ORIGINAL ARTICLE

Bali Medical Journal (*Bali Med J*) 2019, Volume 8, Number 1: 94-100
P-ISSN.2089-1180, E-ISSN.2302-2914

Published by DiscoverSys

Effect of hyperbaric oxygen therapy to IFNγ and TNFα expression in pregnant *Rattus novergicus* infected with *Tachyzoite* of *Toxoplasma gondii*

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ABSTRACT

Background: Hyperbaric Oxygen Therapy (HBOT) is a method of increasing oxygen delivery to tissues. The therapy improves tissue oxygenation and stimulates the formation of H_2O_2 as a secondary messenger for tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and nuclear factor kappa beta phosphorylation (NF-kB) which play an important role in the rapid transcription of a wide variety of genes in response to extracellular stimuli.

Aim: This study aims to determine the effects of Hyperbaric Oxygen therapy in enhancing the expressions of IFNγ and TNFα in pregnant rats infected with *Toxoplasma gondii*.

Methods: This study is an animal study with a 'randomized control group of post-test only design' on 34 *Rattus novergicus* Sprague Dawley rats. Randomly, the rats were divided into four groups. The HBOT treatment group A is pregnant rats infected with tachyzoite received 10 sessions of HBOT 2.4 ATA in 3x30 minutes. B is Pregnant only and received 10 sessions of HBOT 2.4 ATA in 3x30 minutes. C is pregnant and infected with tachyzoite but not received HBOT. And the last, D is pregnant rats only without infection and not received HBOT. Each infected pregnant rats were given a 10^3 Tachyzoite of *Toxoplasma gondii* via intraperitoneal injection. Examinations of IFNγ and TNFα expressions were performed on day-5 after HBOT (HBOT twice a day). Rats that die or experience abortion will be eliminated while rats that still survive will be taken the blood by intracardiac technique. IFNγ and TNFα levels were measured by serum ELISA examination.

Results: The results showed that the HBOT could improve IFNγ (p=0.000), TNF-α (p= 0.02) significantly in the provision of HBOT 2.4 ATA for 3x30 minutes in 10 sessions over five days of therapy.

Conclusion: HBOT can improve the expressions of IFNγ and TNFα, in the provision of HBOT 2.4 ATA for 3x30 minutes, 10 times in 5 days and HBOT administration can prevent abortion in pregnant rats infected with tachyzoite *T. gondii*.

Keywords: *Toxoplasma gondii*, Tachyzoite, Hyperbaric Oxygen Therapy, IFNγ, TNFα

Cite this Article: Nurdianto, A.R., Aryati, Suryokusumo, M.G., Mufasirin. 2019. Effect of hyperbaric oxygen therapy to IFNγ and TNFα expression in pregnant *Rattus novergicus* infected with *Tachyzoite* of *Toxoplasma gondii*. *Bali Medical Journal* 8(1): 94-100. DOI:10.15562/bmj.v8i1.1316

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Received: 2018-08-30
Accepted: 2018-10-14
Published: 2019-4-1

INTRODUCTION

IFNγ is an interleukin that plays a major role in the abortion of pregnant women infected with *Toxoplasma gondii*. 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.[1] Cell-mediated immune responses are essential for against toxoplasmosis.[2] Resistance to *T. gondii* is mainly mediated by type 1 cytokines, such as IFNγ which is central in resistance to *T. gondii* infection, whereas type 2 cytokines, such as IL4 and IL10, are associated with increased susceptibility to infection.[3,4] Susceptibility of the pregnant host to toxoplasmosis may be due to a type 2 cytokine bias that is maintained during gestation.[5] *T. gondii* is a potential stimulus of type 1 cytokines, perhaps reflecting in keeping the host alive during infection. On the other hand, there is the likelihood that strong type 1 response induced early during *T. gondii* infection will induce abortion early in pregnancy.[6,7]

A type 2 cytokine bias has been identified in normal murine placenta and is associated with successful implantation maintenance of early pregnancy, and suppression of local inflammatory responses.[8] On the other hand, type 1 cytokines cause inflammatory immune reactions and graft rejection mechanisms which lead to the abortion of the conceptus.[9]

Cytokine TNFα is released by macrophages when the body is infected with T. gondii that serves to eliminate parasites. In addition, the cytokine also affects other cells that are not infected and cause apoptosis.[10] At the onset of infection, IFNγ is produced by natural killer cells (NK). In this phase involves the innate immune system, NK cells and macrophages. NK cells are the main cells producing IFNγ and will activate macrophages to produce TNFα as microbicides. In the chronic phase, T lymphocytes produce IFNγ in large quantities.[11]

HBOT may suppress the inflammatory process shown in some studies.[12-15] In healthy humans treated with OHB with a dose of 2.8-3 ATA for 45 minutes, the ability of neutrophils in the blood circulation to attach to his target tissue will be temporarily impeded.[16] Administrated HBOT

(2 ATA for 60 minutes) at 12 hours after injury reduced the RNA and protein levels of caspase-3, interleukin- 8 and tumor necrosis factor-α.[14] HBOT improved outcomes and reduced inflammation by increasing anti-inflammatory cytokine interleukin-10,[13,17] and decreasing the level of TNFα.[18] Recently, HBOT significantly increased the expression of heme oxygenase-1, and inhibited the expression of NF-kB in a rat TBI model.[15,19]

Mechanism of HBOT in Pregnant Rats needs to be proven. This study tried to find the influence of HBOT on serum levels IFNγ and TNFα in pregnant rats infected with tachyzoite *Toxoplasma gondii*. The results of this study are expected to explain the mechanism of administration of HBOT in pregnant rats with toxoplasmosis.

MATERIAL AND METHODS

This study is an animal study with a 'randomized control group of post-test only design' on 34 Sprague Dawley rats. Randomly, the rats were divided into four groups with nine rats in each group. The HBOT treatment group A is pregnant rats infected with tachyzoite received ten sessions of HBOT 2.4 ATA in 3x30 minutes. B is Pregnant only and received ten sessions of HBOT 2.4 ATA in 3x30 minutes. C is pregnant and infected with tachyzoite but not received HBOT. And the last, D is pregnant rats only without infection and not received HBOT. Each infected pregnant rats were given a 10^3 Tachyzoite of *Toxoplasma gondii* via intraperitoneal injection. Examinations of IFNγ and TNFα 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 intracardiac blood by intracardiac technique. IFNγ and TNFα levels were measured by serum ELISA examination.

RESULTS

IFNγ data from 4 groups of research conducted homogeneity test obtained significance value of (0.194) which shows the data is homogeneous. Then the results of One Way ANOVA test showed a significant value of (0.000) with significant value when $p < 0.05$. Then the above data was tested again with Pearson correlation test and obtained significance result of $p = 0.041$ indicating that there is a relationship between IFNγ level with TNFα levels in the treatment of HBOT in pregnant rats infected with Tachyzoite infection.

There was a significant correlation between HBOT at IFNγ and TNFα concentrations in pregnant rats infected with *T. gondii* tachyzoite between and within group A, B, C, D. The administration of HBOT can have a significant effect on the elimination of tachyzoite infection in pregnant rats as indicated by $p < 0.000$.

DISCUSSION

T. gondii infected macrophages produce IL12 which activates NK cells to produce IFNγ and stimulates the differentiation of T helper (Th) lymphocytes into Th1 cells. Th1 cells produce IFNγ and IL2. Macrophages as Antigen Presenting Cell (APC) express Major Histocompatibility Complex I (MHC I) so that it was captured by T cell receptors (Cytotoxic T Leucocyte, CTL). The resulting cytokine IL2 induces CTL to produce IFNγ. IFNγ is essential for macrophage activation and to promote macrophage function as microbicides.[10] As the results of the study of cytokines in groups C and A, the significant increment of IFNγ were found chiefly in group A.

Increased dissemination of transplacental tachyzoite is associated with increased IFNγ secretion.[20,21] Increased IFNγ secretion is related to increased ICAM1 molecules that facilitate monocyte migration.[21,22] On the other hand, monocytes are permissive and dominant cells infected by takizoit[23] and facilitate tachyzoite migration to the placenta. Although monocytes will not enter the fetal circulation, it can actively penetrate the placental tissue with its gliding movements and transmigration capabilities.[24] Thus, *T. gondii* has a greater chance of spreading and invading placental tissue associated with an increase in inflammatory cytokines in acute cases. In chronic cases, IFNγ is neutralized to cause fetomaternal tachyzoite transmission through the placenta.[25] Based on the above research, IFNγ has a vital role in the process of elimination of *T. gondii* in the body in optimum concentration as IFNγ has a destructive effect and vice versa at low concentrations can also decrease the elimination activity of *T. gondii*. The results of this study showed that there were no rats that had an abortion with the administration of tachyzoite infection with a dose of 10^3 via intraperitoneal in groups A and C, although there was a much higher concentration of IFNγ compared to the control group (groups B and D). This can be achieved by giving HBOT while can increase IFNγ without causing abortion.

As from the Figure 1, we know if group A had a higher concentration of IFNγ better than group C, D, and B. This showed that HBOT administration in this study in groups A and C could increase the IFNγ which had a vital role in the elimination process of *T. gondii*. As against in group B and D,

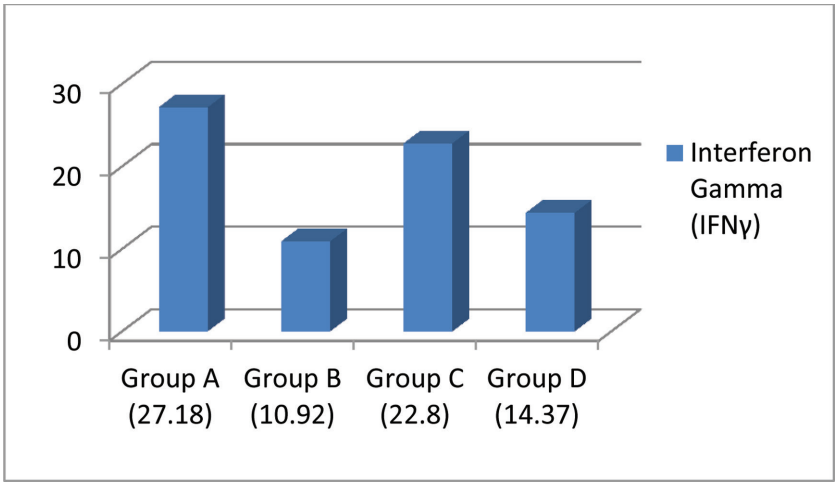

Figure 1 Mean of IFNγ between group

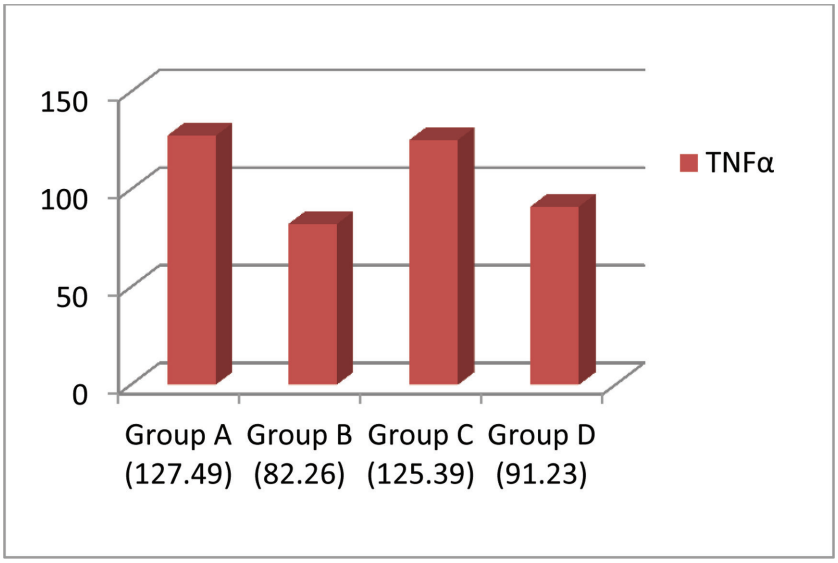

Figure 2 Mean of TNFα between group

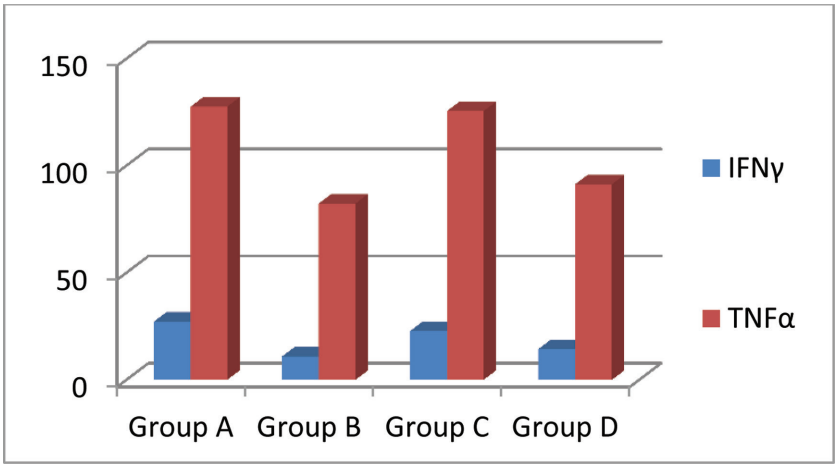

Figure 3 Comparison between IFNγ and TNFα

IFNγ is still in a lower level because the group did not experience *T. gondii* infection, although in a study by Suwanti (2005) which stated that in the first trimester the immunohistochemistry picture shows an increase in the number of IFNγ because the large IFNγ production in the embryo implantation process.[26]

T. gondii evokes a strong cellular immune response toward T helper-1 (Th1) T cells characterized by the creation of Th1 cytokines such as IFNγ and interleukin-2 (IL2).[10] Increased IFNγ as the induced response of cytokine Th1 to the fetal-maternal interface caused fetal rejection. IFNγ is produced by NK cells and CD8+ T cells. There were three alternative pathways of IFNγ synthesis as the host's response to *T. gondii* infection.[27] This was consistent with the results shown by this study where IFNγ in groups A and C was higher than in groups C and D.

T.gondii-infected macrophages produced IL12 which activated NK cells to produce IFNγ and T helper (Th) differentiation into Th1 cells that produce IFNγ and IL2. Macrophages expressed Major Histocompatibility Complex I (MHC I) so that it was captured by the Cytotoxic T Leucocyte receptor, CTL. The resulting cytokine IL2 induces CTL T cells to produce IFNγ. This could be seen in group C which showed IFNγ production was higher than group B and D.

IFNγ production could be conducted in 3 ways. First, *T. gondii* infection in macrophages stimulates macrophages producing IL12, TNFα, IL1, and IL15. IL12 cytokines with IL1β, IL15, and TNFα, stimulate NK cells to produce IFNγ. The cytokine IFNγ then activates the TNFα macrophages. IFNγ synergizes with TNFα induces the expression of intracellular nitric oxide synthase (iNOS), which produces nitric oxide (NO) to kill intracellular *T. gondii*.[27] Second, T. gondii infection in macrophages or APC encourages to produce IL12. APC presents a parasitic peptide through MHC II, so it is recognized by Th cells (CD4+ T cells). The Th cells binding to MHC II produce IL2. IL2 cytokines of Th and IL12 cells of APC induce differentiation from Th into Th1. Th1 cells produce IFNγ.[26] Third, infected macrophages or APCs T. gondii express MHC I recognized by CTL T cells. CTL T cell bonds with APC via MHC I and the presence of IL2 produced Th cells trigger CTL T cells resulting IFNγ.[27]

Rats infected with *T.gondii* showed an increase in the number of femur bone cells expressing TNFα. Previous studies of *T. gondii* infection increased the number of placental decidual macrophages,[26] muscle cells,[28] liver and lymph cells[29] expressing TNFα. Increased TNFα in *T. gondii* infection is a cascade of the body's immune response to eliminate parasites. In this experiments showed that there was an increase of TNFα concentration in serum especially in group A and group C, and it showed that TNFα was important

Published by DiscoverSys | *Bali Med J* 2019; 8(1): 94-100 | doi: 10.15562/bmj.v8i1.1316

Table 1 Test of Homogeneity of Variances

(I) Group TNFα	(J) Group TNFα	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	45.23200*	15.74722	0.007	13.0719	77.3921
	C	-10.68913	16.29993	0.517	-43.9780	22.5998
	D	36.26342*	14.63422	0.019	6.3764	66.1505
B	A	-45.23200*	15.74722	0.007	-77.3921	-13.0719
	C	-55.92113*	16.29993	0.002	-89.2100	-22.6322
	D	-8.96858	14.63422	0.545	-38.8556	20.9185
C	A	10.68913	16.29993	0.517	-22.5998	43.9780
	B	55.92113*	16.29993	0.002	22.6322	89.2100
	D	46.95255*	15.22737	0.004	15.8541	78.0510
D	A	-36.26342*	14.63422	0.019	-66.1505	-6.3764
	B	8.96858	14.63422	0.545	-20.9185	38.8556
	C	-46.95255*	15.22737	0.004	-78.0510	-15.8541

Table 2 Multiple comparison IFNγ

(I) Group IFNγ	(J) Group IFNγ	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	16.352250*	3.552591	0.000	9.09689	23.60761
	C	4.384125	3.677281	0.243	-3.12589	11.89414
	D	10.625761*	3.301495	0.003	3.88321	17.36831
B	A	-16.352250*	3.552591	0.000	-23.60761	-9.09689
	C	-11.968125*	3.677281	0.003	-19.47814	-4.45811
	D	-5.726489	3.301495	0.093	-12.46904	1.01606
C	A	-4.384125	3.677281	0.243	-11.89414	3.12589
	B	11.968125*	3.677281	0.003	4.45811	19.47814
	D	6.241636	3.435311	0.079	-.77421	13.25748
D	A	-10.625761*	3.301495	0.003	-17.36831	-3.88321
	B	5.726489	3.301495	0.093	-1.01606	12.46904
	C	-6.241636	3.435311	0.079	-13.25748	.77421

*. The mean difference is significant at the 0.05 level.

Table 4 ANOVA between and within group

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1236.242	3	412.081	8.163	0.000
Within Groups	1514.508	30	50.484		
Total	2750.751	33			

for immune response to eliminate *T.gondii*. This was consistent with the results of this study which showed that TNFα concentrations in group A and group C also increased with IFNγ increment and this function was used to eliminate *T. gondii* whereas in uninfected groups B and D had high TNFα concentrations. It could be stated that the administration of OHB in this study might increase the concentration of IFNγ and TNFα in serum to eliminate *T. gondii*.

Suwanti (2005) suggest that TNFα increased in conjunction with increased IFNγ in the placenta induce trophoblasts expressing Fas and stimulating Fas more sensitive to apoptosis.[26] This can cause the implantation process to run smoothly, whereas if apoptosis occurs excessively it will cause abortion. Rats in group A were infected and given HBOT, have high IFNγ and TNFα levels did not cause abortion in rats although we can see that very high

IFNγ and TNFα concentrations were compared with groups B and D. Inverted results were shown by group B which had IFNγ and TNFα concentrations are lower than group B.

The leukocyte population in the decidua is dominated by macrophages, NK cells, and CTL T cells, as well as Th cells. So if there is an infection in the decidua, the lymphocytes in the decidua are activated to produce IFNγ.[26] This is consistent with the results of this study which showed high IFNγ results in the group infected with tachyzoite in group C, especially the higher increase in group A given HBOT.

Plasmodium falciparum infection could increase the levels of IFNγ and IL-2 in the placenta. IFNγ and TNFα were associated with poor pregnancy outcomes in the form of fetal loss and weight of infants born small.[30] *P. falciparum* parasites are protozoa belonging to one ordo with T. gondii. Different results were shown by this study where increases in IFNγ and TNFα did not show an abortion during the study.

HBOT administration in this study not only works by increasing the amount of oxygen in the tissue but also produces H_2O_2 which can become a second messenger to activate NF-kB which ultimately can increase the activity of inflammatory responses such as TNFα and IFNγ. IFNγ gave protection against *Toxoplasma gondii* infection through STATI molecules in the JAK / STAT pathway.[31,32] IFNγ would induce INDO formation which then will degrade tryptophan in non-phagocytic cells and induce increased secretion of reactive oxygen intermediate (ROI), nitric oxide (NO) and reactive nitrogen intermediate (RNI) in phagocytic cells. Tryptophan degradation inhibits replication tachyzoite of *T. gondii*.[31] IFNγ also induced synthesis of IGTP and LRG-47 which could control the development of splenocytes.[32]

Tc / CD8 + cells activate NK cells and macrophages by producing a toxic ROI, NO or RNI for tachyzoites through IFNγ production.[10] The authors suggest that a high concentration of IFNγ in group A and C help the immune system to eliminate tachyzoite of *T.gondii*. The sequence of cytokines capable of causing tissue and organ damage when secreted in high quantities is IL-18, IFNγ, IL12 and TNFα.[33] So if the concentration of IFNγ and TNFα is very high and not in optimum concentration (group A, B, C, and D), abortion will happen. But in this case, the authors found that HBOT could increase IFNγ and TNFα concentrations without causing abortion. In this case, serum TNFα levels in group A were higher than in group C. This was in contrast to previous studies in foot ulcer patients with diabetes showing a decrease in TNFα levels in patients after HBOT administration.[34] In the study of giving HBOT therapy to other wounds also showed a reduction in inflammation with a decrease in TNFα, IL10, MMP9, and IL8.

The figure 3 showed that TNFα always followed the increase in IFNγ value but the rise of IFNγ and TNFα values in the four groups had different values, and it might have different implications for the elimination and protection of different powers possessed by *Rattus novergicus* against Toxoplasma gondii infection. There was an effect of giving HBOT to TNFα levels in pregnant *Rattus novergicus* infected with *T. gondii* which was significant between group A and group B ($p = 0.007$), group A with group D ($p = 0.019$), and group B with group C ($p = 0.002$). Even though it was obtained a p-value = 0.545 between group A and group C, it tended to be insignificant. It was found that the increase in the number of TNF α in group A compared to group C was quite significant with the improvement of clinical conditions of the rats.

As from the results of statistical tests, it was found there was an effect of giving HBOT to IFNγ levels in pregnant *Rattus novergicus* infected with *T. gondii* which was significant between group A with group B ($p = 0.000$), group A with group D ($p = 0.003$), and group B with group C ($p = 0.003$). It obtained $p = 0.243$ between group A and group C or it tended to be insignificant. It was found that the increase in the number of IFN group in group A compared to group C was quite significant with the improvement of clinical conditions of the rats and the most important was that the rats had no experience of abortion.

IFNγ and TNFα data were carried out by Pearson statistical test, and the results showed that p-value <0.041 which means there was a correlation between IFNγ and TNFα levels. Increased levels of IFNγ will be followed by TNFα to eliminate *Toxoplasma gondii* in the body of rats. Previous research stated that IFNγ and TNFα both singly and jointly inhibited multiplication and activate macrophage cells to eradicate tachyzoite and to prevent reactivation of bradyzoites.[2,10,31,35,36] According to Ceravolo et al. (1999), TNFα can impede tachyzoite multiplication up to 30%. The IFNγ could impede tachyzoite replication by 54% to 65%. Besides, the combination of IFNγ with TNFα could inhibit tachyzoite replication by 73%.[31] IFNγ also plays a role in the induction of switching from IgM to IgG2a which is very important for the immune response to toxoplasmosis.[22] So the administration of HBOT might provide a good influence for elimination and protection of *Toxoplasma gondii* in pregnant *Rattus novergicus*.

Based on ANOVA test, there was a significant correlation between hyperbaric oxygen therapy at IFNγ and TNFα concentrations in pregnant rats infected with *T. gondii* tachyzoite (between groups A,

B, C, D and within the group). So, the administration of HBOT could have a significant effect on tachyzoite infection in pregnant rats as indicated by p <0.000. During the study, there were no rats that experienced abortion or died while undergoing treatment, besides the results of intra-peritoneal fluid smears of rats group A and C (rats pregnant and infected with tachyzoite) were not found tachyzoite. Based on the results of this study, HBOT could improve the expressions of IFNγ and TNFα, in the provision of HBOT 2.4 ATA for 3x30 minutes with ten sessions in 5 days.

CONCLUSION

HBOT can improve the expressions of IFNγ and TNFα, 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.*

ACKNOWLEDGMENTS

Thanks to the study group of Toxoplasma gondii Faculty of Veterinary Medicine of Airlangga University who are willing to provide Tachyzoite sample for this research. Thank you also to the Department of Hyperbaric Faculty of Medicine Hang Tuah University Surabaya on permission to use Hyperbaric Chamber. Thanks to Bupati of Sidoarjo and Head of Public Health Office Sidoarjo for permission to study.

DISCLOSURE

The author reports no conflicts of interest in this work.

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