



## Research Article

# Elevation of Bcl2 expression in spiralis artery of pregnant *Rattus norvegicus* infected with tachyzoite of *Toxoplasma gondii* with hyperbaric oxygen therapy

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## ABSTRACT

Spiral artery apoptosis plays a role in the process of abortion. Low Bcl2 expression found in abortion and in spiral artery. In pregnancy, Toxoplasmosis infection is found to increase apoptosis in spiral arteries. Hyperbaric Oxygen Therapy (HBOT) improves the expression of Bcl-2. This study aims to determine the effects of Hyperbaric Oxygen therapy in enhancing the expressions of Bcl2 in artery spiralis of pregnant rats infected with tachyzoite of *Toxoplasma gondii*. This is an experiment with a 'randomized control group of post-test only design' on 37 *Rattus norvegicus* Sprague Dawley rats. Rats were divided into four groups. The group A is pregnant rats infected with 10<sup>3</sup> tachyzoites via intraperitoneal injection and received 10 sessions of HBOT 2.4 ATA in 3x30 minutes. Group B is pregnant only and received HBOT. Group C is pregnant and infected with tachyzoite but did not received HBOT. And the last, Group D is pregnant rats with no infection and did not received HBOT. Examinations of Bcl2 expressions were performed on day-5 after HBOT (twice a day). The Bcl2 expression was measured with immunohistochemistry. All data were tested with One-way ANOVA from SPSS 21. There is an increased expression of Bcl2 spiralis artery in the Group A. There was a significant difference between Group A and Group C with a value of p=0.042. HBOT can increase the expression of Bcl2 from the spiral arteries of rats, in the provision of HBOT 2.4 ATA for 3x30 minutes, 10 times in 5 days.



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### ABSTRAK

Apoptosis arteri spiralis berperan dalam proses aborsi. Ekspresi Bcl2 rendah ditemukan pada aborsi dan di arteri spiral. Pada kehamilan, infeksi Toxoplasmosis ditemukan meningkatkan apoptosis pada arteri spiralis. Terapi Oksigen Hiperbarik (HBOT) meningkatkan ekspresi Bcl-2. Ini adalah penelitian eksperimental dengan desain post-test only pada 37 hamil *Rattus norvegicus* Sprague Dawley, maka tikus dibagi menjadi 4 kelompok. Grup A HBOT adalah tikus hamil yang terinfeksi tachyzoite yang menerima terapi 10 sesi HBOT 2.4 ATA dalam 3x30 menit; kelompok B adalah Hamil saja dan tidak mendapat HBOT; kelompok C Hamil dan terinfeksi tachyzoite *T.gondii* tetapi tidak menerima HBOT; dan kelompok D adalah tikus hamil normal. Setiap tikus hamil yang terinfeksi diberi 10<sup>3</sup> Tachyzoite *Toxoplasma gondii* melalui intraperitoneal. Pemeriksaan ekspresi Bcl2 dilakukan pada hari ke-5 setelah HBOT (dua kali sehari). Tikus terbunuh dan kadar Bcl2 Arteri spiralis diukur dengan pemeriksaan Immunohistokimia. Semua data dianalisis dengan uji ANOVA dengan Program SPSS 21. Pada studi ini menunjukkan terapi Oksigen Hiperbarik dapat meningkatkan Ekspresi Bcl2 Arteri Spiralis dengan dosis 2.4 ATA selama 3x30 menit dalam 10 sesi selama 5 hari terapi. Studi ini menyimpulkan bahwa HBOT dapat meningkatkan ekspresi Bcl2 dalam arteri spiralis, dalam pemberian HBO 2.4 ATA selama 3x30 menit dalam 10 sesi.

**Kata kunci** : HBOT, Arteri spiralis, Bcl2, Tachyzoite *Toxoplasma gondii*

### INTRODUCTION

Abortion is defined as the end of a pregnancy before the fetus can live outside the womb, namely before the gestational age of 20 weeks from the date of the first day of last menstruation or fetal weight of fewer than 500 grams. Abortion is an obstetric problem that has not been widely revealed and is one of the causes of maternal and fetal death (Kupesic et al., 2002; Stirrat and Wardle, 2001; Cunningham et al., 2001).

Abortion incidence ranges from 16 to 25% of all pregnancies (Stirrat and Wardle, 2001; Weiss et al., 2004) Bleeding from the birth canal is a symptom in 10-15% of young pregnancies, half of which end in abortions (Cunningham et al., 2001). Most (60%) of abortions occur before 12 weeks' gestation and the rest occurs in the range 12-20 weeks (Hill, 2004; Clark et al., 2001).

The Bcl2 protein levels in abortion are lower than in normal pregnancies. Bcl2 and

Caspase-2 levels in abortion are higher than in normal pregnancies. Factors known to play a role in the occurrence of abortion include infection, chromosomal abnormalities, immunological abnormalities, hormonal abnormalities, or maternal infection (Kupesic et al., 2002; Hill, 2004; Clark et al., 2001; Danihel et al., 2002). One cause of infection is from *Toxoplasma gondii*. There is the likelihood that Th1 response induced early during *T. gondii* infection will induce abortion early in pregnancy.

Abortion is the most common form of embryo death caused by pregnancy failure (De Falco et al., 2004). 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, including apoptosis in spiralis artery that supply blood flow in uteroplacental nutrition and it is associated with low Bcl2 expression (Toder et al., 2002).



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HBOT can improve the expression of Bcl-2, inhibit pASK-1, pJNK, and Bax, and decline Bax/Bcl-2 ratio (Xiao et al., 2015). Elevation the serum concentration of IFN $\gamma$  and TNF $\alpha$  from pregnant rats infected with tachyzoites of *T. gondii*, in the provision of HBOT 2.4 ATA for 3x30 minutes, ten times in 5 days and HBOT administration can prevent abortion (Nurdianto et al., 2019).

Mechanism of HBOT in spiralis artery apoptosis in Pregnant Rats infected with tachyzoite *T. gondii* needs to be proven. This study tried to find the influence of HBOT on Bcl2 expression in spiralis artery of pregnant rats infected with tachyzoite *T. gondii*. The results of this study are expected to explain the mechanism of HBOT administration in pregnant rats with toxoplasmosis.

### METHODS

This study is an animal study with a 'randomized control group of post-test only design' on 37 Sprague Dawley rats. This experiment has ethical clearance from the Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Airlangga University No.777\_KE. Randomly, the rats were divided into four groups (Group A, 9 rats; Group B 8 rats; group C 8 rats; group D 12 rats) with minimum sample in group is 6 sample. The HBOT treatment group A is pregnant rats infected with tachyzoite received ten sessions of HBOT 2.4 ATA in 3x30 minutes. Group B is Pregnant only and received ten sessions of HBOT 2.4 ATA in 3x30 minutes. Group C is pregnant and infected with tachyzoite but not received HBOT. And the last, Group D is pregnant rats only without infection and did not received HBOT (control group). Each infected pregnant rats were given a 10<sup>3</sup> tachyzoite of *T. gondii* via intraperitoneal injection. Examinations of Bcl2 expressions were performed on day-5 after HBOT (HBOT twice a day). Euthanized or aborted rats will be

eliminated while rats that still survive will be taken from the uterus.

### Animal Model

Rats placed at temperatures between 20-23°C to get a chance for a big pregnancy because male mice will be active to impregnate at this temperature. Rats mate at night. The next day the female rat's vagina was examined for the presence of a vaginal plug. If the vaginal plug is positive, it means that the rats have been pregnant for 0.5 days (Clark et al., 2001).

After obtaining the vaginal plug, the female rat will be separated from the male rat then sent to the Bio-Safety Level 2 Lab (BSL2) Faculty of Veterinary, Airlangga University. Before injecting *T. gondii* tachyzoite, the abdomen were touched around the uterus to determine whether the rat is truly pregnant. The first day of pregnancy was calculated based on the first day the rats appeared vaginal plug. Rats which at the time of examination were not found to be pregnant will be left uninfected (Dewi, 2009; Rusdiana 2014).

### HBOT Protocol

The HBOT with 100% oxygen pressure 2.4 ATA for 3 x 30 minutes with 5-minute intervals (2 session) were given per day for 10 sessions in 1 chamber. The administration of HBOT is carried out in different cages per group. During the administration of HBOT, the experimental animal still gets ad libitum food and drink, except in the last session (10th session).

### Immunohistochemistry

Immunohistochemical staining uses streptavidin and biotin. The uterine tissue in embedding paraffin is cut 4-5  $\mu$ m thick. Preparations were depenalized in xylol 2 times for 5 minutes each sample, then respectively entered in absolute ethanol 2 times for 3



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minutes, ethanol 95% 2 times each 3 minutes, and ethanol 70% 3 minutes, finally washed with distilled water. Preparations were dropped with 5-minute K proteinase, washed with phosphate buffer saline (PBS) twice, then dosed with hydrogen peroxidase (H<sub>2</sub>O<sub>2</sub>) 3% for 5 minutes and washed with PBS twice. Each incision was incubated in Bcl2 anti-rat monoclonal antibodies for 30 minutes. Subsequently, incisions were incubated in secondary antibodies labeled with biotin for 30 minutes, washed with PBS twice. Then, the streptavidin-peroxidase was pressed for 15-30 minutes, washed with PBS twice and finally put in a solution of Diaminobenzidine (DAB) substrate for 5-10 minutes. Counterstain used hematoxylin, incubated 30 seconds at room temperature then washed 3 times with distilled water. The preparation is then dried and covered with a glass cover. The Bcl2 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 of the percentage of positive immunoreactive cells with color intensity scores on immunoreactive cells. Data for each sample is the average IRS value observed in 5 (five) different Fields of View at 400x magnification. All of these examinations use an ordinary light microscope, the Nikon H600L brand, which is equipped with a 300 megapixel Fi2 DS digital camera and Nikkon Image System image processing software

**Table 1.** Scoring of Bcl2 expression

No	Group	Average Expression
1	A	3.755556
2	B	2.6
3	C	1.625
4	D	3.383333

## RESULTS

From the results of microscope readings it was found the results of Bcl2 expression from immunohistochemical staining and made as follows group A 3.75556, Group B 2.6, Group C 1.625 and Group D 3.383333 Bcl2 expression is indicated by a dark brown expression. Calculation of scores based on immunohistochemical staining obtained mean Bcl2 expression in the pregnant Rattus novergicus spiral arteries infected with tachyzoite *T. gondii* given HBOT of 3.755556.

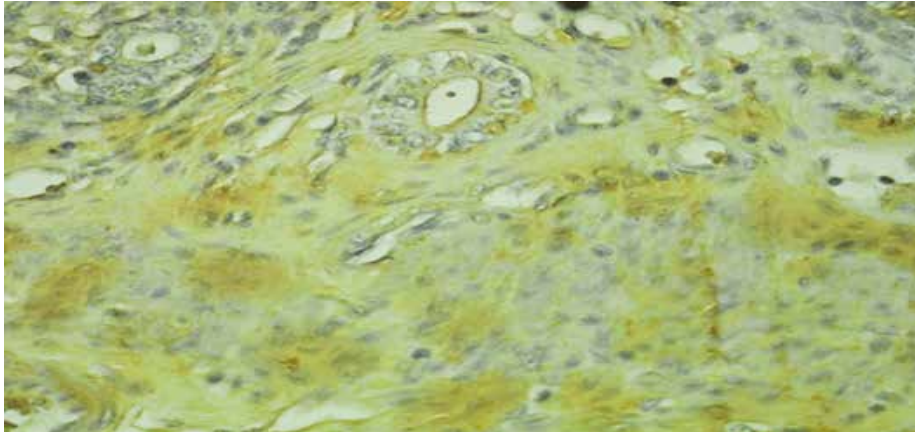
Bcl2 expression is indicated by a dark brown expression. Calculation of scores based on immunohistochemical staining obtained mean Bcl2 expression in the pregnant Rattus novergicus spiral arteries that were not infected with tachyzoite *T. gondii* but were given HBOT of 2.6

Bcl2 expression is indicated by a dark brown expression. Calculation of scores based on immunohistochemical staining obtained mean Bcl2 expression in pregnant Rattus novergicus spiral arteries infected with tachyzoite *T. gondii* without being given HBOT is 1,625.

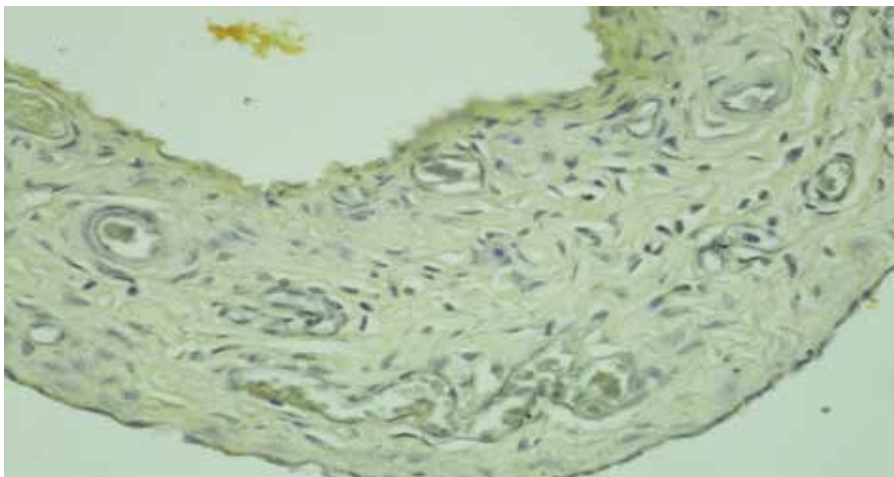
The calculation of the score based on immunohistochemical staining obtained an average expression of Bcl2 in the normal pregnant Rattus novergicus spiral artery is 3.38333

From the graph above, we can see that the expression of Bcl2 in Group C is much lower than the other groups indicating that administration of HBOT can increase the expression of Bcl2 in spiral arteries so that it is expected to reduce the rate of pregnant Rattus novergicus infected with *T. gondii* tachyzoite.

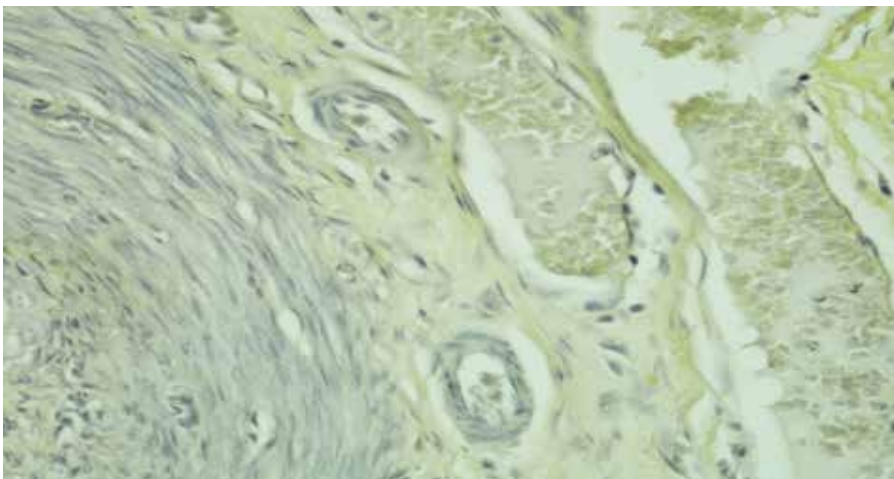
The results in Group C showed that mice



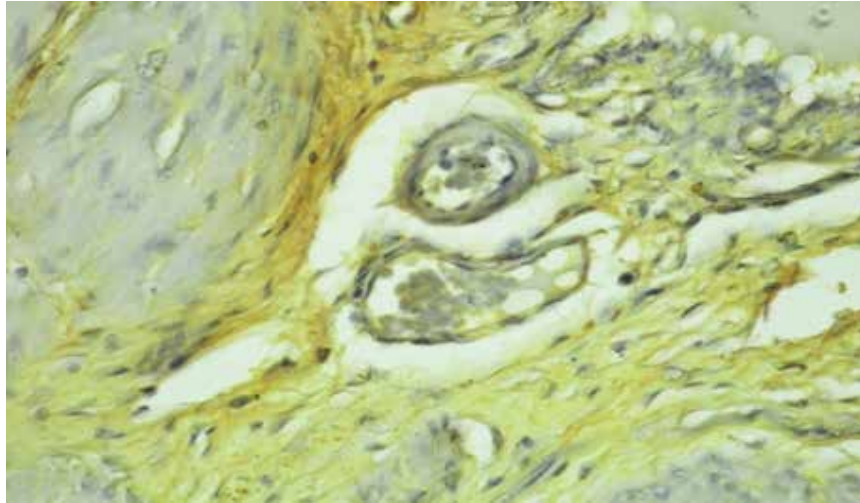
**Figure 1.** IHC staining Bcl2 in spiral arteries *Rattus norvegicus* W1A3 with a 400X magnification microscope.



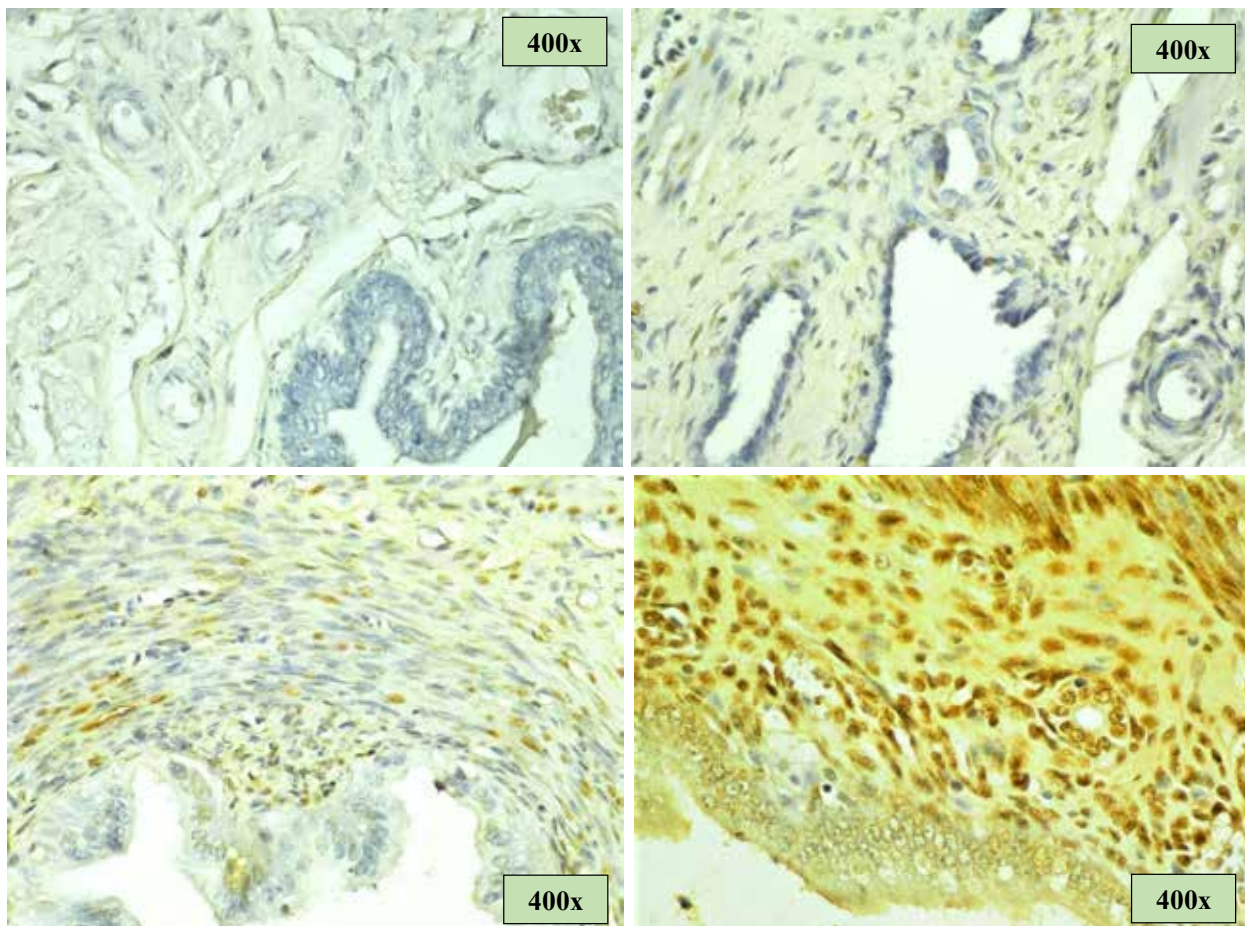
**Figure 2.** The coloration of Bcl2 in the spiral artery *Rattus norvegicus* W1B4 with an enlargement of a 400X microscope.



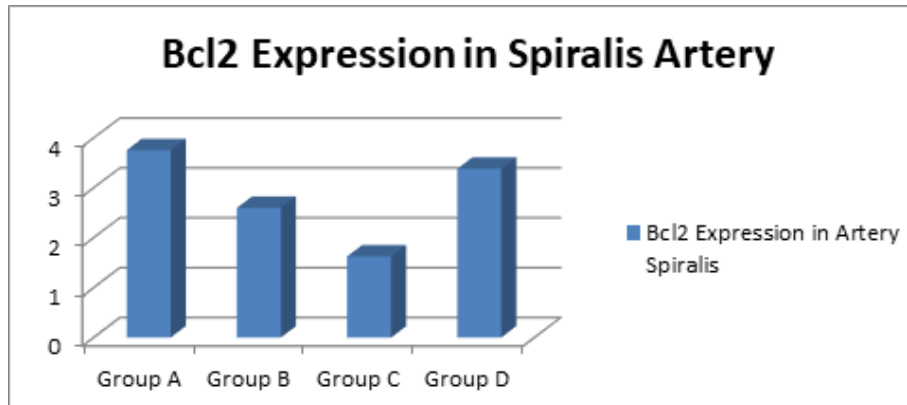
**Figure 3.** Staining Bcl2 in the spiral artery *Rattus norvegicus* W1C2 with an enlargement of a 400X microscope.



**Figure 4.** The coloration of CPI Bcl2 in the spiral arteries *Rattus norvegicus* W1D3. Bcl2 expression is indicated by a dark brown expression



**Figure 5.** Comparison between Bcl2 myometrium spirals (arrow) expression groups expressed by myocyte cells (immunohistochemical staining, 400x magnification; Nikon H600L microscope; 300 megapixels DS Fi2 camera)



**Figure 6.** Bcl2 Expression in Spiralis Artery

From the graph above, we can see that the expression of Bcl2 in Group C is much lower than the other groups indicating that administration of HBOT can increase the expression of Bcl2 in spiral arteries so that it is expected to reduce the rate of pregnant *Rattus novergicus* infected with *T. gondii* tachyzoite.

The results in Group C showed that mice infected with *T.gondii* tachyzoite decreased Bcl2 in their spiral arteries and indicated that *T. gondii* infection had the potential to cause apoptosis in spiral arteries.

From the results of statistical tests using One-way ANOVA SPSS 21, there was a significant difference between Group A and Group C with a value of  $p = 0.042$ .

## DISCUSSION

In this study, there was a lower expression of Bcl2 in Group C or infected *Rattus novergicus* group than Group A, B and D. It can be concluded that administration of HBOT can increase Bcl2 in tachyzoite-infected *Rattus novergicus* spiral arteries and infection of *T.gondii* tachyzoite can downregulate Bcl2 in pregnant rats. This shows that the potential for apoptosis in Group C is greater than Group A, B, and D so that in this study it can be concluded that infection with *T. gondii* can increase the potential for apoptosis in the spiral artery. Because of Bcl-2 as an

anti-apoptosis is located in the cytoplasm of the syncytiotrophoblast and cells in the uterus function including spiral artery to maintain pregnancy and fetal development. A decrease in Bcl-2 protein levels during the first-trimester pregnancy is associated with pregnancy failure (Danigel et al., 2002). Apoptosis occurs through two pathways, which occur in receptors and mitochondria. In the first trimester of pregnancy, the receptor pathway is more influential than the mitochondrial pathway involving three components, namely Fas ligand (FasL), Fas, and Caspase-3 which are mostly located in the cytotrophoblast (De Falco et al., 2004).

It is expected that with the avoidance of spiral arteries from apoptosis, arterial insufficiency in the uteroplacental can be minimized and the decidual cell damage will be avoided.

We know that infection of *T. gondii* induces cytokines produced from Th cell lines, such as  $IFN\gamma$ ,  $TNF-\alpha$ , IL-12 and IL-18 (Abbas et al., 2000; Sibley et al., 2002; Nguyen et al., 2003). *T.gondii* infection in pregnancy can cause activation of the apoptotic pathway due to excessive production of  $TNF\alpha$  that can disturbing remodeling spiral artery with decreases in Bcl2 expression. Failure of remodeling the spiral arteries in results in



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decreased uteroplacental blood flow causing hypoxia and uteroplacental ischemia which results in delayed fetal growth to abortion (Prawirohardjo, 2009).

Spiral arteries have the function of supplying blood to the endometrial lining of the pregnant uterus which extends from the inner endometrium to the decidual cells. Overexpression of Bcl2 blocks TNF-related apoptosis-inducing ligand (TRAIL)-induced Apoptosis (Sun et al., 2001). Three TNF family members are involved in the regulation of vascular cell apoptosis namely Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ), TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). All these factors are expressed or produced by trophoblasts, TNF $\alpha$  binds and activates two different receptors TNF-receptor 1 (TNF-R1) and TNF-R2, both are expressed in endothelial cells and VSMC. Activation of TNF-R1 leads to the recruitment of intracellular adapter molecules, namely TNF-receptor-associated death domain (TRADD), which together with a number of proteins including TRAF2 (TNF-receptor-associated factor 2) activates the JNK pathway and promotes cell survival. However, if TRAF2 recruits the Fas Activating Death Domain (FADD) it will cause pro-caspase 8 to divide and induce apoptosis (Whitley and Cartwright, 2010). The results of immunohistochemical Bcl2 spiral arteries were found that Bcl2 levels in Group A, B, and D were higher than Group C and this explained that in Group A infected with *T. gondii* and getting HBOT can prevent spiral arteries from apoptosis which can cause abortion.

The results of the above study are supported by previous research data which states that administration of HBOT in rats experiencing Traumatic Brain Injury (TBI) can increase anti-apoptotic proteins (Bcl-2 and Bcl-x1) and reduce apoptosis in rat brain cells (Vlodavsky

et al., 2005; Liu et al., 2006; Xu et al., 2012). The administration of HBOT can reduce apoptosis by suppressing mitochondrial-mediated apoptotic pathways by triggering Bcl-2 expression and maintaining intact mitochondria (Palzur et al., 2008).

The administration of HBOT can reduce gene expression that triggers apoptosis such as c-fos, c-jun, Bax and decreases activation of Caspase-3. Conversely, HBOT administration alleviates the decrease in the Bcl-2 anti-apoptotic gene and stimulates the expression of neurotrophic factors (NTFs), such as NGF, BDNF, GDNF, and NT-3 (Barbosa et al, 2015) and this can have a good effect on the fetus and still needs to be proven.

Bcl-2 plays a role in the process of apoptosis and abortion through the mechanism of excessive hypoxia in pregnancy (Savion et al., 2002) and there is an association of NF-kB in the process of apoptosis which results in abnormal growth, fetal abnormalities and abortion (Torchinsky et al., 2004) whereas the administration of HBOT in research rats can increase the amount of dissolved oxygen and reduce hypoxia. In addition, the second messenger (H<sub>2</sub>O<sub>2</sub>) produced by administration of HBOT can activate NF-kB so that the immunological and anti-apoptotic processes will be active, it can explain that HBOT can reduce the rate of apoptosis and abortion of tachyzoite *T.gondii*-infected mice. This is related to a previous study, which stated that there was a decrease in Bcl-2 associated with apoptosis and ended in abortion in experimental animals (Savion et al., 2002).

Bcl-2 is a powerful anti-apoptotic protein that can prevent cells from dying and levels remain high outside the mitochondrial membrane. Bcl-2 can also be used as an early detection of apoptosis (Reed, 2008). When looking at the results of Group C above, it can be concluded that the apoptosis process in the spiral arteries of





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mice infected with tachyzoite *T. gondii* can be prevented by HBOT. This is in line with several previous studies which stated that HBOT can reduce apoptosis by decreasing the expression of HIF 1 $\alpha$ , P53 and BNip3 in addition to increased expression of Bcl2 and Caspase 3 (Zhang et al., 2008). HBOT can reduce apoptosis in studies of mice that experience spinal cord injury by suppressing ASC (Apoptosis-associated speck-like protein) and Caspase 3 (Long et al., 2014). In addition, HBOT increases the expression of Bcl-2 and inhibits pASK-1 (signal-regulating kinase 1 apoptosis) in the skin flap trial. HBOT increases pJNK expression (c-Jun N-terminal kinase pathway) in the skin flap experiment. HBOT decreases Bax (pro apoptosis) expression. HBOT decreases Caspase-3 activity and decreases the ratio of Bax / Bcl-2 in skin flap trials (Xiao et al., 2015).

### CONCLUSION

Tachyzoite *T.gondii* infection in pregnant rats can reduce expression of Bcl2 in spiralis artery. HBOT can improve the expressions of Bcl2 in spiralis artery, in the provision of HBOT 2.4 ATA for 3x30 minutes, ten times in 5 days and HBOT administration can prevent abortion in pregnant rats infected with tachyzoite *T. gondii*.

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### DISCLOSURE

The author reports no conflicts of interest in this work.

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