Toxoplasmosis: Experimental Vaginal Infection in NMRI Mice and Its Effect on Uterin, Placenta and Fetus Tissues

Parvin Dolk Bayat 1, Zahra Eslamirad 2,*, Saeedeh Shojaei 3

1 Department of Anatomy, Arak University of Medical Sciences, Arak, IR Iran
2 Department of Parasitology and Mycology, Arak University of Medical Sciences, Arak, IR Iran
3 Department of Medical Parasitology and Mycology, Tehran University of Medical Sciences, Tehran, IR Iran

*Corresponding author: Zahra Eslamirad, Department of Parasitology and Mycology, Arak University of Medical Sciences, Arak, IR Iran. Tel: +98-864473505, Fax: +98-864473521, E-mail: dr.eslamir@arakmu.ac.ir

Background: Toxoplasma gondii is an important zoonotic pathogen. Vertical transmission of the parasite occurs when females were infected primarily during gestation. This parasite is transmitted to the fetus through the placenta and may cause miscarriage, permanent neurological damage, premature birth and visual impairment. It has been found that mouse is susceptible to Toxoplasma and is particularly an interesting model to the study of congenital infection but whether the entry of Toxoplasma through vagina route is involved in transmission of the parasite to the placenta and fetus or not.

Materials and Methods: In the current experimental study, two 6-8 week NMRI female mice were crossed with one male. The pregnant mice were divided into 2 groups: experimental group that was infected by parasite via intra-vaginal (IV) and control group that received the same volume of normal saline via IV. One mouse from each group was killed on the fifth day after infection. The peritoneal fluid, ovary and uterus of mouse samples were taken and divided into two parts. One part used for PCR and the other was kept in formalin for histological study. These steps were repeated seven times and at least 10 mice in each group (case and control) were studied by molecular and histological methods.

Results: PCR using DNA extracted from the experimental group showed that the parasite existed in tissues of the uterus and placenta but not in the embryos and peritoneal fluid. PCR using DNA extracted from the control group was negative.

Conclusions: Tachyzoite of Toxoplasma and DNA of this parasite were observed in sub mucosa and muscles of the uterus and in the villi of placenta, but not in histological sections of the fetus. Therefore, histological and molecular results were consistent.

Keywords: Infection; Toxoplasma; Vaginal; NMRI Mice

1. Background

Toxoplasmosis is a parasitic disease caused by the protozoan Toxoplasma gondii. T. gondii is an important zoonotic pathogen and has worldwide distribution (1). This disease may result a spectrum of consequences including severe congenital defects, blindness or death (1). Ingesting tissue cysts from undercooked meat, drinking contaminated drinks, or occasionally ingesting oocysts from the environment are the ways for human infection. Although most infections with T. gondii in humans are asymptomatic but in patients such as those with acquired immunodeficiency syndrome, toxoplasmic encephalitis can be life threatening (1). Vertical transmission of the parasite occurs when females are infected primarily during gestation. At this time parasite can be transmitted to the fetus through the placenta and may cause miscarriage, permanent neurological damage, premature birth and visual impairment (2). Severe cases of congenital toxoplasmosis occurs with greater frequency when the mother is infected during the first two trimesters of pregnancy, when it is acquired later, symptoms tend to be subclinical or even lacking in the fetus (3). In recent years numerous animal models have been used to study the pathology of toxoplasmosis, efficacy of vaccines, new drugs for treatment of congenital transmission, chorioretinitis and toxoplasmic encephalitis. It has been found that mouse is susceptible to T. gondii and is particularly an interesting model to the study of congenital infection (4).

Implication for health policy/practice/research/medical education:
It has been proven that T. gondii penetrates into the bloodstream and spreads within the host, but direct entry of the parasite into the uterine tube, uterus tissue and subsequent placenta and fetus have not been studied.
in the correct position. The suspension was inoculated to vaginal canal of female mice by a liquid handling instrument on the 14th day of pregnancy.

3.4. Inoculation Procedure in Brief
Twenty microliters of suspension containing 2 million parasites were prepared. Mouse was kept by a person in the correct position. The suspension was inoculated to vaginal canal of female mice by a liquid handling instrument on the 14th day of pregnancy.

3.5. Sampling
Sampling began 5 days post infection. One mouse from each group was killed on the fifth day after infection. Ovary and uterus of mice were isolated and divided into two parts. One part used for DNA extraction and the other for histological study. This experiment was repeated seven times.

3.6. Tissue Preparation for Light Microscopy (LM)
Uterine tissue (5mm × 5mm), placenta and embryos (total) were fixed in 10% formalin for 48 h. The tissue was briefly washed with saline, and then this tissue passed through a series of ascending concentration of ethanol and rinsing with xylene. The samples were embedded in melted paraffin and the slices with diameter of 3-µm (total) were fixed in 10% formalin for 48 h. The tissue was processed with light microscopy (Nikon, Japan). All chemicals were purchased from Merck (Co. Germany).

3.7. DNA Extraction
Genomic DNA was extracted by utilizing an extraction DNA kit according to manufacturer’s instructions from blood and tissue samples (RTP Bacteria DNA Mini Kit, sterasec). This product was confirmed by electrophoresis on a 0.8% agarose gel.

3.8. Polymerase Chain Reaction (PCR)
Standard primers TOX4 and TOX5 were selected for polymerase chain reaction (PCR) (9). TOX4 (5’- CGCTGCAGGGAGGAAGACGAAAGTTG-3’)

3.3. Procedure of Infecting Animals
The number of parasites per microliter (µl) of suspension of parasite was counted. The mice were inoculated with 2 million parasites by the vagina. It should be noted that in the current study, different concentrations of parasites in identical volume of suspension were used (20 µl). Thus the parasite suspension to a final volume of 20 microliters were used for each mouse, due to the fact that increasing the volume of suspension (more than 20 µl) may lead to parasite penetration into the peritoneal cavity and increase the likelihood of parasite penetration into peritoneal cavity.

2. Objectives
Vaginal route was selected because it was desirable to select a route of infection which perhaps carried out under natural conditions in human.

3. Materials and Methods

3.1. Parasite
In the current experimental study, the RH strain of Toxoplasma gondii was kindly donated to us by Department of Parasitology, Faculty of Health, Tehran Medical Sciences University. Tachyzoites were maintained by serial intraperitoneal passage in young white mouse. Under this condition the mice were ill or dead after 3 to 5 days post infection (pi). Then, peritoneal fluid was collected. Tachyzoites were counted in a Neubauer chamber. These parasites were used for infection of experimental animals via intra-vaginal (IV).

3.2. Mice
All procedures in this study were conducted according to international regulations for animal experimentation and approved by the Institutional Medical Ethics Committee of Arak University of Medical Sciences, Arak, Iran (Accreditation No.90-609-JZ, July 2011). Passage of the parasite was done on young Balb/c mice. Vaginal infection was done on 6-8 week NMRI female mice (Pasteur Institute, Iran). All these mice were housed one per cage in an air-conditioned animal room at an ambient temperature of 23 °C, relative humidity ranging from 55 ± 60%, in a 12-h (on/off light) cycle and with free access to food and water. Female mice were cross with one male. Visible of vaginal plug designated as zero day of pregnancy (EO). The pregnant mice were divided into 2 groups: experimental group that infected by parasite via IV and control group that received the same volume of normal saline via IV. These steps were repeated fourteen times and at least 10 mice in each group (case and control) were studied by molecular and histological methods.

T. gondii for the first time during pregnancy (5). Arantes and Lopes studied artificial insemination of female dogs and rams respectively with T. gondii -positive seminal samples. Their results suggested that T. gondii may be sexually transmitted in domestic dogs and rams (6, 7). It has been proven that T. gondii penetrates in to the bloodstream and spreads within the host, but direct entry of the parasite through mucosa into genital organs has not been studied. Therefore the current study evaluated, whether the entry of T. gondii through vagina route is involved in direct crossing of the parasite into the uterine tube, uterus tissue and subsequent placenta and fetus or not. Then experimental animals were infected through vaginal route and the likelihood of direct transmission of parasite to the female genital tissue and placenta and fetus were studied.

3.3. Procedure of Infecting Animals
The number of parasites per microliter (µl) of suspension of parasite was counted. The mice were inoculated with 2 million parasites by the vagina. It should be noted that in the current study, different concentrations of parasites in identical volume of suspension were used (20 µl). Thus the parasite suspension to a final volume of 20 microliters were used for each mouse, due to the fact that increasing the volume of suspension (more than 20 µl) may lead to parasite penetration into the peritoneal cavity and increase the likelihood of parasite penetration into peritoneal cavity.

3.4. Inoculation Procedure in Brief
Twenty microliters of suspension containing 2 million parasites were prepared. Mouse was kept by a person in the correct position. The suspension was inoculated to vaginal canal of female mice by a liquid handling instrument on the 14th day of pregnancy.

3.5. Sampling
Sampling began 5 days post infection. One mouse from each group was killed on the fifth day after infection. The peritoneal fluid samples were taken before the death. Ovary and uterus of mice were isolated and divided into two parts. One part used for DNA extraction and the other were kept in formalin and sent for histological study. This experiment was repeated seven times.

3.6. Tissue Preparation for Light Microscopy (LM)
Uterine tissue (5mm × 5mm), placenta and embryos (total) were fixed in 10% formalin for 48 h. The tissue was briefly washed with saline, and then this tissue passed through a series of ascending concentration of ethanol and rinsing with xylene. The samples were embedded in melted paraffin and the slices with diameter of 3-µm were produced by microtome. The sections from each paraffin block were stained with Hematoxylin and Eosin (H&E) (8). Finally, sections were sectioned and were photographed with light microscopy (Nikon, Japan). All chemicals were purchased from Merck (Co. Germany).

3.7. DNA Extraction
Genomic DNA was extracted by utilizing an extraction DNA kit according to manufacturer’s instructions from blood and tissue samples (RTP Bacteria DNA Mini Kit, sterasec). This product was confirmed by electrophoresis on a 0.8% agarose gel.

3.8. Polymerase Chain Reaction (PCR)
Standard primers TOX4 and TOX5 were selected for polymerase chain reaction (PCR) (9). TOX4 (5’ - CGCTGCAGGGAGGAAGACGAAAGTTG-3’

www.SID.ir
TOX5 (5′-CGCTGCAGACACAGTGCATCTGGATT-3′)

After optimization of the PCR reaction for pH and MgCl₂ concentration, the PCR reaction was performed in a 25 µl reaction mixture containing 0.5 mM of each primer, 100 mM dNTP (Pharmacia Biotech), 60 mM Tris-HCl (pH 9.0), 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.5 U SuperTaq (Sphaero O). Amplification was performed on an Eppendorof thermocycler by 5 min incubation at 94 °C, followed by 35 cycles of 30 min at 94 °C, 30 min at 58 °C, 30 min at 72 °C and a final 10 min incubation at 72 °C. Accordingly, a 529 bp fragment was constructed. This product was confirmed by electrophoresis on a 0.8% agarose gel.

4. Results

4.1. PCR Results

PCR, using extracted DNA from uterine and placenta of experimental group showed an expected band (estimated 529 bp). But this band was not observed in PCR reactions using extracted DNA from peritoneal fluid and embryos of the same group. In other words, it seems that the parasite existed in tissues of the uterus and placenta but not in the embryos and peritoneal fluid (Figure 1). PCR using DNA extracted from the control group was negative.

4.2. Histologic Results

In the experimental group embryos were dead, although they were alive in control group. Evaluating by light microscope showed that normal saline treatment did not cause any tissue changes in the sections of control group which received it. But in the experimental group which received parasite, the parasite was observed in tissue, also the polymorphic inflammatory infiltration with some apoptic sites and congestion of vessels in both endometrium and myometrium of uterus was observed too (Figure 2).

Furthermore, in sub-mucous and myometrium, tachyzoite of *Toxoplasma gondii* were observed (Figure 3).

In the same group, in the villi of placenta vessels, congestion and polymorphic inflammatory infiltration was observed (Figure 4).

However, the parasite was not detected in fetal tissue sections and no changes were found in fetus tissues. It is noteworthy that in peritoneal fluid samples the parasite was not detected.
The Villi of Placenta With Vessels Congestion and Polymorphic Infiltration in placenta may improve our understanding about barrier structure of the placenta, as well as enhancing our understanding about how *T. gondii* infection affects the placenta and crosses from mother to fetus. Thus whether *T. gondii* infection to pregnant mice, uterus and placenta were infected to toxoplasmosis through the blood but result of the current study showed that the pregnant mice were infected to toxoplasmosis through the mucous of vagina. In the present study, *Toxoplasma gondii* were detected in sub-mucousa and muscular layer of uterus. Therefore, it is possible that parasite enters directly into uterus. Also no parasites were observed in the fetus, therefore it seems that placenta is a good barrier to prevent transmission of the parasite to the fetus, but it remains a question that why fetus are dead. Croy (2003) reported that in infected Swiss-Webster mice the factor possibly involved in resorption is spiral artery vasodilatation. IFN-γ is known to regulate spiral artery dilatation in non-infectious setting (17). Also in current study we observed spiral artery vasodilatation, therefore IFN-γ may be involved in fetal death. An important question remains and that is, Toxoplasma after vaginal insemination has directly entered into placenta or after entering the blood of the mother has penetrated into decidua and then into placenta. In conclusion, NMRI mice present a satisfactory model for research on congenital toxoplasmosis. So far, the study on direct passage of Toxoplasma through the mucosal tissues has not been performed. The current study showed that this parasite possibly can enter into tissue. However, it is recommended that a specific staining method be designed for *Toxoplasma gondii* in tissue. Thus differentiation of parasite from surrounding tissue is done better and more accurately.

### Acknowledgements

This study was emanated from a proposal (No. 607) approved and supported by Arak University of Medical Sciences. Thereby, authors thank Education and Research Department of Arak University. The authors would also...
like to thank Dr. Saeed Babaee for English review, Reza Hajihosein and Behzad Ghorbanzadeh for their technical assistance.

Authors’ Contributions
All authors had equal role in design, work, statistical analysis and manuscript writing.

Financial Disclosure
The authors declare that there exists no conflict of interest.

Funding/Support
The study is funded by Arak University of Medical Sciences.

References
1. Remington JS. Infectious Diseases Of The Fetus And Newborn Infant. 6 ed. Philadelphia: Elsevier Saunders; 2006. pp. 947-1091.
2. Garcia JL, Gennari SM, Navarro IT, Machado RZ, Sinhorini IL. Toxoplasma gondii: isolation of tachyzoites rhoptries and incorporation into Iscom. Exp Parasitol. 2004;108:1-6.
3. Boothroyd JC. Toxoplasma gondii: 25 years and 25 major advances for the field. Int J Parasitol. 2009;39:939-46.
4. Freyre A, Falcon J, Mendez J, Rodriguez A, Correa L, Gonzalez M. Refinement of the mouse model of congenital toxoplasmosis. Exp Parasitol. 2006;109:54-60.
5. Elsaid MM, Martins MS, Frezard F, Braga EM, Vitor RW. Vertical toxoplasmosis in a murine model. Protection after immunization with antigens of Toxoplasma gondii incorporated into liposomes. Mem Inst Oswaldo Cruz. 2001;96(4):99-104.
6. Arantes TF, Lopes WD, Ferreira RM, Pieroni JS, Pinto VM, Sakamoto CA, et al. Toxoplasma gondii: Evidence for the transmission by semen in dogs. Exp Parasitol. 2009;123(2):190-4.
7. Lopes WD, da Costa AJ, Santana LF, Dos Santos IS, Rossane WM, Lopes WC, et al. Aspects of toxoplasma infection on the reproductive system of experimentally infected rats (Ovis aries). J Parasitol Res. 2009;
8. Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc. 2008.2008;pdb prot4986.
9. Homann WL, Versammen M, De Braekeleer J, Verschueren H. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR. Int J Parasitol. 2000;30(1):69-75.
10. Pappas G, Roussos N, Falagas ME. Toxoplasmosis snapshots: global status of Toxoplasma gondii seroprevalence and implications for pregnancy and congenital toxoplasmosis. Int J Parasitol. 2009;39(12):1385-94.
11. Robbins JR, Zeldovich VB, Poulchanski A, Boothroyd JC, Bakardjiev AI. Tissue barriers of the human placenta to infection with Toxoplasma gondii. Infect Immun. 2002;70(2):248-28.
12. Pezerico SB, Langoni H, da Silva AV, da Silva RC. Evaluation of Toxoplasma gondii placental transmission in BALB/c mice model. Exp Parasitol. 2009;123(1):168-72.
13. Menzies FM, Henriquez FL, Roberts CW. Immunological control of congenital toxoplasmosis in the murine model. Immunol Lett. 2008;115(3):39.
14. Shiono Y, Mun HS, He N, Nakazaki Y, Fang H, Furuya M, et al. Maternal-fetal transmission of Toxoplasma gondii in interferon-gamma deficient pregnant mice. Parasitol Int. 2007;56(1):141-42.
15. Biaucourt A, Garcia AG. Pathogenesis and pathology of hematogenous infections of the fetus and newborn. Pediatr Pathol Mol Med. 2002;21(4):353-9.
16. Barragan A, Sibley LD. Migration of Toxoplasma gondii across biological barriers. Trends Microbiol. 2003;11(9):426-30.
17. Gray BA, Esadeg S, Chantakru S, van den Heuvel M, Paffaro VA, He H, et al. Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. J Reprod Immunol. 2003;59(2):275-91.