Molecular Study of INF gamma Gene in Aborted Placenta Induced by Brucella Melitensis in Ewes

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Abstract. Brucella melitensis is an important cause of ovine abortion in several sheep rearing countries, The immunological control of the pathogen in the placenta or by the fetus could be the key to determine the mechanism of abortion and/or transplacental transmission to the fet. This work was performed to detect INF gamma gene sequence in the DNA of AL-ousi breed, and in association with ovine abortion caused by Brucella melitensis. The cytokine gene expression, analyzed by PCR, was performed to explore the immunological regulatory mechanisms of IFN-γ on and its effect on abortion. The results indicated the presence of amplified fragment around ~340 bp in length compared to the ~55 bp that was predicted. Also, the level of DNA expression was the highest in placenta of normal delivered group and the lowest in aborted placental.

Introduction

Interferon gamma (INF-γ) is a pleiotrophic cytokine which plays an important central role in immunity against intra cellular pathogen. Brucella is an obligate intracellular gram-negative bacterium. It causes abortion in ruminants and humans. The ability of the host to mount a cell-mediated response is important in immunological control of this organism, so that protection is strongly associated with gamma interferon (IFN-γ) production.

In humans abortion results from primary gestational infection after direct contact with infected ruminant material. However, primary gestational infection or a persistent, subclinical infection established prior to pregnancy can be the causes of abortion in ruminant.

This link between disease and pregnancy represents the ability of the organism to exploit the immunological and/or physiological status of the pregnant host during this period. The semiallogeneic fetus, is not rejected by the maternal immune system indicating that this immune system does not react in a manner predicted by the self-non self model of immune activation. In term this prompts the suggestion that the maternal immune system is suppressed during pregnancy.

On the other hand, it has subsequently been shown that there is immune modulation during pregnancy which is manifested by a decreased production of IFN-γ and a bias towards Th2-type or regulatory cytokines in the placenta. This production of inflammatory cytokines, particularly at the maternofetal interface, is incompatible with successful pregnancy.
Trophoblast cells are a key component of the placenta. They form an interface between the fetus and mother. Certain intracellular mechanisms operate to regulate the trophoblastic response to cytokine exposure. By this, the critical balance of invasiveness into maternal decidua is maintained while avoiding immunological rejection. There is both in vitro and in vivo evidences that IFN-γ is unable to induce class II expression in trophoblast cells. The immunological control of pathogens at the maternofetal interface is different from that in the periphery. Together, the intracellular and extracellular regulatory mechanisms that are in place to prevent maternal allo-rejection render this tissue particularly susceptible to invasion by certain intracellular organisms, such as *brucella spp*, *C. abortus* and *Toxoplasma gondii*. The presence of inflammatory cytokines, such as tumor necrosis factor alpha, interleukin 2, and IFN-γ, can lead to abortion (23).

It is therefore possible that abortion mediated by infectious agents represents a form of immunological amputation of the infected fetal unit for the preservation of the mother (8).

**Material and Methods**

A total number of 12 placenta were collected from ewes at AL-Najaf city including 8 aborted and 4 term delivered placenta. Fresh placental tissue was subjected to total DNA extracted using the Genomic DNA Mini Extraction Kit tissue (KAPA BIOSYSTEM, USA) following the manufacturer’s instructions. Absorbance at 260 nm and 280 nm was measured.

DNA concentration was calculated according to equation. The purity was measured by calculating the ratio of absorbance at 260 nm to that at 280 nm. Two methods of amplification were conducted, namely conventional PCR and RT-PCR and the results of both were compared. The KAPA 2G Robust hot start ready mix (KAPA BIOSYSTEM USA) was used for amplification in the conventional PCR methods. The primers sequences were as followed: the forward primer: 5’- GAA TAC CTG GAC TAT GCC GA -3’, the reverse primer ‘- CC TCA CTT CCC TAC ATC CCT -3’.

Primers sequences for IFN-γ were according to Smeed *et al.* [10] and according to the Gene Bank database of the National Center for Biotechnology Information (X52640).

Experiments of primer optimization for both types of PCR techniques were first conducted.

The conventional PCR amplification was performed in a 25-μL final reaction volume. The reaction mixture included 3 μL template DNA, 0.4 μL of each forward and reverse primers, 4 μL (50 x) PCR mix and 20 μL nuclease free water. The conventional PCR was run in the VeriTeeg thermal cycler, applied biosystem using the following reaction conditions: an initial single denaturation at 95°C for 10 min, followed by denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec and elongation at 72°C for 10 min with a final extension at 72°C for 5 min. The amplification was completed after 35 cycles. The PCR products were separated by electrophoresis using 1% agarose gel and were revealed by ethidium bromide staining. The fragment length was suspected to appear at a size of 55 bp.

The RT-PCR amplification were run in 20 μL containing 10 μL KAPA SYBER FAST qpcr Master Mix (2 X), 0.4 L forward primer (10 M), 0.4 L Reverse primer (10 M), 0.4 dUTP (10 M), 0.4 ROX High/Low, 0.4 μL KAPA RT Mix (50 x), and 3 μL template DNA made up to 20 μL with deionized water. The cycling conditions for all genes were as follow: 10 min at 95°C, 40 cycles of 30 seconds at 95°C, 1 min at 55°C, and 1 min at 72°C followed by a melt curve starting at 65°C rising to 94°C at 0.3 per second.
Results:

The results of conventional PCR revealed amplification product in all sample of placenta obtained from aborted except one and normally delivered placenta except one as well. However, the expected band was not detected at the 55 bp size. Instead it was detected in the size of about 340 bp. figure 1

![Figure 1](image)

Figure (1) The DNA amplification products of INF gamma in the placenta from different aborted and normal delivered ewes amplified by conventional PCR. Lane 1: 2 kb DNA ladder marker Lane 2,3,4,5: PCR amplification product of INF gamma in aborted placenta; Lane 6,7,8: PCR amplification product of INF gamma in normal delivered placenta. The product size is 340 bp.

Real time PCR results revealed amplification of all the samples as well. The Ct values of samples ranged from 18.65 to 24.97 and only one sample showed no Ct value. Those for normal delivered placenta ranged from 16.85 to 18.48 and only one sample did not show Ct value. Table (1)

| No | Normal placenta | Aborted placenta |
|----|-----------------|------------------|
| 1  | 16.85           | 18.65            |
| 2  | 17.23           | 19.23            |
| 3  | 18.48           | 21.67            |
| 4  | No Ct           | 22.10            |
| 5  | No Ct           |                  |

Discussion

IFN-γ in restricted quantities is considered one of the essential factors for healthy pregnancy (24). It is essential for the maintenance of the decidual cells integrity and the opening of the implantation window (1) and in low doses it is beneficial for the progress of pregnancy (1).

However, over expression of IFN-γ during pregnancy is detrimental. Some studies reported that IFN-γ could inhibit the secretion of progesterone (18), induction of apoptosis of placental cells and the expression of placental antigen MHC could also be mediated by IFN-γ (26).

In the present study, amplification of IFN gamma gene was first conducted by conventional PCR. However, the presence of a larger amplification products than the expected one made the results questionable. For this reason the RT-PCR was conducted using the same primer set. The same samples yielded amplification in the RT-PCR experiment. The range of Ct values was nearly similar in both the normal deliveries and aborted placenta DNA samples. This suggests that all the samples except one in
each group yielded good DNA amount and the presence of the gene was documented in all. The difference in the size of the amplification products of the conventional PCR necessitates doing further molecular studies like polymorphism studies and gene sequence to determine the presence or not of a mutation. Expression studies are also important to rule out IFN gamma as an agent to enhance or mediated abortion.

Further analyses are also required to define the molecular mechanisms involved in IFN-γ-stimulated abortion. IFN-γ may trigger an autocrine cascade in trophoblast giant cells. In mouse, IFN regulatory factor – 1(IRF-1) is highly expressed in the mouse trophoblast [1, 17]. This transcription factor is induced by INF gamma and has the function of activating the promoters of many interferon-regulated genes [17], in turn, these determine the responsiveness of the trophoblast to INF gamma. Many studies showed the presence of INF gamma producing cells in the pregnant uterus (2, 10, 4), and suggest that INF gamma can be beneficial to pregnancy if secreted at appropriate times, concentration and location (2).

At low dose (100 IU/MI) INF gamma increases total cell numbers in cultured ectoplacental cones and promotes their differentiation. INF gamma has been associated with direct effects on the viability of cultured term trophoblast of human source (28). However, INF gamma is considered an abortion-inducing factor in high doses (22).

In mice, high, in vivo doses of INF gamma (3x 10^5 IU/mL) were shown to be deleterious to early embryos (15, 5) and in vitro treatments reduced trophoblast outgrowth, limiting invasive potential. Mechanism participating in INF gamma induced fetal death have not been clearly defined and its quite probable that other types of cells, in addition to trophoblast cells, participate in the in vivo outcome.

Clark et al. found [6, 7] that an abnormal increase in endogenous IFN-γ (1,000 IU) combined with high TNF-α (2,000 IU) on gd 7.5 led to abortion within 48 h. These cytokine levels correlated with strong expression of fgf2 prothrombinase in decidua as well as in trophoblast suggesting a maternal vascular etiology with thrombosis and ischemia. It is therefore likely that the concentration of IFN-γ, its balance with other pro- and anti-inflammatory cytokines, and the stage of gestation at which is, produced are fundamental in defining whether IFN-γ plays a physiological or pathological role during pregnancy.

IFN-gamma was the cytokine that showed the highest upregulation at the materno-foetal interface in infected animals, especially in the caruncle. The role of this cytokine in the maternal placenta would have a crucial importance in the transmission of the pathogen to the fetus. In naturally infected animals, production of IFN-gamma has been related to protection against abortion (21), although as indicated previously, excess of IFN-gamma could result in death by exaggerated immunopathological reactions.

A precise balance of immunological mechanisms prevents rejection of the fetus by the maternal immune system (13). Any disturbance of this equilibrium, As what happens when there is production of the pro-inflammatory cytokines IFN-α and TNF-alpha at the materno-fetal interface. This in turn suggests that the ovine fetus is especially susceptible to pathogens, such as Brucella, that elicit a host pro-inflammatory immune response (13).

Thus, abortion appears to be triggered by a fetal cell mediated immune response to Brucella, provoked by the predilection of Brucella, for chorionic epithelial cells, leading to the destruction of these cells and to the release of LPS. The toxic sequelae include placental thrombosis and infarction, and the destruction of chorionic epithelial cells leads to disruption of the hormonal control of pregnancy.

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