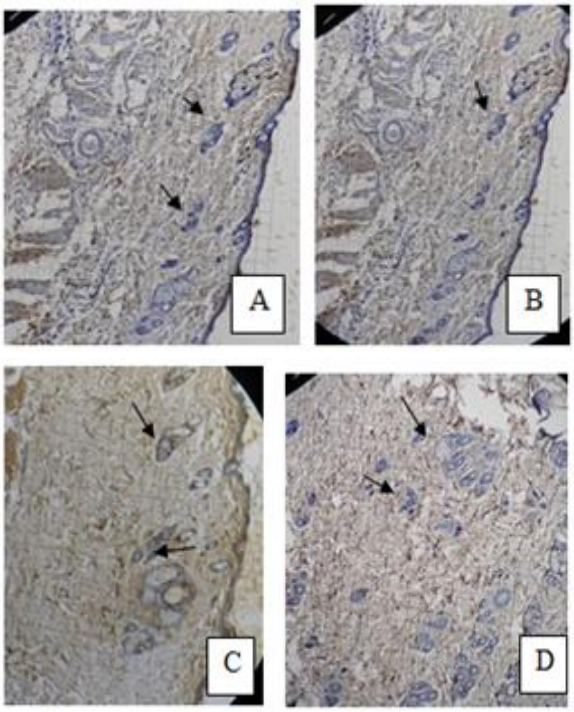


Figure 5. FGF expression on immunohistochemistry. A = Normal Group (K0), B = Treatment Group -1 (P1), C = Treatment Group -2 (P2), D = Treatment Group -3 (P3) using binocular microscope with 400 times enlargement. *arrows point to FGF

**NF-κβ Expression**

This study observed a decreased activation of NF-κβ in the P3 group which was treated with combination therapy of metformin-OHB ($p < 0.05$) when compared to the normal group (K0) and to the groups that were not treated (K1 and P1). Interestingly, there were no significant differences ($p > 0.05$) when comparing the decreased NF-κβ expression of the P3 to the P2 group, which rats was given metformin therapy (Figure 2). We assumed that hyperglycemic was generated due to the increased of ROS in mitochondria of β-pancreatic cells. Subsequently, once hyperglycemic is generated, the formation of non-enzymatic glycation will take place and induce the covalent bonds between the aldehyde groups of glucose and free amino groups of the protein. The reaction leads the formation of AGEs, which is known as free radicals. AGEs affect the release of pro-inflammatory mediators, NF-κβ. This state is predicted to be the reason of prolonged wound healing process in hyperglycemic patient compared to the normal one. Due to this condition, increased NF-κβ may further increase oxidative stress. Treatment of hyperglycemia with metformin together with OHB, was able to reduce the level of NF-κβ and induce the level of FGF due to several mechanisms. Combination therapy of Metformin and OHB also increased pO2 thus induced activation of PMN, macrophage and fibroblast cells. Activation of fibroblast cells could decrease inflammatory cytokines stimulated by NFκβ (Isoda et al 2006).

**FGF Expression**

Fibroblast Growth Factor (FGF) is playing important role in the wound healing process. This healing process is greatly influences by the hyperglycemic condition. Once the hyperglycemic has been controlled, the healing process of the wound will be hastened due to the increased level of FGF. In this study, we did not see the faster healing condition in the group treated with combination therapy, or with metformin alone, compared to the normal group. This might be due to the hyperglycemic condition of the animals that was not controlled yet. As this study was not conducted to heal the hyperglycemic condition, therefore we could not see the increased FGF level in the treated group compared to the normal group, as theoretically mentioned.

**Metformin and OHB in reducing inflammation in Type-2 DM**

It was assumed that metformin has the ability to improve the sensitivity of insulin receptor via the Insulin Receptor Substrate (IRS) by activating cyclic Adenomonophosphat dependent Protein Kinase (c-AMPK). Moreover, metformin has also vasodilator effect by increasing endothelial nitric oxide synthase (eNOS) production (Davis et al 2006). As the vasodilator, metformin may reduce hypoxia that occurred during infection. Furthermore, hyperbaric oxygen therapy (HBO) acts by suppressing the pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ level (Graves et al 2006). Additionally, HBO reduces the activity of fibroblast on rat periodontal ligament (Graves et al 2006). Interestingly, our study support previous study which stated that combination of metformin with HBO worked synergistically to generate eNOS production and perform hypoglycemic effect (Dewi et al 2010), thus, resulting better treatment outcome of DM type 2 patient suffering from gangrene.

**CONCLUSION**

Combination of metformin and hyperbaric oxygen therapy in wounded hyperglycemia rats hastens the wound healing process due to: 1) the decreased of NF-κβ activation, in groups with combination therapy compared to non therapy; 2) the increased of FGF

expression, in groups with combination therapy compared to non therapy groups.

**ACKNOWLEDGEMENTS**

We would like to express our sincere gratitude to Lembaga Penelitian dan Pengabdian kepada Masyarakat (LPPM) Widya Mandala Catholic University Surabaya for awarded the Interdisciplinary Grant and to the Dean of Medical Faculty and the Dean of Faculty of Pharmacy, Widya Mandala Catholic University Surabaya for the support and facilities. Also a special thank you for Dra. Tania Hariadi for helping of the tissue handling. Moreover, we would also send a big thanks for our students, Giovanni and Sisilia as good laboratory co-workers.

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