



## **Tumour necrosis factor- $\alpha$ (TNF- $\alpha$ ) in mycotic aborted placenta : an evaluation by Immunohistochemistry**

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### **Abstract:**

the present study aims to study histopathological changes in the mycotic aborted and normal placenta of sheep ,in addition to study the levels of TNF-a in mycotic aborted and normal placenta by immunohistochemistry assay (IHC)

Seventy five , samples of aborted placenta and twenty five of normal placenta were collected , during November 2017 to May 2018 from certain regions of AL-Najaf city .

Placental samples were fixed in 10% neutral buffer formalin for 72 hrs for histopathological examination, paraffin embedded tissue belong to mycotic aborted and normal group were used for assessment of TNF alpha by immunohistopathological study was carried out to determine TNF protein in the placenta sample.

Histopathological examination showed suppurative necrotized placentitis, in addition to congestion of the blood vessels and fibrin deposition in the inter-villous space as well as calcium deposition in necrotized the necrotic area and fibrosis of stromal area in certain chorionic villi and sloughing and desquamation of syncytiotrophoblast.

Result of IHC of TNF alpha protein showed significant elevation in TNF alpha protein in inflammatory cell (macrophage) of aborted sheep placenta (increasing of intensity and score), while no changes could be observed for pro-inflammatory molecules in the control sample (normal delivery).

**Key words :** TNF alpha - sheep – fungal - abortion - immunohistochemistry

### **تقييم (TNF- $\alpha$ ) في المشيمة المجهضة بسبب الاصابات الفطرية بواسطة الكيمياء المناعية**

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### **الخلاصة :**

الدراسة تناولت دراسة التغيرات المرضية في مشيمة هذه الحيوانات ,بالإضافة إلى معرفة التغيرات الجينية لمورث ( TNF alpha) باستعمال تقنية ( immunohistochemistry) في نماذج مشائم الأغنام المجهضة للإصابات الفطرية و مشائم الأغنام الوالدة طبيعياً .

جمعت 75 عينة من مشائم الأغنام المجهضة و 25 من مشائم الأغنام الوالدة طبيعياً في محافظة النجف الاشراف للفترة من تشرين الثاني/2017 ولغاية شهر ايار/2018 . أرسلت النماذج إلى مختبر الأمراض /كلية الطب/جامعة بغداد .

استعمل النسيج المجمد المأخوذ من مشيمة الحيوانات المجهضة والوالدة طبيعياً لدراسة (TNF alpha) عن طريق معرفة المعلومات ولتحديد كمية وغزارة بروتين (TNF alpha) المنتج من مشيمة الحيوانات المجهضة والوالدة طبيعياً بطريقة (IHC). أظهرت التغيرات المرضية في مشيمة الأغنام المجهضة للإصابات الفطرية وجود التهاب فيحي مشيمي متموت فضلاً عن احتقان الأوعية الدموية وترسب الكالسيوم في أنسجة المشيمة , كذلك وجود انسلاخ تموتي لخلايا الارومة المخلاوية التغذوية (syncytiotrophoblast) في أنسجة مشيمة الحيوانات المجهضة .

نتائج فحص (IHC) لبروتين (TNF alpha) تم تسجيل زيادة ملحوظة في بروتين (TNF alpha) في الخلايا الالتهابية (البلعمية) العائدة إلى مشائم الحيوانات المجهضة، في حين لم تكن هنالك أي تغيرات ممكنة حاصتها في زيادة كمية إنتاج هذا البروتين في مشائم الأغنام الولادة طبيعياً.

**الكلمات المفتاحية:** TNF- $\alpha$  . أجهاض . فطريات . أغنام . كيمياء مناعية

## Introduction

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is known to be one of the most versatile cytokines. It serves as a normal mediator of tissue homeostasis, it has pathophysiological effects at high concentrations and it is expressed in various tissues. (1)

TNF- $\alpha$  is secreted from a variety of gestational cells such as placental fragments, amnion and chorion-decidua, upon stimulation with bacteria or lipopolysaccharides. TNF- $\alpha$  stimulates macrophages to produce IL-12 that activates NK cells, NK cells produce INF- $\delta$  that causes differentiation of Th0 into Th1 which produce INF- $\delta$ . these cytokines induces destruction of trophoblast and lead to abortion. (2).

However, there are different etiology that induce abortion such as stress factors, pharmaceuticals, nutritional deficiency and toxic plant, in addition to biological agents, bacteria, viruses, parasites and mycotic agents (3).

To get a better understanding of the role of TNF- $\alpha$  in mycotic abortion in sheep, we investigated the expression of this cytokine using immunohistopathological techniques in addition to study the pathological lesions in mycotic aborted placenta.

## Materials And Methods

The present work was carried out at the unit of medical genetics and genes, Department of Pathology, College of Medicine.

### Samples :

Seventy five (75) samples of aborted sheep placenta were collected from different areas of Al-Nagaf city, during November 2017 to May 2018, these samples were divided into histopathological and immunohistochemical study.

### Histopathological examination:

Histological examinations were performed on placenta, that collected during the course of the study, these samples were fixed in 10% buffered formalin for 72 hrs, the samples were routinely processed and stained with Haematoxylin and Eosin (HE)

for histopathological examination according to (4).

### Immunohistochemical analysis for the detection of TNF alpha antigen in paraffin embedded sections

Paraffin- embedded sections from each specimen were cut at 4  $\mu$ m, mounted on glass and dried overnight at 37°C. The tissue sections were deparaffinized in xylene (2×10 min) and dehydrated through graded alcohol (100 % , 2× 5 min) and 95 % and 70 % for 5 min). Endogenous peroxidase activity was blocked using (1.5 %) H<sub>2</sub>O<sub>2</sub> for 10 minutes.

Tissue samples were heated to retrieve antigens in citric buffer (pH 9.0) at 100 °C for 10 min. The sections were incubated with mouse monoclonal antibody against TNF alpha (diluted 1:50 over night at 37 °C. Then sections were washed in buffer solution and covered with Biotinylated secondary antibody for 2 hours at 37°C, AB enzyme was applied for 30 min at 37°C.

Further processing of the sections for detection was performed using the dextran-polymer method and diaminobenzidine (DAB; Sigma). After being washed, the sections were counter stained with Mayer's hematoxylin, washed in water, and successively immersed in graded ethanol solutions and xylene before cover slipping. All samples were processed under the same conditions.

### Scoring:

When counting the number of positive cells in the staining tissues samples, at least 10 high-power fields were chosen randomly on each section. Additionally, the number of macrophage was counted in the fields. All immunostained sections were examined by the same two observers with a ×400 objective under the light microscope (Olympus Bx50; Olympus Optical Co, Ltd, Tokyo, Japan) for evaluating TNF alpha expressions.

In the abortion and normal delivery specimens TNF alpha expression in

macrophages was evaluated by counting 100 cells of each section. TNF alpha expression was quantitatively assessed as 0 (no stained cells), score 1 (from 1-25 positive cells), score 2 (from 26-50 positive cells), score 3 (from 51-75 positive cells) and score 4 (from 75 and over). The intensity was scored as 0 (absence), (low), (moderate), or (high). The pattern and intensity of staining in the different cell types of placenta samples was evaluated by two independent observers using a light microscope at a magnification of 200 (20 x objective and 10 x ocular).

The presence of lesions in the placental samples was investigated in formalin fixed tissue samples embedded in paraffin from those cases that were tested positive for abortion (tables 3.1 and 3.2). In cases where serial sections were used for H&E and IHC section comparisons could be made between lesion sites and IHC labeling. Histological evaluation of the placenta was compromised occasional necrosis of endothelial cells.

However, immunohistochemical labeling allowed the identification of TNF alpha antigen in all cases studied. Lesions were visible in all samples, after the likely lesion sites were identified by the IHC labeling.

## Results and Discussion

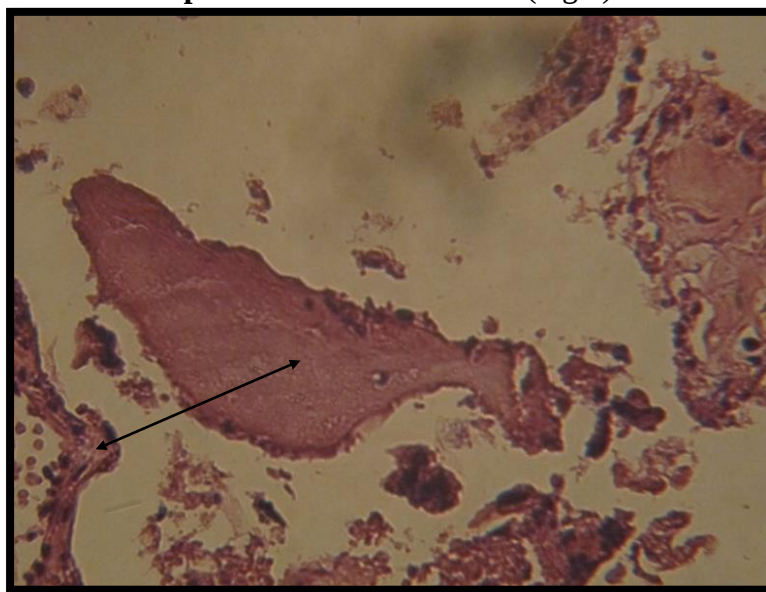
### Macroscopic appearance of the placenta

Cotyledons of the placenta expressed congestion, necrosis, and opacity. No clear gross lesions were noticed in the examined organs of the fetus. These findings are in agreement with (4) who reported haemorrhagic necrotizing placentitis associated with thickened necrotic cotyledons in abortion of cattle.

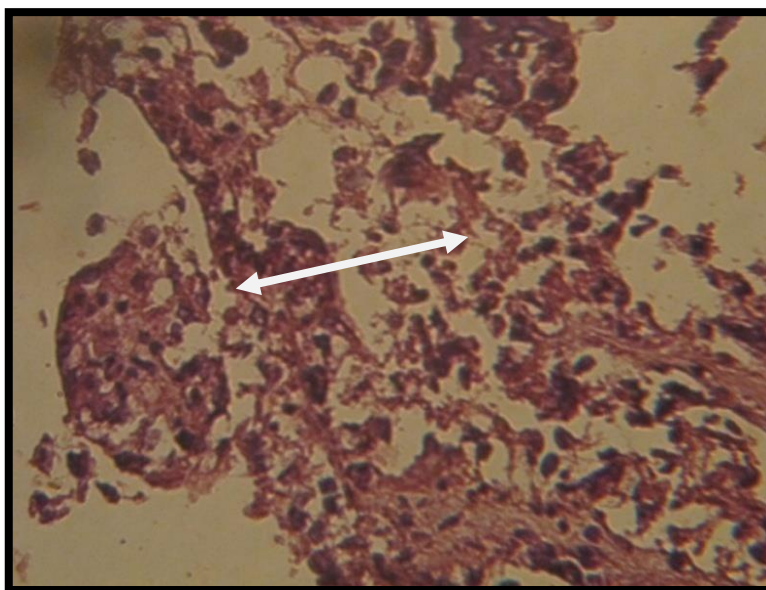
### Histopathological Examination:-

The presence of lesions in the placental samples was investigated in formalin fixed tissue samples embedded in paraffin from those cases that had tested positive for abortion.

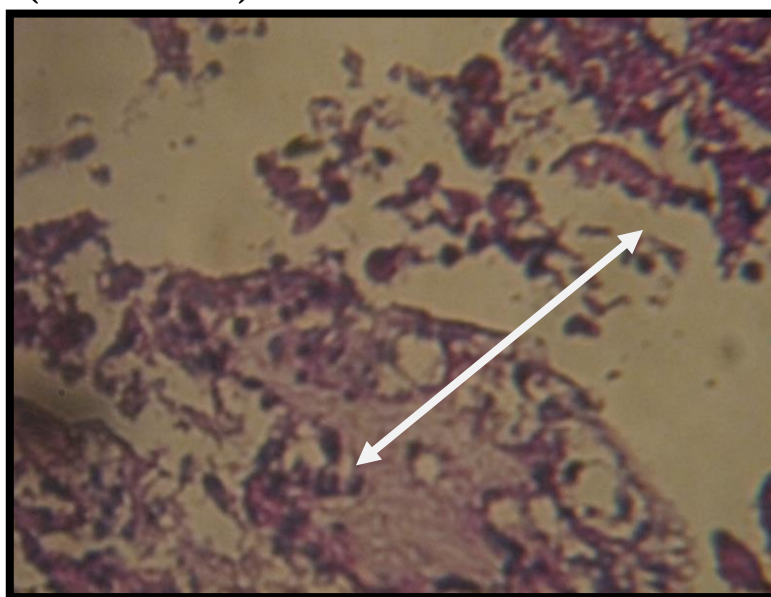
The main lesions were characterized by necrosis of chorionic villi with congestion of the blood vessels (Fig:1) as well as necrosis and sloughing of trophoblastic cells of the chorionic villi with neutrophils infiltration in the villous stroma (Fig:2). In another section, it was reported congestion of blood vessels with neutrophils in their lumen in the chorionic villi stroma (Fig:3) and in the necrotic area of the chorionic villi (Fig:4), necrosis of trophoblasts with fibrin deposition with inflammatory cells infiltrating in the intervillous space were seen (Fig:5). In other animals, congestion of blood vessels with neutrophils in their lumen and calcium deposition in the placental plate were noticed (Fig:6).



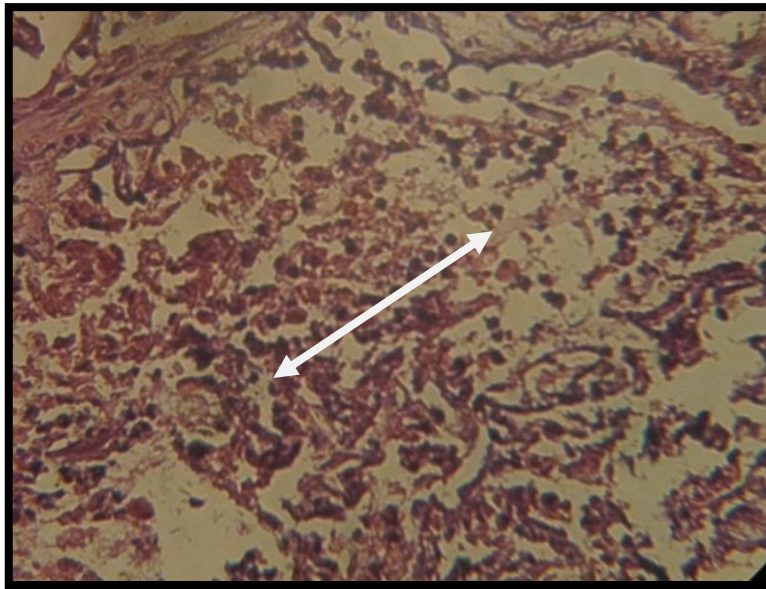
**Fig:1. Histopathological section in the placenta of aborted sheep shows necrosis of chorionic villi with congestion of the blood vessels (H&E stain 40X).**



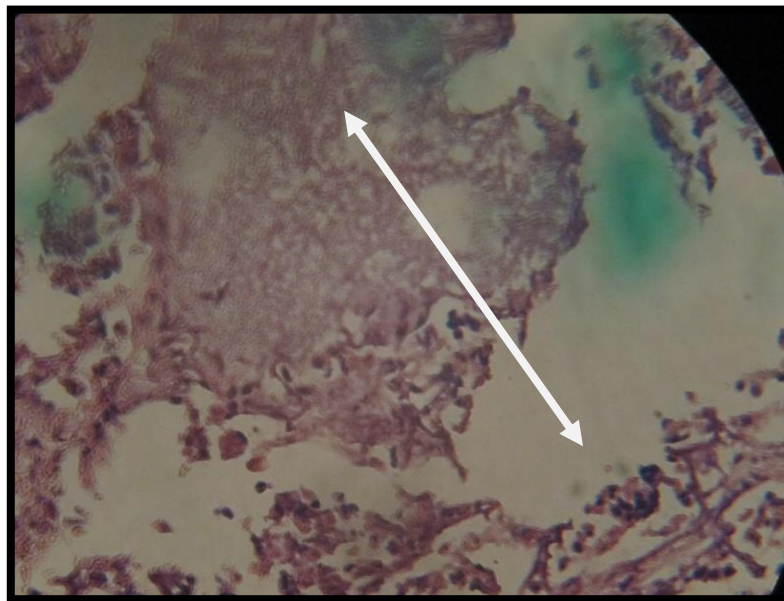
**Fig:2.Histopathological section in the placenta of aborted sheep shows necrosis and sloughing of trophoblastic cells of the chorionic villi with neutrophils infiltration in the villous stroma (H&Estain 40X).**



**Fig:3.Histopathological section in the placenta of aborted sheep shows congestion of blood vessels with neutrophils in their lumen in the chorionic villi stroma (H&Estain 40X).**

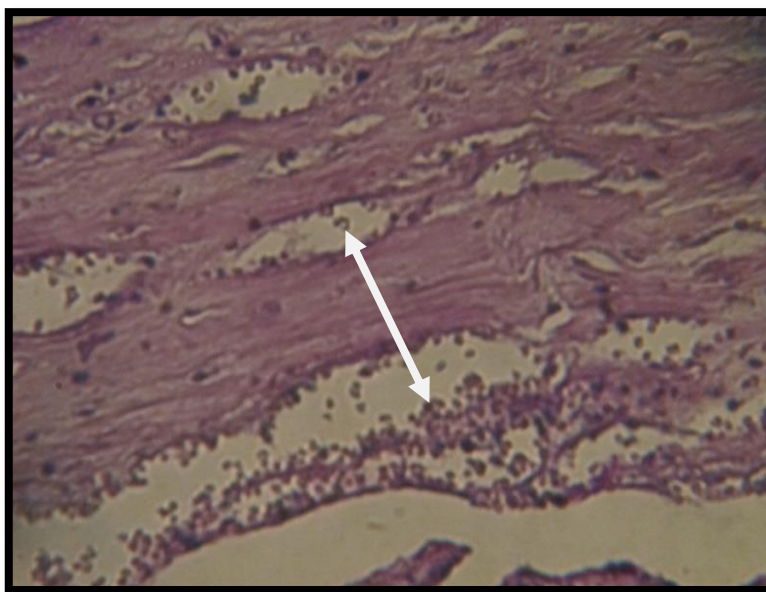


**Fig:4.**Histopathological section in the placenta of aborted sheep shows severe necrosis of cotyledon ,congestion of blood vessels with neutrophils infiltration in the necrotic area (H&Estain 40X).



**Fig:5** Histopathological section in the placenta of aborted sheep shows necrosis of trophoblasts with fibrin deposition in the chorionic villi with inflammatory cells infiltratin in the intervillous space (H&E stain 40X).





**Fig:6 Histopathological section in the placenta of aborted sheep shows congestion of blood vessels ,with neutrophils in their lumen in the plate connective tissue (H&E stain 40X)**

Presence of necrosis of chorionic villi with neutrophils infiltration was demonstrated. This result may be due to thrombus that occluded the blood stream and lead to ischemia and necrosis . Necrotic tissue attracts neutrophils (5) .

The presence of dystrophic calcification in necrotic placentitis( Fig 6) agrees with the results of (6 )who showed calcium deposition in placental infection .Severe congestion of the blood vessels was found in the chorionic villi stroma , neutrophils infiltration, sloughing and desquamation of trophoblasts , (Fig 2) this results may indicate that infection is acute of trophoblast which plays an essential role in cell degeneration .

### **Immunohistochemistry**

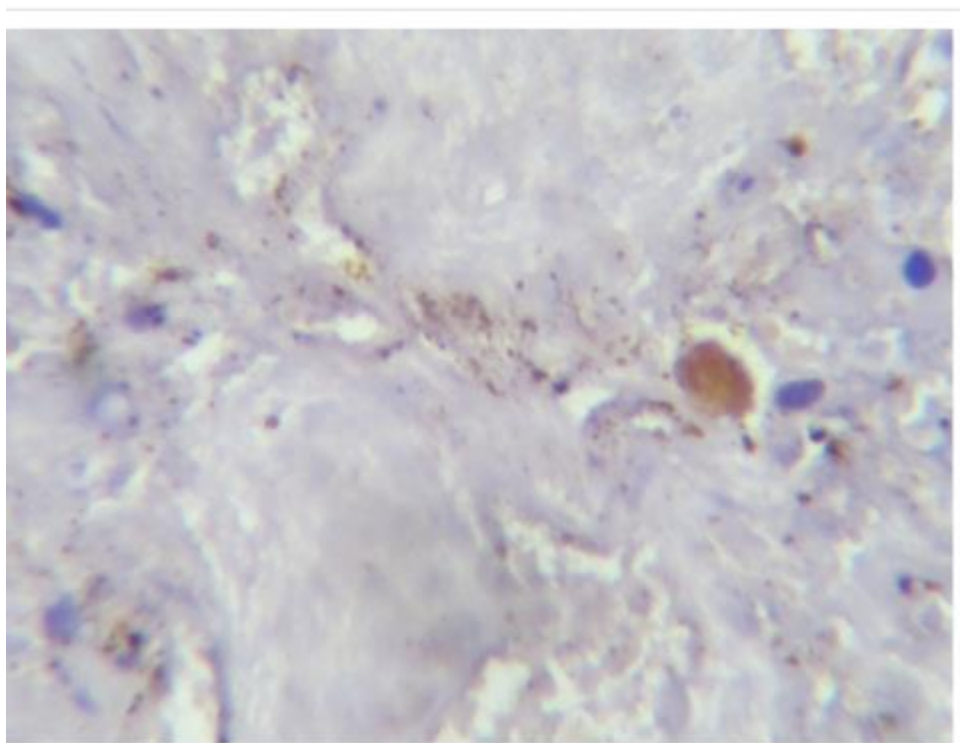
#### **Immunohistochemical detection of placental TNF- $\alpha$**

Five microscopic fields of each tissue were evaluated in each case.. Two observers examined the sections independently, and positive fine brown cytoplasmic granularity and/or surface membrane expression were recorded as positive. Figure( 7) shows the immunohistochemical detection of TNF alpha in macrophage of placental tissue .

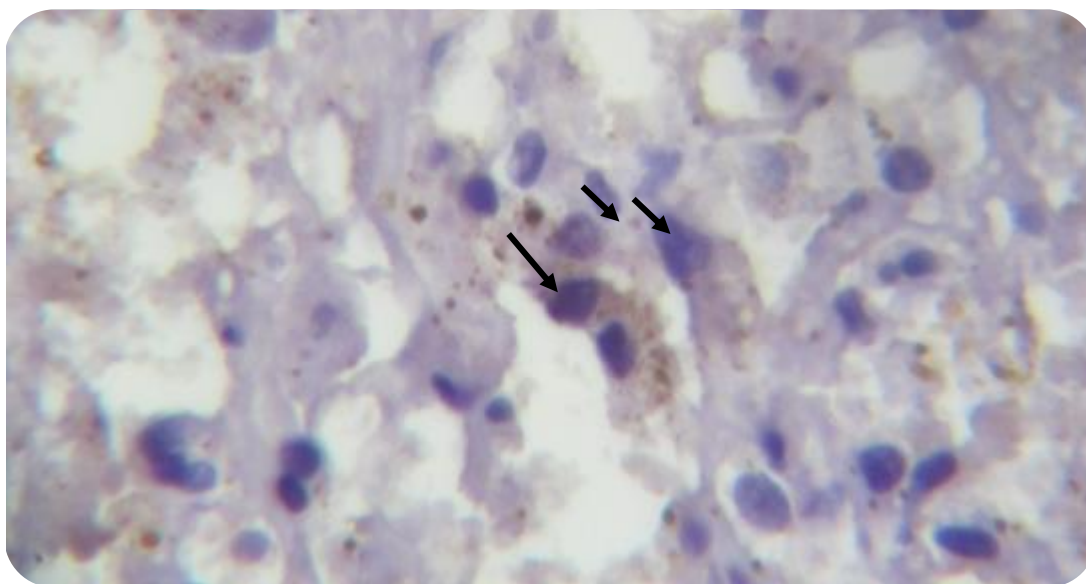
The distribution of TNF- $\alpha$  in control placentas was less than in the aborted ones. The arrows show the immunostaining positive cells and the arrowheads point to the negative cells..The score analysis of the protein in both groups revealed that TNF- $\alpha$  expression increased in aborted placenta than normal delivery.

#### **Histological and immunohistochemical analysis of the placental samples**

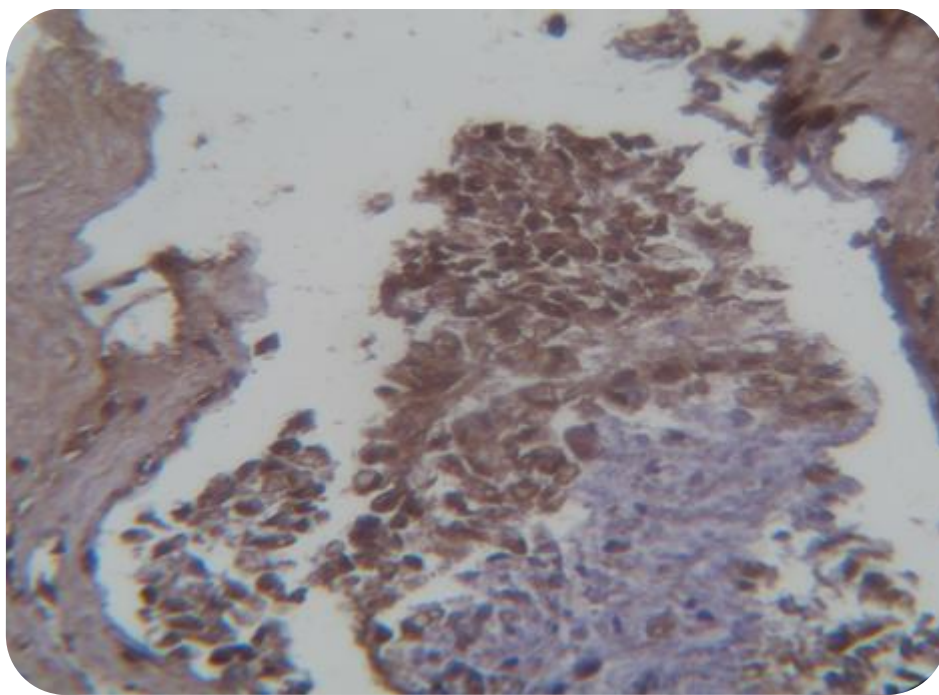
The distribution of the TNF alpha was studied by immunohistochemical study . In cases where serial sections had been used for H&E and IHC section comparisons could be made between lesion sites and IHC labeling. Histological evaluation of the placenta was compromised by occasional necrosis of endothelial cells . However, immunohistochemical labelling allowed the identification of TNF alpha antigen in all cases studied. Lesions were visible in all samples, after the likely lesion sites had been identified by the IHC labeling.



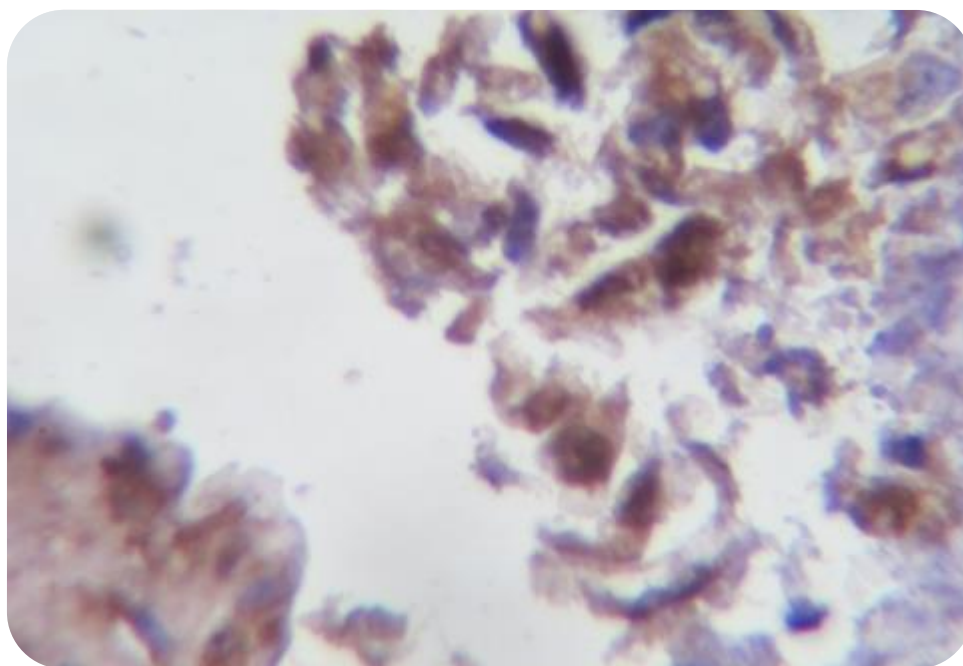
**Fig( 7):Placenta section from normal delivery ewes show TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 2, Moderate Intensity.(IHC).Stained by DAB-chromogen (Brown) and counter stained by Hematoxyline(Blue).40X.**



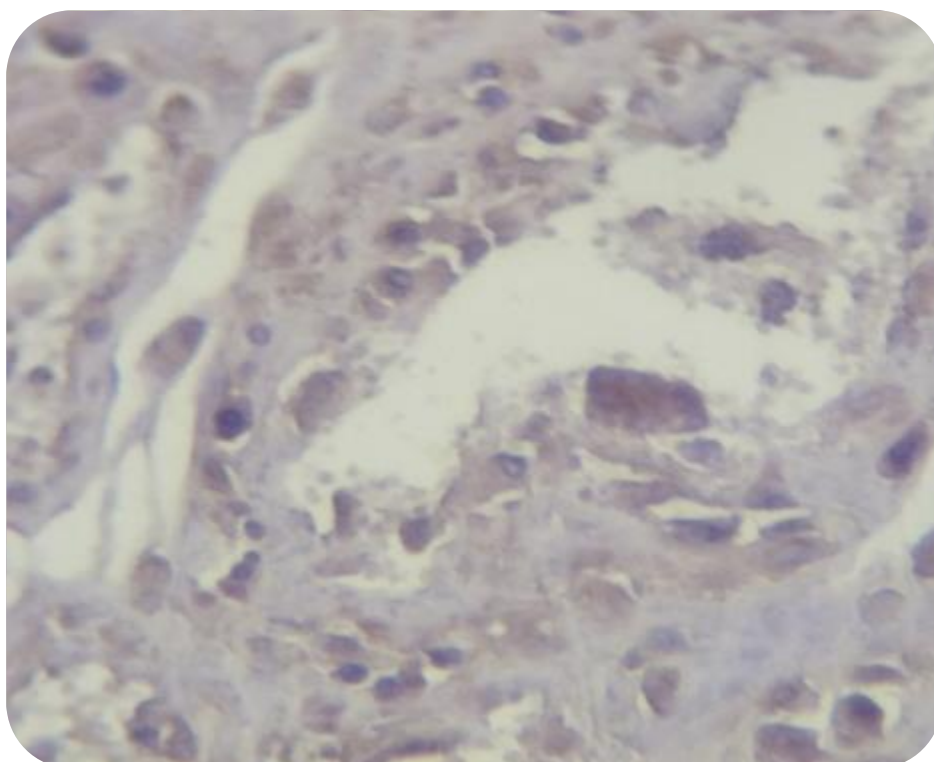
**Fig(8):Placenta section from normal delivery ewes show TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 1, Moderate Intensity.(IHC).Stained by DAB-chromogen (Brown) and counter by Hematoxyline(Blue).40X.**



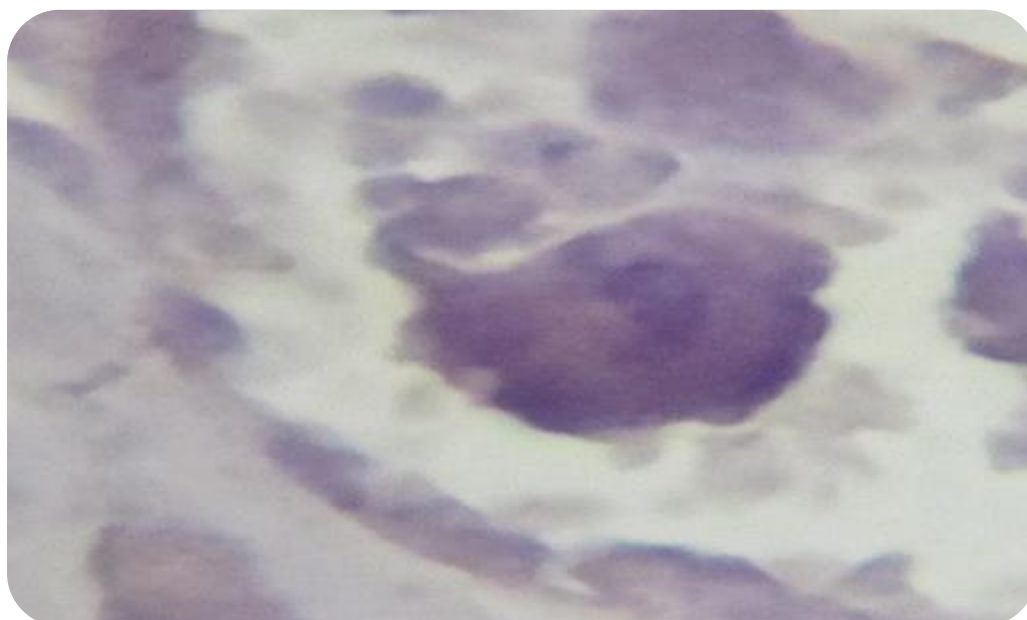
**Fig (9 ):Placenta section from aborted sheep show TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 3,high Intensity.(IHC).Stained by DAB-chromogen (Brown) and counter stained by Hematoxyline(Blue).40X.**



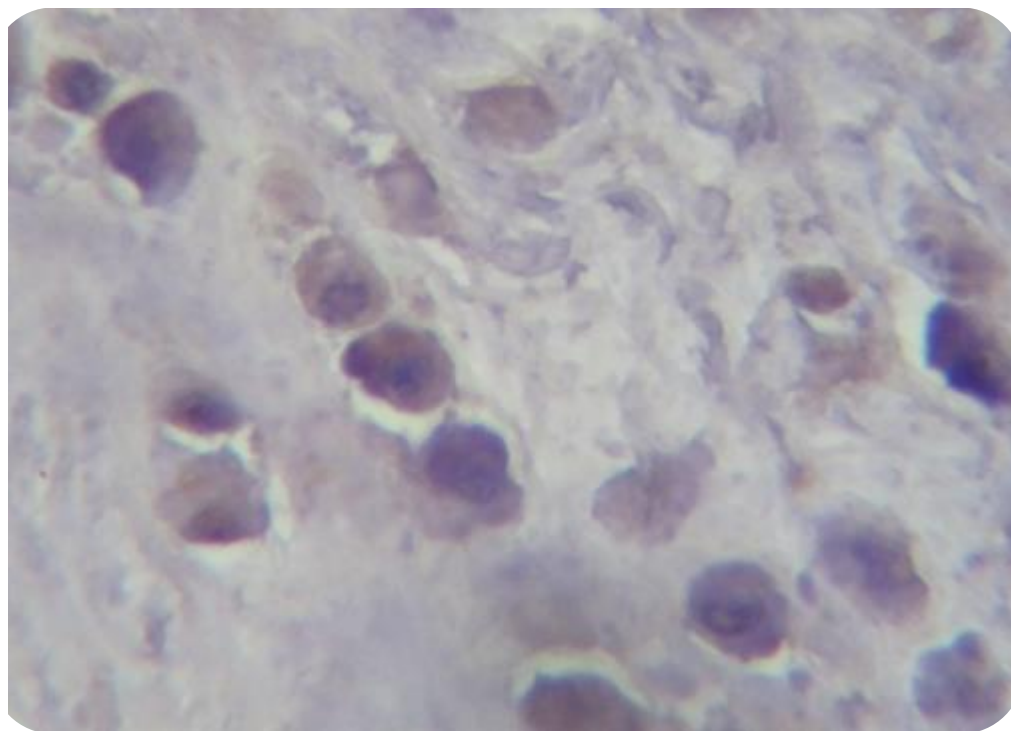
**Fig (10 ):Placenta section from aborted sheep show TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 3,high Intensity.(IHC).Stained by DAB-chromogen (Brown) and counterstained by Hematoxyline(Blue).40X.**



**Fig(11):Placenta section from aborted sheep showTNF-alpha expression (cytoplasmic staining) of Macrophage .Score 4, high Intensity.(IHC).Stained by DAB-chromogen (Brown) and counterstained stained by Hematoxyline(Blue).40X.**



**Fig(12 ):Placenta section from aborted sheep show TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 4,high Intensity.(IHC).Stained by DAB-chromogen (Brown) and counterstained by Hematoxyline(Blue).40X.**



**Fig(13)A section OF Placenta from AN aborted sheep show TNF-alpha expression (cytoplasmic staining) of Macrophage .Score 4,high Intensity.(IHC).Stained by DAB-chromogen (Brown) and counter stained by Hematoxyline(Blue).40X.**

The positive staining was detected mainly in macrophages, as brown, finely granular or sometimes diffused intracytoplasmic staining. Macrophages were large, dense, and their cytoplasm was filled with heavily stained amorphous material. In general, cells in the inflammatory foci revealed more intense reaction than isolated macrophages and neutrophils which were negative or had weak cytoplasmic reaction.

A semi-quantitative IHC analysis of TNF- $\alpha$  in animals of abortion group and normal pregnancy group was made.

The expression of TNF- $\alpha$  in mycotic aborted placental tissue was detected in all cases (100%). The expression of TNF- $\alpha$  in macrophage found in sections of abortion group was markedly stronger than that in normal delivery group. TNF- $\alpha$  immunoreactivity in the placenta of mycotic aborted ewes was observed in the necrotic areas, in macrophage, in addition to placental cytotrophoblast cells, while Syncytial trophoblast cells cannot be immunophenotypically proposed as having a macrophage-like characteristic, release of TH 1 cytokines by them suggests that they

can process the antigen and take part in its modulation.

TNF alpha immunopositivity was high among the necrotic cells in the placenta of mycotic aborted ewes (fig 9, 12, 13). Whereas, in the control placental tissues from normal delivered ewes, TNF alpha immunopositive cells were markedly few and, and there was a significant difference between the mycotic aborted animals and the control normal delivery.(fig 8)

(7) explained that the TNF- $\alpha$ , which produced mainly by activating monocytes/macrophages in amnion, chorionic and decidual tissue, triggered prostaglandin F<sub>2</sub> and E<sub>2</sub> biosynthesis by the decidua and amnion which they lead to induce uterine contractions and abortion.

There is some support for the hypothesis that these cytokines stimulate uterine contractions. Both IL-1 and TNF $\alpha$  stimulate arachidonic acid release and prostaglandin production in human myometrial cells, thus stimulating myometrial contractions and ripening of the uterine cervix (8).

Higher concentration of TNF- $\alpha$  may result in abortion by: promoting apoptosis of atrophic cells, elevation of the synthetic rate

of PG E2, excitement of smooth muscle of uterus, stimulation of Th1 type of immunologic reaction, rejection of embryonic tissues, activation of coagulative system leading to thrombosis of trophic vessels in placenta, etc. It is demonstrated by some authors that spontaneous abortion is associated with the abnormal increase of serum TNF- $\alpha$  protein(9).

Inflammatory cytokines such as IL-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are readily detected in maternal serum, placenta, and fetus after activation of the innate immune response in pregnant animals, and this cytokine can directly pass maternal through the placenta (10).

However, the role of TNF- $\alpha$  in inducing abortion was recorded by (11) who showed that IL-10 modulates resistance to inflammatory stimuli by down regulating expression of pro-inflammatory cytokines TNF Alpha ,IL-6,IL-1A and IL-12 acting to protect against inflammation-induced pathology in the implantation site (12).

While the result in the second abortion showed less intensity of TNF alpha in placental tissue in IHCs than the first abortion because there is high cellular and humeral response enough to reduce the number of micro organism reach the placenta .

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