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Evaluation of antinuclear antibodies in pregnant women with abortion with toxocariasis and toxoplasmosis in Iran

İran'da toksokariyaz ve toksoplazmozlu düşük yapan gebelerde antinükleer antikorların değerlendirilmesi

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ABSTRACT

Objective: *Toxoplasma gondii*, *Toxocara* spp, and antinuclear antibodies (ANAs) have been reported as a cause of abortion. To determine the prevalence rate of T. gondii, *Toxocara* spp, and ANAs in aborted women, and to investigate the association between infection and the number of the sociodemographic estimated risk factors.

Methods: This study was carried out in aborted women referred to the Qaem hospital in Mashhad city (Razavi Khorasan Province). Serum samples were examined for the presence of Antinuclear Antibody (IgG antibodies), anti-*Toxoplasma gondii* IgG and IgM antibodies, and IgG anti-toxocariasis spp antibodies by ELISA (Enzyme-Linked Immunosorbent Assay) kit. Data like contact with animals' cats and dogs, age, and Type of cause of abortion (in most cases) were collected as risk factors for the prevalence of infection.

ÖZET

Amaç: *Toxoplasma gondii*, *Toxocara* spp ve antinükleer antikorlar (ANA'lar) düşük nedeni olarak rapor edilmiştir. Bu çalışmanın amacı düşük yapan kadınlarda *T. gondii*, *Toxocara* spp ve ANA prevalans oranını belirlemek ve enfeksiyon ile sosyodemografik olarak tahmin edilen risk faktörlerinin sayısı arasındaki ilişkiyi araştırmaktır.

Yöntem: Bu çalışma, Meşhed şehrinde (Razavi Horasan Eyaleti) Qaem hastanesine sevk edilen düşük yapan kadınlarda gerçekleştirildi. Serum numuneleri ELISA (Enzyme-Linked Immunosorbent Assay) kiti ile Antinükleer Antikor (IgG antikorları), anti-Toxoplasma gondii IgG ve IgM antikorları ve IgG anti-toksokariazis spp antikorlarının varlığı açısından incelendi. Kedi ve köpek gibi hayvanlarla temas, yaş ve düşük nedeni (çoğu vakada) gibi veriler enfeksiyon prevalansı için risk faktörleri olarak toplandı.

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Results: Out of 162 total samples of pregnant women were collected, 28% were positive for *T. gondii* IgG, and 2% IgM, anti-*Toxocara* spp IgG antibodies were detected in 12% and Antinuclear antibodies detected in 31% in pregnant women with abortion. Co-infection of *T. gondii* and *Toxocara* spp detected in 9%, also ANAs detected in cases with *T. gondii* in 24% and 10% with *Toxocara* spp. ANAs showed positivity with co-infection of *T. gondii* and *Toxocara* spp in 8%. This study showed a significant association between infection of each *T. gondii*, *Toxocara* spp, and ANAs with age and animal contact such as cats and dogs.

Conclusion: These study findings indicated that there is a relatively high prevalence of *T. gondii*, *Toxocara* spp, and ANAs in pregnant women with abortion. The coexistence of two agents or of one with ANAs increases the risk of abortion. All of them showed association with age and contact with domestic animals like cats and dogs.

Key Words: Antinuclear antibody, toxoplasmosis, toxocariasis, pregnant women, abortion

Bulgular: Toplam 162 gebeden alınan örneklerin %28'inde *T. gondii* IgG, %2'sinde IgM pozitifliği, %12'sinde anti-*Toxocara* spp IgG antikorları ve abortus yapan gebelerin % 31'inde Antinükleer antikorlar tespit edildi. Olguların %9'unda *T. gondii* ve *Toxocara* türlerinin ko-enfeksiyonu, *T. gondii* olgularında %24 ve *Toxocara* spp. olgularında %10 oranında ANA'lar tespit edildi. *T. gondii* ve *Toxocara* türlerinin ko-enfeksiyonunda ise ANA pozitifliği %8 oranında bulundu. Bu çalışma, hem *T. gondii*, hem *Toxocara* spp. enfeksiyonları ve hem de ANA'ların, olguların yaşı ve kedi, köpek gibi hayvanlarla teması arasında önemli bir ilişki olduğunu göstermiştir.

Sonuç: Bu çalışma bulguları, düşük yapan gebe kadınlarda *T. gondii*, *Toxocara* spp. ve ANA'ların prevalansının nispeten yüksek olduğunu göstermiştir. İki ajanın beraber veya her birinin ANA'larla birlikte bulunması düşük riskini artırır. Bunların hepsinin olguların yaşı ve kedi, köpek gibi evcil hayvanlarla temasıyla ilişkili olduğunu gösterdi.

Anahtar Kelimeler: Antinükleer antikor, toxoplasmosis, toxocariasis, hamile kadınlar, abortus

INTRODUCTION

Autoimmune disorders are most common in women 6-10 times than men (1). The number of autoantibodies reported as a cause of early pregnancy rejection in 30% of women (2). Among those antibodies, antinuclear antibodies (ANAs) are a group of autoantibodies that targeted nuclear and cytoplasmatic antigens (3). The immunologic linkage between the mother and the embryo is a pivotal factor for pregnancy completing (4, 5) and supposed that variation in this immunologic parameters can cause significant complications including recurrent abortion (6). The mechanism by which ANAs lead to abortion is not known well but according to a hypothesis, ANAs cause an inflammation in the uterus which be not convenient for implantation of the embryo(7). There is growing attention in investigating the association between autoimmune diseases and infection with parasites like *Toxoplasma gondii* and *Toxocara* spp (8, 9). Toxoplasmosis is a common zoonotic infectious disease caused by *Toxoplasma gondii* which recognized as the third most prevalent cause of mortality from foodborne infection, and the most common protozoan infectious agent causing abortion (10-12) and main infection route is through contact with infected cats' feces (13). Globally , toxocariasis is known as one of the most prevalent zoonotic infectious disease caused by the larval stages of *Toxocara canis* and *Toxocara cati*, the common roundworms of dogs and cats, respectively (14, 15). Humans can be infected by the occasional ingestion of embryonated *Toxocara* spp via contaminated soil or food (16, 17) or by the ingestion

of encapsulated larvae in the tissues of meat and/or raw or undercooked viscera of chicken, ducks, and cattle infected with *T. canis* larvae (18, 19). The prevalence of toxocariasis infection in pregnant women is infrequent, while in case of infection is possible to cause implications in reproductive health (20, 21). The present study was performed to investigate any potential association between *T. gondii*, *Toxocara* spp infection, and antinuclear antibodies by assessing the seroprevalence diagnosis and their estimated risk factors in pregnant women with abortion in Mashhad city Iran.

MATERIAL and METHOD

Sampling strategy

This study was performed from 26 June 2018 to 17 January 2018 in pregnant women with abortion referred to the Qaem hospital in Mashhad city (Razavi Khorasan Province).

Immunoserology

A total of 162 pregnant women with abortion (81 cases) and Healthy pregnant women (81 case) were enrolled in this study. All stages of the experiment were conducted in the specialized immunology laboratory. About 2-4 mL of whole blood samples were taken from each participant using venipuncture. The samples were allowed to clot and centrifuged at 1000 g for 3 minutes in order to the separation of sera (Because the samples were to be analyzed for the presence of Antinuclear Antibody (ANA), the samples were placed immediately at 4 °C after centrifugation). Samples were stable for 14 days at 4 °C and for 21 days at -20 °C for ANA detection (22). In addition, the hemolysis serum samples were not tested. Sera samples were screened for anti-*Toxoplasma gondii* (IgG, IgM), anti-*Toxocara* spp (IgG) antibodies, and Antinuclear Antibody (IgG) using ELISA kit (EUROIMMUN, Germany for Toxoplasmosis, Antinuclear Antibody (ANA), and IBL, Germany for Toxocariasis). The kit has sensitivity and specificity of >98%. According to the contents of the kit and the results of the ELISA reader (ELx800, manufactured by BioTek).

Statistical analysis

Data analysis for this study was performed using the SPSS software version 20 (SPSS, Chicago, IL, USA). Associations between the presence of Antinuclear Antibody (ANA), Toxocariasis, and Toxoplasmosiss were evaluated by Pearsons chi-square test.

The study was approved by the Zahedan University of Medical Sciences Regional Research Ethics Committee.(Date: 24.01.2021 and Number: IR.ZAUMS.REC.1399.487).

RESULTS

Seroprevalence of ANA

ANAs were detected in 31% of pregnant women with abortion. A positive ANA was recorded and showed a significant association with age of women (p=0.005), a positive correlation has been detected between infection rate and age. The causes of abortion have been reported and showed a significant association with ANAs (p=0.042). In addition to the role of contact with dogs or cats and the prevalence of infection (p=0.001) (Table 1). The incidence rate of infection varied in pregnant women with abortion and healthy pregnant women (Figure 1). also, the association between ANAs with the number of abortions (Figure 2).

Seroprevalence of *Toxoplasmosis*

Out of 81 pregnant women with abortion, 28 % showed specific IgG for *T. gondii*. With Negative IgM. The age of these women showed a significant association with the prevalence rate of infection (P=0.001). The causes of abortion showed association with Toxoplasmosis (P=0.033) especially endometrial cancer. Also, contact with cats was associated significantly (P=0.002) (Table 2), while IgM detected only in 2% with IgG Negative without significant association with age, cause of abortion, and animal contact. Also, 8% of cases showed positive for both IgG and IgM with a significant association with both cause of abortion and animal contact (P=0.045, 0.03 respectively).

Table 1. The presence of Antinuclear Antibody (ANA) in pregnant women with abortion

Variables	Samples N (%)	Positivity N(%)	Prevalence Ratio	P-value
Age				**0.005**
20-29	27 (33.34%)	05(16.13%)	1	
30-39	35 (43.20%)	11(35.48%)	1.05	
40-49	09 (11.11%)	05(16.13%)	2.1	
50 or more	10 (12.35%)	10(32.26%)	4.38	
Total	81 (100%)	31(100%)		
The cause of abortion				**0.042**
Hypertension	15(18.52)	05(16.14)	1	
Bleeding	16(19.76)	03(9.68)	0.63	
Molar Pregnancy	13(16.05)	04(12.90)	1.1	
Ectopic Pregnancy	11(13.58)	02(6.45)	0.62	
Abnormal uterine bleeding (AUB)	06(7.40)	03(9.68)	1.84	
Uterine fibroids	03(3.70)	01(3.22)	1.2	
Uterine rupture	09(11.11)	06(19.36)	2.6	
Endometriosis	01(1.23)	01(3.22)	3.6	
Placenta Accreta	02(2.47)	02(6.45)	3.7	
cervical cancer	02(2.47)	02(6.45)	3.7	
Endometrial cancer	02(2.47)	02(6.45)	3.7	
Other cause	01(1.24)	00(00)	0	
Contact with dog and cat				**0.001**
Yes	37(45.68)	25(80.65)	1	
No	44(54.32)	06(19.35)	0.34	

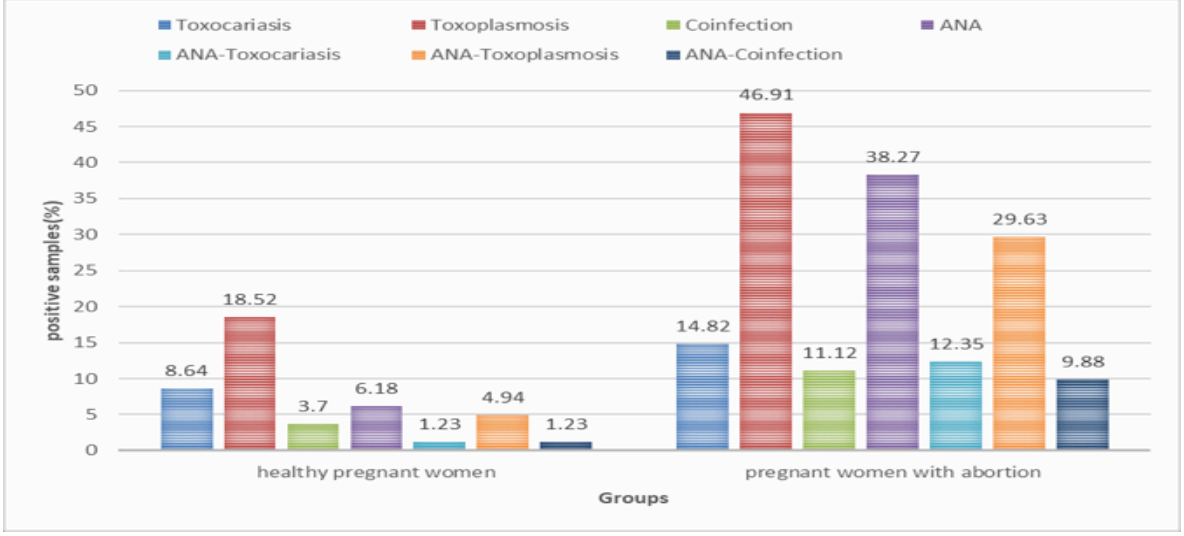

Figure 1. Comparison of infection prevalence and the presence of Antinuclear Antibody (ANA) in pregnant women with abortion and healthy pregnant women

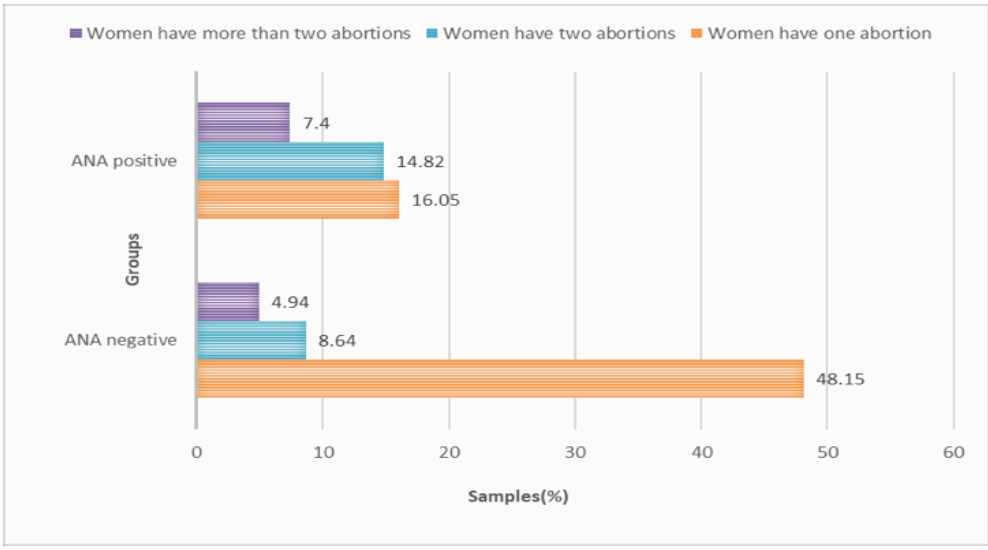

Figure 2. Analysis of all pregnant women with abortion for the presence or absence of Antinuclear Antibody (ANA) based on the number of abortions

Table 2. Prevalence of *Toxoplasmosis* (IgG positive and IgM negative) in pregnant women with abortion

Variables	Samples N (%)	Positivity N (%)	Prevalence Ratio	P-value
Age				**0.001**
20-29	27 (33.34)	04(14.28)	1	
30-39	35 (43.20)	08(28.56)	0.75	
40-49	09 (11.11)	06(21.43)	2.85	
50 or more	10 (12.35)	10(35.73)	4.95	
Total	81 (100)	28(100)		
The cause of abortion				**0.033**
Hypertension	15(18.52)	03(10.71)	1	
Bleeding	16(19.76)	04(14.30)	0.93	
Molar Pregnancy	13(16.05)	01(3.57)	0.27	
Ectopic Pregnancy	11(13.58)	03(10.71)	1.04	
Abnormal uterine bleeding (AUB)	06(7.40)	04(14.30)	2.75	
Uterine fibroids	03(3.70)	01(3.57)	1.30	
Uterine rupture	09(11.11)	07(25)	3.45	
Endometriosis	01(1.23)	01(3.57)	3.98	
cervical cancer	02(2.47)	01(3.57)	2.02	
Endometrial cancer	02(2.47)	02(7.13)	4.03	
Adnexa of uterus	01(1.24)	01(3.57)	3.97	
Other cause	02(2.47)	00(00)	0	
Contact with dog and cat				**0.002**
Yes	37(45.68)	25(89.29)	1	
No	44(54.32)	03(10.71)	0.2	

Seroprevalence of *Toxocariasis*

Out of 81 pregnant women with abortion, 12 % showed specific IgG for *Toxocara* spp. The age of these women showed a significant association with the prevalence rate of infection (P=0.037). Also, contact with cats or dogs was associated significantly (P=0.004) (Table 3).

Seroprevalence of co-infection between Toxocariasis and Toxoplasmosis Seropositivity of Toxocariasis and Toxoplasmosis co-infection were detected in 9% of pregnant women with abortion. (Table 4) shows there was a significant relation between co-infection with age (P=0.014) and contact with an animal like cats or dogs shows a significant association, while the cause of abortion is not associated with co-infection.

Table 3. Prevalence of *Toxocariasis* infection (IgG positive) in pregnant women with abortion

Variables	Samples N (%)	Positivity N (%)	Prevalence Ratio	P-value
Age				**0.037**
20-29	27 (33.34)	01(8.33)	1	
30-39	35 (43.20)	05(41.65)	1.08	
40-49	09 (11.11)	03(25.01)	3	
50 or more	10 (12.35)	03(25.01)	2.7	
Total	81 (100)	12(100)		
The cause of abortion				**0.451**
Bleeding	16(19.76)	03(25)	1	
Molar Pregnancy	13(16.05)	01(8.33)	0.55	
Ectopic Pregnancy	11(13.58)	01(8.33)	0.67	
Abnormal uterine bleeding (AUB)	06(7.40)	02(16.68)	2.84	
Uterine rupture	09(11.11)	03(25)	3	
Placenta Accreta	02(2.47)	01(8.33)	4.09	
Adnexa of uterus	01(1.24)	01(8.33)	8.27	
Other cause	23(28.39)	00(00)	0	
Contact with dog and cat				**0.004**
Yes	37(45.68)	08(100)	1	
No	44(54.32)	00(00)	0	

Seroprevalence of ANAs with *Toxoplasmosis*

Seropositivity for ANAs was detected in addition to the presence of *T. gondii* in 24% of women with abortion. (Table 5) shows there was a significant association with age, cause of abortion especially cervical cancer and Endometrial cancer, and contact with an animal like cats or dogs also shows significant relation (P= 0.002, 0.004,0.001) respectively.

Seroprevalence of ANAs with *Toxocariasis*

(Table 6) shows that 10% of pregnant women with abortion have seropositivity for both ANAs and Toxocariasis. Significant relation of infection with age and animal contact was reported (P=0.009, 0.002 respectively). The cause of abortion did not show a significant association, but abortion due to Abnormal uterine bleeding (AUB) shows the highest prevalence cause.

Seroprevalence of ANAs with co-infection of (Toxocariasis and Toxoplasmosis) Seroprevalence (Table 7). It had been found that the positivity of co-infection and ANAs in 8% of pregnant women with abortion. In addition to showing the significant relation with age with a high prevalence rate in age group (40-49) and animal contact (P=0.003, 0.004 respectively).

Table 4. Prevalence of *Toxoplasmosis* and *Toxocariasis* infection in pregnant women with abortion

Variables	Samples N (%)	Positivity N (%)	Prevalence Ratio	P-value
Age				**0.014**
20-29	27 (33.34)	00(00)	0	
30-39	35 (43.20)	04(44.44)	1	
40-49	09 (11.11)	02(22.22)	2.5	
50 or more	10 (12.35)	03(33.34)	4.3	
Total	81 (100)	09(100)		
The cause of abortion				**0.301**
Bleeding	16(19.76)	01(11.11)	1	
Ectopic Pregnancy	11(13.58)	01(11.11)	0.9	
Abnormal uterine bleeding (AUB)	06(7.40)	02(22.22)	3.9	
Uterine rupture	09(11.11)	03(33.34)	4.4	
Placenta Accreta	02(2.47)	01(11.11)	5.3	
Adnexa of uterus	01(1.24)	01(11.11)	6.6	
Other cause	36(44.44)	00(00)	0	
Contact with dog and cat				**0.003**
Yes	37(45.68)	09(100)	1	
No	44(54.32)	00(00)	0	

Table 5. The presence of Antinuclear Antibody (ANA) and *Toxoplasmosis* in pregnant women with abortion

Variables	Samples N (%)	Positivity N (%)	Prevalence Ratio	P-value
Age				**0.002**
20-29	27 (33.34)	06(25)	1	
30-39	35 (43.20)	03(12.50)	0.35	
40-49	09 (11.11)	05(20.83)	2.7	
50 or more	10 (12.35)	10(41.67)	6.1	
Total	81 (100)	24(100)		
The cause of abortion				**0.004**
Hypertension	15(18.52)	02(8.34)	1	
Bleeding	16(19.76)	02(8.34)	0.5	
Molar Pregnancy	13(16.05)	02(8.34)	0.6	
Ectopic Pregnancy	11(13.58)	02(8.34)	0.75	
Abnormal uterine bleeding (AUB)	06(7.40)	03(12.48)	2.3	
Uterine fibroids	03(3.70)	01(4.16)	1.45	
Uterine rupture	09(11.11)	06(25)	3.3	
Endometriosis	01(1.23)	01(4.16)	4.2	
Placenta Accreta	02(2.47)	01(4.16)	2.4	
cervical cancer	02(2.47)	02(8.34)	4.5	
Endometrial cancer	02(2.47)	02(8.34)	4.5	
Other cause	01(1.24)	00(00)	0	
Contact with dog and cat				**0.001**
Yes	37(45.68)	23(95.84)	1	
No	44(54.32)	01(4.16)	0.06	

Table 6. The presence of Antinuclear Antibody (ANA) and *Toxocariasis* infection in pregnant women with abortion

Variables	Samples N (%)	Positivity N (%)	Prevalence Ratio	P-value
Age				**0.009**
20-29	27 (33.34)	00(00)	0	
30-39	35 (43.20)	04(40)	1	
40-49	09 (11.11)	03(30)	3.8	
50 or more	10 (12.35)	03(30)	3.3	
Total	81 (100)	10 (100)		
The cause of abortion				**0.601**
Bleeding	16(19.76)	02(20)	1	
Molar Pregnancy	13(16.05)	01(10)	0.66	
Ectopic Pregnancy	11(13.58)	01(10)	0.8	
Abnormal uterine bleeding (AUB)	06(7.40)	02(20)	3.45	
Uterine rupture	09(11.11)	03(30)	4.3	
Placenta Accreta	02(2.47)	01(10)	4.9	
Other cause	24(29.63)	00(00)	0	
Contact with dog and cat				**0.002**
Yes	37(45.68)	10(100)	1	
No	44(54.32)	00(00)	0	

Table 7. The presence of Antinuclear Antibody (ANA) and Coinfection in pregnant women with abortion

Variables	Samples N (%)	Positivity N (%)	Prevalence Ratio	P-value
Age				**0.003**
20-29	27 (33.34)	00(00)	0	
30-39	35 (43.20)	02(25)	1	
40-49	09 (11.11)	03(37.50)	5.1	
50 or more	10 (12.35)	03(37.50)	4.5	
Total	81 (100)	08 (100)		
The cause of abortion				**0.317**
Bleeding	16(19.76)	01(12.50)	1	
Ectopic Pregnancy	11(13.58)	01(12.50)	1	
Abnormal uterine bleeding (AUB)	06(7.40)	02(25)	4.5	
Uterine rupture	09(11.11)	03(37.50)	4.95	
Placenta Accreta	02(2.47)	01(12.50)	5.8	
Other cause	37(45.68)	00(00)	0	
Contact with dog and cat				**0.004**
Yes	37(45.68)	08(100)	1	
No	44(54.32)	00(00)	0	

DISCUSSION and CONCLUSION

Abortion is a public health problem, currently, the clinical and experimental research studying the reasons for abortion has been increased as a try to solve this problem (23). In this study, we investigated the seroprevalence of each antinuclear antibodies, Toxoplasmosis, and Toxocariasis among healthy pregnant women and pregnant women with the abortion of Mashhad city. Immunological responses may lead to infertility and miscarriage in a number of cases. Any disruption in the normal immunological association between mother and conceptus is well acted as the main risk factor for abortion. ANAs positivity reported as one of the autoimmune factors that causing 30% of fetal rejection cases (24). Shoenfeld et al in 2006 reported the association between autoimmune disorders in pregnant women and their history of recurrent abortion (25). Among parasitic infections, toxoplasmosis and toxocariasis are recognized as globally distributed zoonotic diseases (26).Toxoplasmosis in pregnant women has a hazardous effect it may cause abortion. A number of previous studies have reported the association between *T. gondii* infection with unexplained abortion (27, 28). In this study, we estimated the prevalence rate of IgG for *T. gondii* in 162 (81 healthy pregnant women, 81pregnant women with abortion) in Mashhad, Iran. In agreement with several studies in Iran, the prevalence of *T. gondii* was 31-32% were reported (29-31). As shown in our results section, 28% of women were IgG positive which was less than last reports from Iran(>40%) (32, 33). Toxocariasis infection in pregnant women has a harmful effect which may reach to pregnancy loss. The overall *Toxocara* IgG prevalence among pregnant women with abortion in our study was 12%, which is lower than that of a previous study conducted in Ilam province in western Iran, IgG antibody was detected in (17.99 %) (34) and higher than the study conducted in Brazil which reported infection only in 6.4% of pregnant women with abortion (35). A number of variables have shown their association with Antinuclear antibody (ANA) and infection of *Toxoplasma gondii* and *Toxocara* spp and such as age, domestic animal contact, and cause of abortion. Our study findings showed a significant association between age and ANAs positivity with high prevalence in the groups (50 or more) and (40-49) with (4.38, 2.1 fold respectively). In agreement with Satoh et al in 2012 , ANA positivity associated with age, infection peak at age 40 to 49 years, (36) and seroprevalence of *T. gondii* IgG with age (37) was reported. Also in agreement with several studies that reported an increase in seroprevalence of *T. gondii* IgG with age(38-40). Our study findings showed a significant association between *Toxocara* IgG and age. Domestic animal contact such as cats and dogs has been recognized as the main transmission route for infection of both *T. gondii* and *Toxocara* spp (41, 42). In agreement with Lappin in 2010 IgM for *T. gondii* was detected only in 2% of pregnant women with abortion was reported (41). Our study results showed that contact with domestic animals such as cats or dogs significantly associated with seroprevalence of *T. gondii* IgG. In agreement with Cong et al in 2014, the association between toxocariasis and contact with dogs and cats was observed (42). Our study finding showed that the seroprevalence of *Toxocara* IgG was significantly associated with domestic animal contact. In addition to our study findings showed a significant association of ANAs with domestic animal contact. Abortion causes varied and a number of them showed association with variables that have been studied in the present study. Seroprevalence of *T. gondii* IgG in our study showed a statically significant association of this infection with abortion causes especially Endometrial cancer, Endometriosis, and Adnexa of uterus respectively. Also, Seroprevalence of *Toxocara* spp in this study showed association with both bleeding and uterine rupture as an abortion cause without significant association. *Toxoplasma gondii* and *Toxocara* spp are very different parasites, one a protozoan and the other a helminth, both organisms sharing the same transmission through contaminated

soil. Cats and dogs both excrete feces in soil and humans ingest soil during work (43). According to a 2017 study, the prevalence of *T. gondii* and *Toxocara* spp had similar risk factors equal to the present study(20). ANAs positivity was detected in 24% of pregnant women with abortion with seropositive *T. gondii* IgG, a significant association with age, cause of abortion, and contact with cats have been observed. Similarly, ANAs positivity was detected in 10% of pregnant women with abortion with seropositive *Toxocara* spp, association with age, and contact with dogs and cats . In addition to the presence of ANAs in cases with co-infection of *T. gondii* and *Toxocara* spp in 8%, relation with age and contact with the animal are statically significant.

In conclusion, the current study revealed that 31%, 28%, 12% of Antinuclear Antibodies, *Toxoplasma gondii*, *Toxocara* spp respectively. These study findings indicated that there is a relatively high prevalence of *T. gondii*, *Toxocara* spp, and ANAs in pregnant women with abortion. The coexistence of two agents or of one with ANAs increases the risk of abortion. All of them showed association with age and contact with domestic animals like cats and dogs.

ETHICS COMITTEE APPROVAL

* The study was approved by the Zahedan University of Medical Sciences Regional Research Ethics Committee. (Date: 24.01.2021 and Number: IR.ZAUMS.REC.1399.487).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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