

Efek Proteksi dari Terapi Oksigen Hiperbarik terhadap Ekspresi Bcl-2 Myometrium *Rattus norvegicus* Bunting yang Terinfeksi oleh Tachyzoite *Toxoplasma gondii*

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Abstrak

Terapi oksigen hiperbarik (HBOT) dapat meningkatkan hantaran oksigen ke jaringan dan menstimulasi pembentukan H_2O_2 sebagai perantara dalam fosforilasi *nuclear factor kappa beta* (NF- κ B) yang sangat penting dalam transkripsi gen anti apoptosis. Penelitian ini bertujuan untuk mencari pengaruh HBOT pada peningkatan ekspresi Bcl-2 di myometrium tikus yang terinfeksi *Toxoplasma gondii*. Penelitian ini adalah penelitian eksperimental murni dengan *randomized control group of post-test only* dan menggunakan 37 *Rattus norvegicus* Sprague Dawley bunting. Tikus secara acak dibagi menjadi empat grup. Grup A merupakan tikus bunting terinfeksi dan mendapatkan HBOT. Grup B merupakan tikus bunting sehat dan tidak terinfeksi tachyzoite serta mendapat HBOT. Grup C merupakan tikus bunting terinfeksi dan tidak mendapatkan HBOT. Grup D merupakan kontrol negatif yakni tikus bunting sehat tanpa mendapatkan HBOT. Tikus yang terinfeksi diberikan 10^3 tachyzoite *T.gondii* melalui injeksi intraperitoneal. Ekspresi Bcl-2 diperiksa dengan immunohistokimia. Semua data dianalisa dengan menggunakan uji ANOVA melalui aplikasi SPSS 21. Terdapat perbedaan yang signifikan pada ekspresi Bcl-2 antara grup A dan grup C karena $p < \alpha$ ($p < 0.017$). HBOT dapat meningkatkan ekspresi Bcl-2 myometrium tikus terinfeksi maupun tidak terinfeksi dengan pemberian HBOT 2.4 ATA selama 3x30 menit, 10X terapi yang terbagi menjadi 2X sehari selama 5 hari.

Kata Kunci: Bcl-2, HBOT, Myometrium, Tachyzoite, *Toxoplasma gondii*

Protective Effect of Hyperbaric Oxygen Therapy to Bcl-2 Expression in the Myometrium of Pregnant *Rattus norvegicus* Infected by Tachyzoite of *Toxoplasma gondii*

Abstract

Hyperbaric Oxygen Therapy (HBOT) can increase oxygen delivery to tissues and stimulate the formation of H_2O_2 as a secondary messenger for phosphorylation of nuclear factor kappa beta (NF- κ B) which plays an important role in the transcription of the anti-apoptotic gene. This study aimed to determine the effects of Hyperbaric Oxygen Therapy (HBOT) in enhancing the

expressions of Bcl-2 in the myometrium of pregnant rats infected by *Toxoplasma gondii*. This study was an experimental study with a randomized control group of post-test only and designed by 37 pregnant *Rattus norvegicus* Sprague Dawley. Randomly, the rats were divided into four groups. Group A is infected pregnant rats that exposed by 10 sessions of HBOT 2.4 ATA in 3x30 minutes. Group B is non-infected pregnant rats and exposed by 10 sessions of HBOT 2.4 ATA in 3x30 minutes. Group C is infected pregnant rats without any exposure. Group D is non-infected pregnant rats without any exposure. Each infected pregnant rat was given a 10^3 tachyzoite of *T.gondii* by intraperitoneal injection. Bcl-2 expressions were measured through immunohistochemistry. All data were analyzed using ANOVA test through SPSS 21 program application. There was a significant difference in Bcl-2 expression between Group A and Group C because $p < \alpha$ ($p < 0.017$). HBOT can increase the expression of Bcl-2 from infected and not infected rat myometrium, in the provision of HBOT 2.4 ATA for 3x30 minutes, twice a day for 5 days.

Keywords: Bcl-2, Hyperbaric Oxygen Therapy, Myometrium, Tachyzoite, *Toxoplasma gondii*

INTRODUCTION

Abortion is the end of a pregnancy before the fetus is sufficiently developed to be able to live outside the womb which is before the gestational age of 20 weeks from the date of the first day of last menstruation or fetal weight less than 500 grams. Abortion is still an obstetric problem that has not been widely revealed and is one of the causes of maternal and fetal death (Moscrop, 2013; Jeve and Davies, 2014; Ghasemi *et al*, 2016).

Bleeding from the birth canal is a symptom in 10-15% of young pregnancies, half of which end in abortion (Jeve and Davies, 2014; Weiss *et al*, 2004). Most (60%) abortions occur before 12 weeks of gestation and the rest occur in the range of 12-20 weeks (Jeve and Davies, 2014). Increased abortion also occurs in uterine abnormalities, diabetes mellitus, hypothyroidism, cardiac abnormalities,

chronic lung disease, increased body mass index, women who smoke, drink alcohol, and others (Ghasemi *et al*, 2016; Hill *et al*, 2004).

Factors that are known to play a role in the occurrence of abortion include chromosomal abnormalities, immunological abnormalities, hormonal abnormalities, or maternal infections including *T. gondii* infection. The majority of abortions within the positive group for *T. gondii* fall in 12 weeks (41%) followed by 8 (15%) and 10 (12%) weeks of gestational age (Al-Fertosi and Juma, 2006). There is a likelihood that strong type 1 response induced early during *T. gondii* infection will induce abortion early in pregnancy (Nurdianto *et al*, 2019a).

Abortion is the most common form of embryonic death caused by early pregnancy failure (Clark *et al*, 2001). The biomolecular mechanism underlying this

abortion is not fully understood. There is evidence that shows an increase in uteroplacental apoptosis and in the reproductive organs associated with abnormalities in pregnancy (PrabhuDas *et al*, 2015). B cell lymphoma 2 (Bcl-2) protein levels in abortion are lower than in normal pregnancies, whereas Caspase-3 protein levels in abortion are higher than in normal pregnancies.

Abortion in pregnancy with *T. gondii* infection is found to be a lower expression of Bcl-2 compared to normal pregnancy. Bcl-2 plays a role in the apoptosis process and abortion through excessive hypoxic mechanisms in pregnancy (Savion *et al*, 2002). HBOT can reduce the cellular hypoxia by reducing the production of HIF-1 α (Choudhury, 2018).

The previous study revealed that HBOT can improve the expressions of Interferon Gamma (IFN γ) and Tumor Necrosis Factor alpha (TNF α), in the provision of HBOT 2.4 (1 atmosphere absolute/ATA) for 3x30 minutes, ten times in 5 days and HBOT administration can prevent abortion in pregnant rats infected by tachyzoite *T. gondii* (Nurdianto *et al*, 2019a). HBOT mechanism in myometrium apoptosis in pregnant rats infected by tachyzoite *T.gondii* needs to be proven. This study tried to find the influence of HBOT on Bcl-2 expression in the

myometrium of pregnant rats infected with tachyzoite *T.gondii*. The results of this study were expected to explain the mechanism of administration of HBOT in pregnant rats with toxoplasmosis.

MATERIAL AND METHOD

This study was a study of 37 *Sprague Dawley Rattus norvegicus* with a post-test only that design randomized control group. This research was performed through ethical clearance No.777_KE from Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Universitas Airlangga. Rats were divided into four groups and given treatment in groups A, B and C while group D is the control group. Group A is infected pregnant rats and exposed by 10 sessions of HBOT 2.4 ATA in 3x30 minutes. Group B is non-infected pregnant rats and exposed to HBOT. Group C is infected pregnant rats without any exposure. Group D is non infected pregnant rats without any exposure. Each infected pregnant rat was given a 10³ tachyzoite of *T. gondii* by intraperitoneal injection. Examination of Bcl-2 was performed on day-5 after HBOT (twice a day) with Monoclonal antibody Bcl-2 (10C4) SANTA CRUZ Biotechnology Inc, Dallas, Texas, United States of America. Euthanized or aborted rats had been eliminated, while rats that still survive had been sacrificed to take the myometrium.

Bcl-2 Expressions were measured using immunohistochemistry.

Animal Model

Rats placed at temperature between 20-23°C. Rats copulated at night and the next day. The female rats were examined for the presence of vaginal plug. If the vaginal plug is positive, it means that the rats have been pregnant for 0.5 day (Clark *et al*, 2001) The female rat was separated and sent to the Bio-Safety Level 2 Laboratory (BSL2) Faculty of Veterinary Medicine, Universitas Airlangga. Before injecting tachyzoite, the abdomen was touched around the uterus to determine whether the rat is truly pregnant. The first day of the pregnancy was calculated based on the first day of the rats having vaginal plug. Rats which at the time of examination were not found to be pregnant, was left uninfected (Dewi, 2009).

HBOT Protocol

Rats got HBOT with 100% oxygen pressure with doses 2.4 ATA, 3x30 minutes and 5-minute intervals (2 sessions) every day for 10 sessions. The administration of HBOT was carried out in different cages every group (in one chamber). The rats still got ad libitum food and drink when getting HBOT (Rusdiana; 2014).

Immunohistochemistry

Immunohistochemistry staining used streptavidin and biotin. The uterine tissue

in embedding paraffin was cut into 4-5 µm thick. Preparations were depinalized in xylol 2 times for 5 minutes in each sample, the respectively entered in absolute ethanol 2 times for 3 minutes, ethanol (95%) for 2 times each 3 minutes and ethanol (70%) for 3 minutes, and eventually washed using distilled water. Preparations were dropped with 5 minutes K proteinase, washed with phosphate buffer saline (PBS) for twice, then dosed with hydrogen peroxidase (H₂O₂) (3%) for 5 minutes and washed using PBS for twice. Each incision was incubated in Bcl-2 anti-rat monoclonal antibodies for 30 minutes. Subsequently, slice were incubated in secondary antibodies labeled with biotin for 30 minutes, washed using PBS for twice. Then the streptavidin-peroxidase was pressed for 15-30 minutes, washed using PBS for twice and finally put in a solution of diaminobenzidine (DAB) substrate for 5-10 minutes. Counterstain used hematoxylin, incubated for 30 seconds at room temperatures then washed for 3 times using distilled water.

This histopathological examination was intended to determine the expression of myometrial Bcl-2. Bcl-2 expression in each sample was assessed semiquantitatively according to the modified Remmele method (Novak *et al*, 2007), where the Remmele scale index

(ImmunoReactive Score/IRS) was the result of multiplication between the percentage of positive immunoreactive cells with color intensity scores on immunoreactive cells. Data for each sample were averaged in IRS value observed in 5 (five) different view fields at 400x magnification. All of these examinations used ordinary light microscopes from the Nikon H600L brand (Nikon Corp., Tokyo, Japan), equipped by a Fi2 DS 300-megapixel digital camera (Nikon Corp., Tokyo, Japan), and Nikon Image System image processing software (Nikon Corp., Tokyo, Japan). All immunohistochemistry were processed through ImageJ application to count the scale bar. All result from immunohistochemistry expression of Bcl-2 myometrium was checked using One way ANOVA SPSS 21 program application (IBM corp., New York, United States).

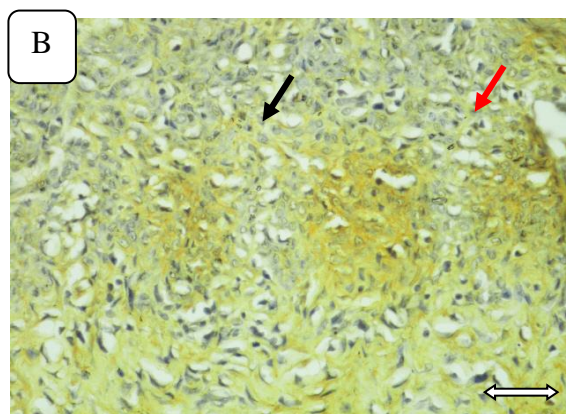
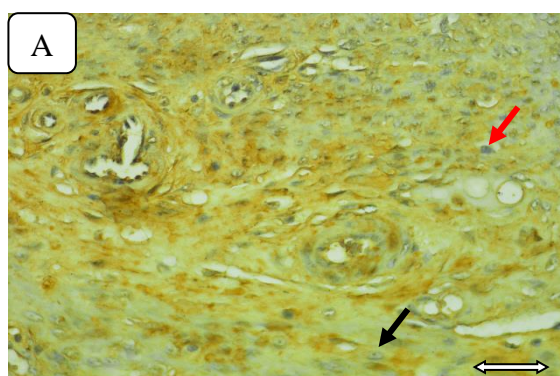
RESULTS

The results of IRS calculation of Bcl-2 expression in myometrial samples that have been treated using immunohistochemical staining shows the following results:

Table 1. Scoring of Bcl-2 Expression

| Group | Average Expression of Bcl-2 (IRS) |
|-------|-----------------------------------|
| A | 5.11 |
| B | 4.23 |
| C | 2.58 |
| D | 4.20 |

Comparison between Bcl-2 myometrium spirals (arrow) expression groups expressed by myosit cells (immunohistochemical staining, 400x magnification; Nikon H600L microscope; 300 megapixels DS Fi2 camera). White marker scale is in 1 μm



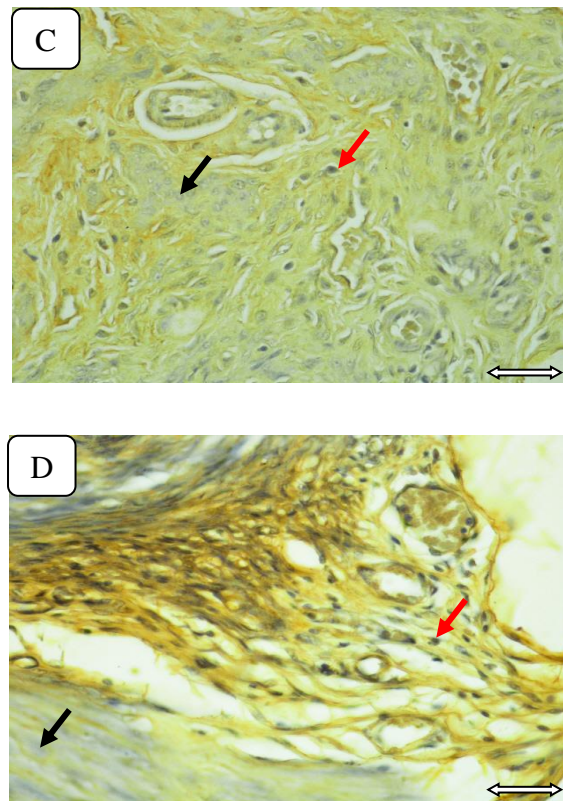


Figure 1. Expression of Bcl-2 Myometrium by Immunohistochemical Staining Red arrows indicate Bcl-2 expression in myocytes cells, while black arrows indicate cells do not express Bcl-2.

Figure A shows that the expression of high Bcl-2 in myometrium layer of pregnant rats infected by tachyzoite which is shown in brown. Figure B shows that the expression of high Bcl-2 in the pregnant myometrium layer of non-infected rats. Figure C shows that Bcl-2 expression decreased in myometrium layer of

pregnant rats infected by tachyzoite without HBOT shown in small are of brown color. Figure D above shows that the expression of high Bcl-2 in the myometrium layer of non-infected without any exposure rats, which is shown as brown.

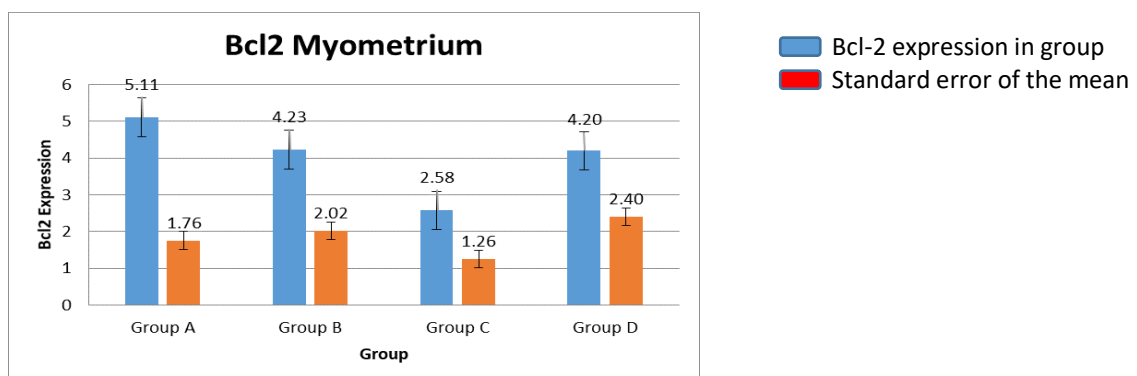


Figure 2. Expression of Bcl-2 in Myometrium among Groups
 Bcl-2 expression in group

From the Figure 2, we can concluded that the expression of Bcl-2 myometrium in group A and B were high, indicating that administration of HBOT could increase the expression of Bcl-2 from pregnant rats infected with tachyzoite.

Based on the statistical test results ANOVA through SPSS 21 program application, it was found that there was a significant difference between the administration of HBOT between Group A and Group C with $p < 0.017$ which means that administration of HBOT in pregnant rats infected by tachyzoite could increase the expression of Bcl-2 in the myometrium.

DISCUSSION

Pregnancy failure is closely related to an increase in apoptosis, which is controlled by the Bcl-2 family and the caspase cascade (Sun and Zhang, 2017; Bartlett, 2017; Toder *et al*; 2002). *T.gondii*-infected pregnancies abortion is often resulted from apoptosis and abortion due to excessive IFN γ production that can down regulate Bcl-2 expression, Fas Ligand (FasL), and protein 53 (p53) (Nurdianto *et al*, 2019a; Nurdianto *et al*, 2019b; Sun *et al*, 2007; Dockrell, 2003; Begum-Haque *et al*, 2009).

Bcl-2 plays a role as anti-apoptosis and abortion through hypoxia in pregnancy and located in the cytoplasm of the

myometrium (Savion *et al*, 2002; Kelly and Strasser, 2011). A decrease in Bcl-2 protein levels during pregnancy is associated with pregnancy failure (Danihel *et al*, 2002). Whereas, the administration of HBOT in research rats can increase the amount of dissolved oxygen (Hyperoxia).

Bcl-2 is a proto-oncogene which can inhibit apoptosis including myometrium (Kelly and Strasser, 2011; Opferman and Kothari, 2017) and this protein increased by giving HBOT as in group A (5.11). We can compare it to Bcl-2 levels in group C (2.58) which was much lower than group A. There was a decrease in Bcl-2 associated with apoptosis and end in abortion in animal studies (Savion *et al*, 2002), we can see this in group C and it can be concluded that the process of apoptosis in the myometrium of rats infected by tachyzoite could be prevented by HBOT.

The results of immunohistochemical Bcl-2 myometrium found that Bcl-2 levels in group A, B, and group D were higher than group C. This result explained that in group A, HBOT could prevent myometrium from apoptosis. These results are supported by previous research data which state that administration of HBOT can increase anti-apoptotic proteins (Bcl-2 and Bcl-xl) and reduce the expression of genes that trigger apoptosis such as c-for, c-jun, Bax and Caspase 3 (Liu *et al*, 2006;

Vlodavsky *et al*, 2006; Xu *et al*, 2012; Barbosa *et al*, 2015; Torchinsky and Toder, 2004). The HBOT can reduce apoptosis by suppressing mitochondrial-mediated apoptotic pathways by triggering Bcl-2 expression and maintaining intact mitochondria involving three components, including FasL, Fas, and Caspase-3 (Palzur *et al*, 2008; De Falco *et al*, 2004; Huppertz *et al*, 2006).

The process of apoptosis in the placenta and myometrium is associated with abnormal pregnancies (Jerzak and Bischof, 2002). Research in mice shows that the process of apoptosis causes cell death in the placenta and the occurrence of miscarriage (Jerzak and Bischof, 2002; Savion *et al*, 2002). In this study, we can see that tachyzoite infection in pregnant rats without HBOT therapy has decreased the expression of Bcl-2 myometrium in group C (2,58) while in the group D, Bcl-2 levels are (4.2). This explains that tachyzoite posed a risk of apoptosis in the myometrium which has the potential to cause fetal abortion.

Excessive apoptosis in the uterus may result in pregnancy failure and most likely to occur if Bcl-2 levels also decrease dramatically as in group C. The possibility of abortion in group C was greater than group A, B, and D which has a higher Bcl-2

level (Toder *et al*, 2002; Jerzak and Bischof, 2002; Savion *et al*, 2002)

CONCLUSION

HBOT can improve the expressions of Bcl-2 in myometrium, in the provision of HBOT 2.4 ATA for 3x30 minutes, ten times in 5 days.

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DISCLOSURE

The author reports no conflicts of interest in this work.

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