

The Role of Immunologic Factors In Abortions Observed in Sheep and Goats

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KEY WORDS: Abortion, Immunology, Sheep, Goat

ABSTRACT

Abortions and the economic losses they bring about is an important issue in sheep and goat husbandary. Despite many studies conducted on sheep and goats, the underlying reasons behind abortions could not be identified. In the present study, the role of immunologic factors in abortions seen among sheep and goats has been evaluated. The frequencies of anti-sperm antibodies (ASA), anti-zona pellucida antibodies (AZPA), and anti-ovary antibodies (AOA) in the sera of sheep and goats who suffered abortion or had healthy births were determined. The study group consisted of 133 sheep and 101 goats of various breeds and ages that had suffered abortion, and the control group consisted of 101 sheep and 131 goats that had healthy births; 10 ml blood was taken from each subject once. Anti-zona pellucida antibodies in sera were

determined by commercial ELISA test kits. Anti-sperm antibodies and AOS were also detected by the ELISA test, which had been previously developed and modified by various investigators. Eventually, the obtained data were compared between the groups. In conclusion, the frequencies of ASA, AZPA, and AOA in sheep were detected as 0.85%, 0.43%, and 1.23% respectively and the same frequencies in goats were 1.29%, 0.00%, and 0.86%, again respectively. Whereas ASA were 0.75% and AOA were 1.50% in sera of sheep that suffered abortion, ASA and AOA values in sera of goats that suffered abortion were 1.98% and 1.98%, respectively. Anti-zona pellucida antibodies could not be determined in blood sera of animals (both sheep and goats) that suffered abortion. Moreover, in blood sera of both sheep and goats that suffered abortion or had healthy birth, no statistically significant difference regarding the frequency of those 3 antibodies was detected.

INTRODUCTION

Despite all the studies and investigations, no significant success has been achieved in the prevention of abortions in sheep and goat husbandry. Alongside a high number of bacterial, viral, and parasitic agents that cause abortion, the failure to achieve a sufficient vaccination regimen against those agents plays an important role as well. However, abortive pathology may still occur in cases where all the precautions are taken and all diagnostic laboratory methods are used. Consequently, there is a trend towards utilizing precautions and measures not just against the common causes of abortion, but also for conditions occurring as a result of immunologic factors. For this reason, investigations should be conducted on abortions of immunologic pathology along with studies performed on common viral, bacterial, and parasitic causes of abortion in animals such as sheep and goats, which show a high frequency of abortion. At this point, the associations between humoral and cellular immune systems and abortions should be evaluated separately. Generally, studies on the association between reproductive and humoral immunity, including the antibody-antigen reactions, have been based on a wide array of events ranging from fertilization to abortion. Among the events occurring in the reproductive system as a result of antigen-antibody reactions, ASA, AZPA, and AOA can be identified as playing an important role.^{1,2}

Despite many studies conducted on the formation of ASA, AZPA, and AOA and their effects on fertility, particularly in animals, only a few investigations have been performed on associations between abortion and those antibodies.

Anti-sperm antibodies have been found in the blood sera of females that suffered abortion. Those studies suggest that these antibodies have to reach high concentrations in order to cause an abortion. There are various hypotheses on how ASA cause loss of pregnancy. One such hypothesis proposes that several spermatozoa in the ejaculate contain

antibodies that lead to prevention and loss of pregnancy. Another hypothesis proposes that certain animals have sperm antigens in their placenta and /or on their blastocytes and that those act as inhibitors of embryonic development. Moreover, it's possible that ASA could develop against embryonic antigens and cause several disruptions in the immunosuppressive system.^{3,4} Naz⁵ reported abnormal embryonic cleavage in the in-vitro fertilization of females that exhibited ASA presence in their sera. Yamamoto et al⁶ determined that antibodies forming against spermatozoons cause congenital malformation in pregnant mice.

Furthermore, ASA has been reported to form and have an elevated titer in abortions associated with such bacteria in animals that experienced experimental or natural *Brucella*.^{7,8}

Currently, the structural characteristics of Zona Pellucida (ZP) have been observed in many mammalian, and the glycoproteins of ZP are known to be 40-90% similar in all the animals. ZP glycoproteins obtained from pigs are used in studies on ZP, particularly in human medicine. While the homology between human and pig ZPs is shown to be 78.79%, it is known as 84.3% between cow and pig ZPs.^{9,10}

Natural formation of those antibodies against ZP glycoproteins in females is explained by autoimmunity. Because ZP forms during the final phases of ontogenesis, it bears a strong antigenic property. Moreover, due to its potential for a high level of absorption, autoimmunity occurring against this protein is recognized as normal.¹¹ However, the extent to which those antibodies have an effect over the reproductive activities of animals, and the reason they are not effective on all the animals remains unknown.

Rote et al¹² reported that recurrent abortions might be associated with AZPA.

The ovary was thought previously to be either an immune target or an organ with autoantigens.¹³⁻¹⁵ Esfendiari et al¹³, reported presence of AOA in 3 sheep that had exper-

rienced ovary transplantation. The previous studies showed that these antibodies exercise a negative influence over reproduction by: 1. producing a negative effect on follicle functions and oocyte maturation, 2. closing the oocyte surface and inhibiting the sperm penetration to ZP, 3. destroying ZP with regard to cytotoxic effects, 4. blocking blastocyte hatching from ZP, 5. inhibiting implantation.¹³⁻¹⁵ Despite past inquiries, no data could be found in the literature on association of AOA and abortions seen among animals.

In the present study, we aimed to determine the ASA, AZPA and AOA frequencies in sheep and goats that suffered abortion, without investigating the viral and parasitic factors.

MATERIALS AND METHODS

In the present study, 10 ml of blood was drawn once from 133 sheep that aborted (age range: 2-8 years; 100 Akkaraman, 33 Awassi breed) and 101 goats that aborted (age range: 2-6 years; 11 Damascus goat, 90 domestic crossbreds). Again, 10 ml of blood was drawn for once from the control group including 101 sheep that gave healthy births (age range: 2-8 years; 77 Akkaraman, 24 Awassi breed) and 131 goats that again gave healthy births (age range: 2-7 years; domestic crossbreds). Sera were separated from the blood samples following routine procedures and those sera were kept at -20 °C until the date of the test.

Analysis of Anti-Zona Pellucida Antibodies

The presence of AZPA in sera of animals was determined by commercial ELISA test kits (DRG Instruments GmbH, Anti-Zona Pellucida Antibodies ELISA, Germany).¹⁶

Analysis of Anti-Ovarian Antibodies, Extraction of Protein and ELISA

Protein was extracted from homogenized normal ovaries with a commercial kit (EZ-RNA total RNA-DNA-Protein isolation kit; Biological Industries Co., Israel). The concentrations of obtained protein were determined according to the instructions of the Bradford Assay (SIGMA, Co., St. Louis,

MO, USA). The ELISA was adapted from Risvanli et al.¹⁷ The ovarian homogenate was diluted to a pH of 9.6 carbonate buffer to a concentration of 100 mg/ml. 100 ul of this solution was added to every second well of a 96 well microtitre tray and incubated overnight at +4°C. Plain carbonate buffer (pH 9.6) was added to the remaining wells. All wells were then emptied and washed with 100 ul/well PBS containing 0.02% tween-20 to block non-specific binding, 100 ul of 10% horse serum was added to each well and incubated for 2 h. The plate was re-washed and 100 ul ovine/goat serum diluted 1/200 with carbonate buffer was added to all pits. After 2 h at RT the plate was re-washed and peroxidase labeled ovine/goat anti-Rat IgG (SIGMA, Co., St. Louis, MO, USA) diluted 1/5000 and left for 2 h at RT. Following a further 6 washes, chromogen solution (0.1 M citrate-phosphate buffer containing 1 mg/mL O-phenyldiamine and 0.003% H₂O₂) was added to each well and left for fifteen minutes in the dark at RT. Dropping 100 ul 1M H₂SO₄ to each well stopped the reaction. The plate was read at 450 nm in an ELISA reader (BIO-TEK ELX800). All serum samples were tested in duplicate. As for the AOA, the OD values higher than the identified cut off values according to the 5x standard deviation of absorbance values that obtained from negative serum examples in ELISA were assessed as seropositive.

During the determination of AOA, 5 male animals for each were used as negative controls.

Analysis of Anti-Sperm Antibodies

The presence of sperm antibodies was determined by ELISA.¹⁸ Washings determined the presence of sperm antibodies and dilutions were made with phosphate-buffered saline (PBS) containing 0.02% Tween-20, pH 7.2. Incubations were carried out at room temperature for 1 h. Antigen was prepared by washing ram and billy goat ejaculate twice in PBS and then re-suspending the sperm at a concentration of 5x10⁶ spermatozoon/mL, in PBS containing 0.25% gluteraldehyde. One hundred microlitres of

this suspension was added to each of the test wells of 96- well plate coated with poly-L-lysine (100 µg/mL) in 0.01 M bicarbonate coating buffer. As a negative control, PBS containing only 0.25% glutaraldehyde was put in some wells. Blockings were done to the wells with the addition of 100 mL of PBS containing 10% horse serum albumin (Sigma Chemical. Co., St. Louis, MO.). Serum samples were diluted 1 to 50 in PBS and were tested in duplicate. As a secondary antibody, ovine/goat anti-rat IgG conjugated with horseradish peroxidase (SIGMA, Co., St. Louis, MO, USA) was used. Following a further 6 washes, chromogen solution (0.1 M citrate-phosphate buffer containing 1 mg/mL O-phenyldiamine and 0.003% H₂O₂) was added to each well and left for 15 minutes in the dark at RT. The reactions were stopped with addition of 100 mL of 1 M H₂SO₄ to the wells, and absorbance was determined at 450 nm wave length (BIO-TEK ELX800). The mean absorbance value of negative sera plus 5 standard deviations were considered as cut off value point for a positive response. The results obtained were distributed, and the frequencies of the aforementioned antibodies were defined in light of the statistical calculations.

Statistical Analyses

Fisher's chi-square test was employed for statistical assessment of the results.¹⁹ Because the frequency in sera exhibiting no antibodies (AZPA in sheep with abortion, AZPA in goats with abortion, AZPA in goat control group, and AOA in goat control group) was zero, statistical analyses couldn't be carried out.

Table 1. Distribution of results within sheep.

Antibodies	Sheep				Total (n=234)	P	
	Aborted (n=133)		Control (n=101)				
ASA	1	0.75	1	0.99	2	0.85	-
AZPA	0	0.00	1	0.99	1	0.43	
AOA	2	1.50	1	0.99	3	1.28	-

- No significant differences between groups ($P > 0.05$).

ASA: Anti-sperm antibodies, AZPA: Anti-zona pellucida antibodies, AOA: Anti-ovary antibodies.

RESULTS

Distribution of the results obtained from sheep and goat sera is shown in Table 1 and 2. No statistically significant difference was found with regard to the analyzed 3 antibodies between the animals that gave healthy birth, constituting the control group, and the animals who suffered abortion ($P > 0.05$). While the frequencies of ASA, AZPA, and AOA in sheep were 0.85%, 0.43% and 1.23% respectively; the same frequencies in goats were 1.29%, 0.00% and 0.86% respectively.

DISCUSSION

The continuation of a pregnancy depends on the maternal immune system, which develops a defense mechanism against fetal antigens. Chhabra and Ohri²⁰ reported that with regard to this immune system, trophoblast and sperm antigens stimulate macrophages and lymphocytes leading to formation of a toxic environment for the embryo that may cause recurrent abortions. The development and continuation of pregnancy in ruminants depends on the relation between the immune system and the conceptus. Within scope of this relationship, both anti-sperm and anti-conceptus immune responses play a role.² Therefore, while investigating the causes of abortions in ruminants, immune factors should be considered.

Erguven et al²¹ determined ASA in 28.3% of 63 female patients who suffered habitual abortion, spontaneous abortion, and intra-uterine fetal death. Their study reported that, in addition to other factors, these antibodies might be the reason behind early abortions.

In a study conducted on blood sera of 683 females, ASA was found to be lower in healthy females. Therefore, antibodies were proposed as factors playing a role in recurrent abortions.²² More-

Table 2: Distribution of results within goats.

Antibodies	Goat						P
	Aborted (n=101)		Control (n=131)		Total (n=232)		
	n	%	n	%	n	%	
ASA	2	1.98	1	0.76	3	1.29	-
AZPA	0	0.00	0	0.00	0	0.00	
AOA	2	1.98	0	0.00	2	0.86	

- No significant differences between groups ($P > 0.05$).

ASA: Anti-sperm antibodies, AZPA: Anti-zona pellucida antibodies, AOA: Anti-ovary antibodies.

over, in a study conducted by Zhang²³, ASA has been reported as one of the important causes of recurrent spontaneous abortion in females. Daya²⁴ reported that while investigating the causes of recurrent spontaneous abortions, the ASA in blood sera might be useful in finding the reason(s) for abortions. Anti-sperm antibodies may be used as an indicator of deficiencies in maternal genital canal immunosuppressor mechanisms. Consequently, recurrent abortions occurring as a result of these antibodies may be treated with immunotherapy.²⁵

However, investigators claim that ASA does not have a significant role in pregnancy loss, and further comprehensive studies are required in order to reveal the activities of those antibodies.²⁶ Check et al²⁷ reported that ASA does not play a very important role in etiology of spontaneous abortions.

In a study applying ELISA conducted by Waziri and Fayemi²⁸ on 235 goats, 15.2% of females and 9.8% of males were found to be positive for ASA. Another study by Fayemi²⁹ performed on 1021 billy goats showed 47.21% of animals were positive for ASA. In the same study, the rate of ASA-positive animals among animals positive for Trypanosoma infection was 62.12%. Moreover, Trypanosoma infection rate was 75.12% among ASA-positive animals. Due to those results, the authors proposed an association between Trypanosoma infestations and ASA formation. While Paolicchi et al⁸ determined ASA was present in 85.7% of all rams experimentally inoculated with Brucella ovis via conjunctival route, the same rate was 71.4% for rams inoculated with Brucella

ovis via preputial route. In light of those results, they reported that genital lesions associated with Brucella ovis may cause ASA in rams. In the present study, while ASA was found to exist in 1.29% of 232 goats included in the investigation, this rate elevated to 1.98% in animals that suffered abortion. Those rates were 0.85% and 0.75% for sheep, respectively. Previous studies in the literature reported that conditions such as Brucellosis and Trypanosoma infestations increase the formation of ASA.^{8,29} Moreover, there are many hypotheses on effects of ASA over reproductive activities and formation mechanisms. In the present study, the frequencies of those antibodies were determined without considering the viral and parasitic causes of the abortions in animals and without applying any experimental procedures.

Rote et al¹² reported that AZPA might have a role in recurrent abortions. In the present study, AZPA was detected in only 1 sheep, which had a normal birth; none of the goats included in the study has exhibited this antibody. In one study, pregnant mice subjected to injection of anti-testicle sera obtained from rabbits showed a fetal malformation frequency of 27.3%. Congenital disorders seen in pregnant mice subjected to injection of anti-testicle sera are believed to be associated with antibody development against spermatozooids.⁶ Despite all of these investigations, no study on association of AOA and abortion in animals was located by the authors.

In conclusion, ASA, AZPA, and AOA were determined to be present at low levels in the

blood sera of sheep and goats included in the present study. No difference was determined with regard to frequencies of the aforementioned antibodies between animals that suffered abortion and animals that had healthy births. Finally, the authors conclude that evaluation of abortion rates, which continue to be an important issue in sheep and goat husbandry, should include immunologic factors, and further investigations should be designed.

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