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RESEARCH ARTICLE

The expression of cytokines and inflammatory bio-markers of Uterus muscles of patients with infectious and Non-infectious vaginitis

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Abstract

The present study aimed to utilize the established immunohistochemical techniques to detect the presence of inflammatory cell infiltrate of cytotoxic T cells, macrophages, perforin, T helper 1 cells, T helper 2 cells and T regulatory cells and T helper 17 in uterus biopsies of patients with infectious and non-infectious vaginitis . CD8 and perforin positive cells were present in the Uterus section from patients with vaginitis in all patients 9/9 (100%) in whom 4/9 (44%) had a mean score of > +3 and 5/9 (56%) had a mean score of >+2 for both CD8 & Perforin . However the patients control (non-infectious vaginitis) showed also CD8 and perforin positive cells but at lesser extent in whom 4/6 (67 %) had a mean score of >+2 and 2/6 (33.%) had a mean score of >+1 for CD8 while 5/6 (83 %) had a mean score of >+1, 1/6 (17 %) had a mean score of >+3 for perforin. While the bio-markers (CD68 , Foxp3 , Gata3 , Tbet , Th17) when non-significant in both infectious and Non-infectious vaginitis

INTRODUCTION

Bacterial vaginosis (BV) is the most common lower genital tract syndrome worldwide among women of child-bearing age, affecting 29.2 % [95 % confidence interval (CI) 27.2– 31.3] of reproductive age women in the USA. BV is a polymicrobial clinical syndrome characterised by an increased vaginal pH and replacement of vaginal lactobacilli by predominantly anaerobic microorganisms such as ***Gardnerella ,Atopobium and Bacteroides*** **spp**., and is strongly associated with several adverse health outcomes, including preterm labour, pelvic inflammatory disease, human papillomavirus and human immunodeficiency virus (HIV) acquisition (Wang *et al*. , 2014) Understanding of the vaginal immune response to BV has been studied but it is incomplete. In contrast to the inflammatory responses induced by vaginal ***Trichomonas vaginalis*** or Candida infections, the large increases in Gram-negative bacteria characteristic of BV typically produce minimal overt inflammation. Multiple investigations have consistently shown BV to be associated with increased genital tract concentrations of the proinflammatory cytokine IL-1 . The measurement of other proinflammatory cytokines, however, including tumour necrosis factor (Fichorova *et al*. ,2009). In a nother study it has shown that the immunoinflammatory response to trichomoniasis has been most studied in pregnant women. ***Trichomonas vaginalis*** positive pregnant women with bacterial vaginosis (Nugent 7–10) had increased vaginal IL-1β and neutrophils compared with bacterial vaginosis alone (Cauci *et al*., 2007). Increased cervical IL-8 and alpha-defensin have been found in pregnant infected women (Simhan *et al*., 2007).

From the literature reviews above it appears that definition of inflammatory cell infiltrate in vagina from infectious Vaginitis and non-infectious Vaginitis infection has not well defined and most of the studies of immunoinflammatory response to vaginitis and vaginosis have been investigated in the blood circulation and not in tissue level. So , the study was performed to utilize the established immunohistochemical techniques to detect the

presence of inflammatory cell infiltrate of cytotoxic T cells (CD8), macrophages (CD68), perforin, T helper 1 cells (Tbet), T helper 2 cells (Gata 3) and T regulatory cells (Foxp3) and T helper 17 cells (Th17) in vagina biopsies of patients with vaginitis infected with bacterial and non-bacterial infection and compared with vaginitis without infection

MATERIALS AND METHODS

Patients : Uterus vagina biopsies were obtained from patients with vaginitis infected with bacterial (*Megasphaera* spp, *Atopobium* spp. , *Gardnerella* spp. *Mobiluncus* spp. *Bacteroides* spp. *Mycoplasma hominis* , *Lactobacillus acidophilus*) and non-bacterial (*Trichomonas vaginalis*, *Candida albicans*) . Inflammatory cells infiltrate were studied in 15 vagina biopsies. 9 patients had vaginitis (15 females, with a median age of 21 years) and 6 patient had uninfected vaginits were studied.

Tissue biopsies : Vagina uterus punch biopsies were taken intra vagina with a 1.4 mm diameter Menghini needle and consisted of 3-5 mm uterus tissue cylinders. All tissues are fixed in 10 % neutral-buffered formalin and embedded in paraffin. 4 µm thick sections were cut from each paraffin block and were obtained for clinical purpose and the remaining tissue was used for this study. Punch biopsies were formalin fixed and then embedded in paraffin cut and stored until use.

Principle of immunohistochemical techniques

Immunohistochemical Technique 1 :The Novolink^{TM} Polymer Detection Systems (Envision technique)The Novolink^{TM} Polymer Detection Systems are used for the visualization of mouse IgG, mouse IgM and rabbit IgG primary antibodies. The positive cells were visualized by using a polymeric conjugate system to recognize mouse and rabbit immunoglobulins. polymer-based immunohistochemical methods utilize a technology based on a polymer backbone to which multiple antibodies and enzyme molecules are conjugated. In this technique (polymer system) 70 enzyme molecules and about 10 antibodies are conjugated to dextran backbone. This allows the entire immunhistochemical staining procedure, from primary antibody to enzyme, to be accomplished in a single step (Chilosi et al. , 1994) . The Novolink^{TM} Polymer Detection Systems (Envision technique)
Paraffin-embedded tissues were deparaffinized with xylene and dehydrated with ethanol and then the antigen was demasked by boiling the section in Tris-EDTA buffer pH 9 and micrwaved at 800 Watt (25 minutes). Endogoenous peroxidase was neutralized using 3% Hydrogen Peroxidise(see apendx 2).To reduce non-specific binding, biopsies were incubated with Protein Block .The biopsies were then incubated with monoclonal antibody (anti-human IL-17 from R&D Systems) for one hour at room tempertaure at 1:30 dilutions .A post primary block solution to enhance penetration of the subequent polymer reagent.. To detect any tissue-bound primary antibody the sections were incubated with secondary antibody Novolink ^{TM}polymer(mouse and rabbit immunoglobulins) for 30 minutes. Peroxidase activity was developed by using a mixture of DAB and DAB substrate buffer the reaction with the peroxidase produces a visible brown precipitate at the antigen site then washed with PBS. Finally, biopsies were counterstained with hematoxylin and prepared for microscopic examination by placing a cover slip on the liver biopsies on the slide.

Immunohistochemical Technique 2
Avidin-Biotin complex technique
Avidin-biotin technique is a very sensitive immunohistochemical technique. Avidin posses four binding sites for biotin. In this technique secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and avidin-biotin peroxidase complex Avidin is a glycoprotein found in egg white, has an extraordinary affinity for the small molecule vitamin,biotin. Covalently coupling biotin to immunoglobulin or peroxidase molecules gives them the ability to bind avidin molecules(Hsu *et al.* ,1981) . *Avidin-Biotin immunoperoxidase technique*
Breifly, antigen was demasked by boiling the sections in Sodium citrate buffer pH 6 and microwaved at 600 W (2 x 5minutes).Endogoenous peroxidase was neutralized using Peroxidase Block for 30 minutes, Non-specific background staining was inhibited by 10 minutes incubation of the biopsies with 10 % horse serum. After washing avidin -biotin blocking reagent was applied for 30 min to block endogenous biotin. The sections were then incubated with a polyclonal goat anti-human IL-17 antibody for one hour at room temperature at 1:30 dilution..The sections were then incubated with biotin conjugated secondary antibody anti-goat (Horse anti-goat IgG, diluted 1/50) for 30 minutes. Immune reactivity was visualized by avidin-biotin- peroxidase complex kit reagents. Peroxidase activity was developed by using mixture of DAB substrate solution for 8 minutes then washed and placed in PBS. Finally

biopsies were counterstained with hematoxylin and prepared for microscopic examination by placing a cover slip on the liver biopsies on the slide.

Microscopic analysis : Inflammatory cells infiltrate (cytotoxic T cells (CD8), macrophages (CD68), perforin, T helper 1 cells (Tbet), T helper 2 cells (Gata 3) and T regulatory cells (Foxp3) and T helper 17 cells (IL17)cells) were detected using light microscopy. Positive cells were counted in vagina section (uterus histology area). The percentage of positive cells was calculated and expressed semi quantitatively, with"0" equalling no positive cells, "1" minimal (<5%), "2" moderate (5-10%), "3-4 "abundant (>10%) quantities of positive cells (Hussain *et al.* , 1994) .

Statistical analysis : statistical analysis was performed using the SPSS (SPSS Inc., Version 10.0, and Chicago, Illinois, USA) statistical Software. The differences between infectious vaginitis and non-infectious vaginitis patients in respect of demographical, histological and clinical features were compared using the t-test as appropriate. Data were presented as mean ± standard deviation (SD). Two-sided p values <0.05 were considered as statistically significant.

Results and discussion

The study used two imunnohistochemical techniques to detect inflammatory cell infiltrate in the vagina section obtained from patient with vaginitis . CD8, perforin,T-bet, Foxp3, CD68 and Gata3 positive cells were visualized in the tissue obtained from vagina by using Envision technique. While IL-17 positive cells were visualized by using Avidin Biotin complex system . The study examined the presence of inflammatory cell infiltrate (cytotoxic T cells (CD8), macrophages (CD68), perforin, T helper 1 cells (Tbet), T helper 2 cells (Gata 3) and T regulatory cells (Foxp3) and T helper 17 cells (Ih17) positive cells in patients with infectious vaginitis using immunohistochemical staining and compared with control group (non-infectious vaginitis). The distribution of inflammatory positive cells infiltrate results are shown in Table (1).

CD8 positive cells expressed staining on the cell membrane,(Fig. 1) while perforin staining expressed in the cytoplasmic staining (Fig. 2).CD8 and perforin positive cells were present in the Uterus section from patients with infectious vaginitis in all patients 9/9 (100%) in whom 4/9 (44%) had a mean score of > +3 and 5/9 (56%) had a mean score of >+2 for both CD8 & Perforin (Table 2). However the patients control (non-infectious vaginitis) showed also CD8 and perforin positive cells but at lesser extent in whom 4/6 (67 %) had a mean score of >+2 and 2/6 (33.%) had a mean score of >+1 for CD8 while 5/6 (83 %) had a mean score of >+1, 1/6 (17 %) had a mean score of >+3 for perforin. The comparison of positivety CD8 and perforin positive cells was made between two groups (infectious vaginitis versus Non-infectious vaginitis) . The scoring levels of CD8 and perforin positive cells were significantly elevated in patients with infectious vaginitis (mean 2.4± SD0.5) than in patients with non-infectious vaginitis (1.7±0.5) (p=0.007). Similarly scorin levels of perforin was also significantly elevated in patients with infectious vaginitis (2.4± SD 0.5) than in patients with non-infectious vaginitis (1.3±0.8) (p=0.003) (Table 2) .

Cytotoxic T cells have implicated in the pathogenesis of infectious diseases. Increased levels of CD8 has been studies in a number of infectious human diseases including bacterial, fungus and parasite infection (Leigh et al. , 2006).On the other hand The role of CD8+ T cells has been studies as well in Tuberculosis and it is believed that CD8 cells involve their cytolytic activity mediated by cytotoxic granules. CD8+ Tcells contribute to protective response against TB (Prezzemolo *et al.* , 2014).In the present study we analysis expression of Cd8 T cell in patients with infectious vaginitis in comparison with non-infectious vaginitis . This expression also increased in the cases infectious vaginitis than non-infectious vaginitis and the high levels inflammatory cytokines may contribute the activation of cell subpopulation that interleukin 17 but there is no-significant differences between to group (infectious vaginitis and non-infectious vaginitis) . our finding suggest at the possibility the CD8 contribute to resistance to bacterial infection .

It has been found that perforen independent cytotoxicity was reponsible for most of the CD8 mediated protection in secondary infection and in adoptive transfer experiments (Broek *et al.*, 2014) . These findings have been shown when testing the expression of perforin and its role in the pathogenesis of bacterial vaginitis .

We investigated transcription gene factors (Tbet, Foxp3 and Gata3) representing T helper 1 (Fig. 3) , T regulatory (fig. 4)and Th2 (Fig.5) and cells respectively in the vagina biopsy tissue. The staining of all transcription genes were expressed in the nucleus of positive cells .Tbet and Foxp3 positive cells were present in almost sections of infectious vaginitis and control group in whom 1/9 (11 %) hade mean score 0 ,2/9 (22%) had a mean score of >+1 and + 5/9(56)had meane score >+2 , 1/9 (11%) hade mean score >+3 for Tbet , and Foxp3 respectively in infectious vaginitis group compared with the control group non-infectious vaginitis being present in whom 1/9 (11 %) hade mean score 0 , 1/9 (11%) had a mean score of >+1 , 7/9 (78%) hade mean score >+2 . 3/6 (50%)hade mean score >+1 , 1/6 (17%) who had a mean score of >+2 and 2/6 (33%) who had mean score >+3 for Tbet and foxp3. 2/6(33%) who hade mean score of –Ve , 2/6 (33%) who hade mean score >+1 , 2/6 (33%) who hade mean score >+2 (table 2). The comparison of positivity of Tbet and Foxp3 between two groups were not significantly different P< (0.3) , (0.06) respectively

Foxp3 was initially characterized as a specific key intracellular marker of CD25+CD4+ T regulatory cells (TRegs)12 ,Its expression has been reported in more cell subsets including a minor subset of CD8+CD25+T cells13 (Devaud *et al.* , 2014).

Gata3 positive cells were present in the uterus section in both groups (infectious vaginitis and non-infectious vaginitis) but most of the sections showed either negative or occasionly presence of Gata 3 positive cells except in a few cases showed only 4/9 (44%) who had mean score –Ve , 5/9 (56%) who hade mean score >+1 or +2 and 2/6 (33%) had a mean score of -Ve , 3/6 (50%) who hade mean score +1 , 1/6 (17%) who hade mean score >+2 , However the comparison of positivity of Gata3 between two groups were not significantly different P< 0.4 (Table 2) **.**Gata-3 plays a central role in Th2 responses both in vitro and in vivo (Zhu *et al*., 2004) **.**GATA-3 belongs to the GATA family of transcription factors. It regulates luminal epithelial cell differentiation in the mammary gland.(Hosein *et al*. ,2006) **.**

CD68 positive cells expressed staining on the cell membrane (Fig. 6) . CD68 positive cells were present in the vagina section from patients with vaginitis in all patients 9/9 (100%), 6/9(67%) who hade mean score >+3 , 3/9(33%) who hade mean score >+2 however in non-infectious vaginitis we found 1/6 (17%) who had mean score >+3 , 5/6(83%)who had mean score >+2 .The comparison of positivity of CD68 between two groups were not significantly different P < 0.4 (Table 2) . During bacterial infection, professional phagocytes are attracted to the site of infection, where they constitute a first line of host cell defense. Their function is to engulf and destroy the pathogens. Thus, bacteria must withstand the bactericidal activity of professional phagocytes, including macrophages to counteract the host immune system (Lovewell *et al*., 2014).

Th17 positive cells expressed staining in the cytoplasmic staining (fig. 7) Th17 positive cells were present in the vagina section from patients with infectious vaginitis 6/9 (67%) who hade score –Ve ,3/9 (33%) who hade score + or ++ Ve ,and in non-infectious vaginitis we found 4/6 (67%) who hade score –Ve and 2/6 (33%) who hade score >+2 However the comparison of positivity of IL17 between two groups were not significantly different P< 0.4 (Table 2) .The IL-17 family comprises cytokines that participate in inflammatory responses and in the pathogenesis of many inflammatory disorders. The IL-17 cytokines show high homology to IL-17A (16% to 50% of amino acid sequence identity), (Zaragoza *et al*., 2014) .

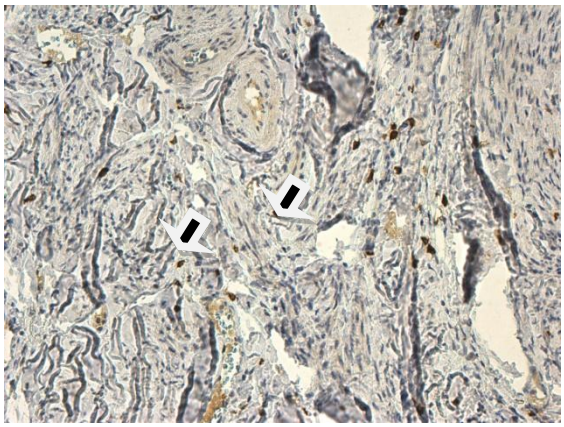
Tabl 1- The distribution of inflammatory positive cells infiltrate results

Viginitis	CD8	Perforin	T-bet	FoxP3	CD 68	Gata 3	IL-17
361 / 14	Ve+++	Ve+++	Ve+	Ve ++	Ve+++	Ve-	Ve+
366	Ve++	Ve++	Ve+	Ve -	Ve+++	Ve+	Ve-
364	Ve+++	Ve++	Ve-	Ve +	Ve++	Ve-	Ve-
332 / 14	Ve++	Ve+++	Ve ++	Ve ++	Ve+++	Ve ++	Ve-
294 14	Ve+++	Ve++	Ve+++	Ve ++	Ve+++	Ve++	Ve ++
192 / 14	Ve++	Ve++	Ve ++	Ve ++	Ve ++	Ve -	Ve -
170 / 14	Ve++	Ve++++	Ve ++	Ve ++	Ve++	Ve-	Ve -
169 / 14	Ve++	Ve++	Ve ++	Ve ++	Ve+++	Ve+	Ve+
253 / 14	Ve+++	Ve+++	Ve ++	Ve ++	Ve+++	Ve +	Ve-

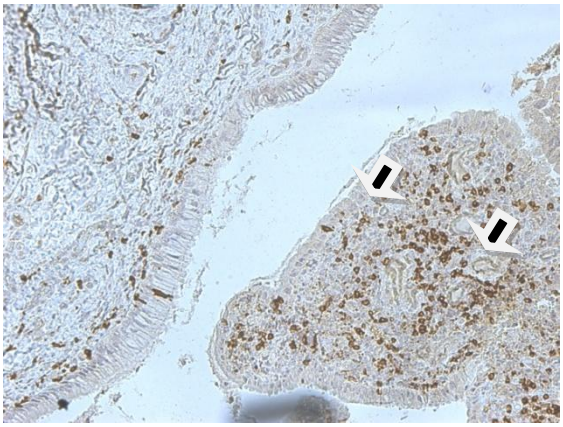
Non-Viginitis	CD8	Perforin	T-bet	FoxP3	CD 68	Gata 3	IL-17
276 / 14	Ve++	Ve+	Ve +	Ve +	Ve ++	Ve ++	Ve-
198	Ve+	Ve+	Ve+++	Ve ++	Ve++	Ve -	Ve ++
251 / 14	Ve++	Ve+	Ve ++	Ve -	Ve+++	Ve-	Ve-
99 / 14	Ve++	Ve+++	Ve+++	Ve ++	Ve ++	Ve +	Ve ++
1367	Ve+	Ve+	Ve +	Ve -	Ve++	Ve+	Ve-
425 / 14	Ve ++	Ve+	Ve+	Ve+	Ve ++	Ve +	Ve-

Table 2. Distribution of Inflammatory Bio-markers in the Vagina of Patient With Vaginitis

Bio-markers	Infectious Vaginitis (Mean± standard deviation)	Non-infectious Vaginits	P < Value
CD8	**2.4**±0.5	1.7±0.5	0.007
Perforin	**2.4**±0.5	1.3±0.8	0.003
T-bet	**1.6**±0.8	1.8±0.9	NS(0.3)
Foxp3	**1.6**±0.7	1.0±0.8	NS(0.06)
CD68	**2.55**±0.5	2.16±0.4	NS(0.07)
Gata3	**0.77**±0.8	0.83±0.7	NS(0.4)
IL-17	**0.4**±0.7	o.6±1.0	NS(0.3)

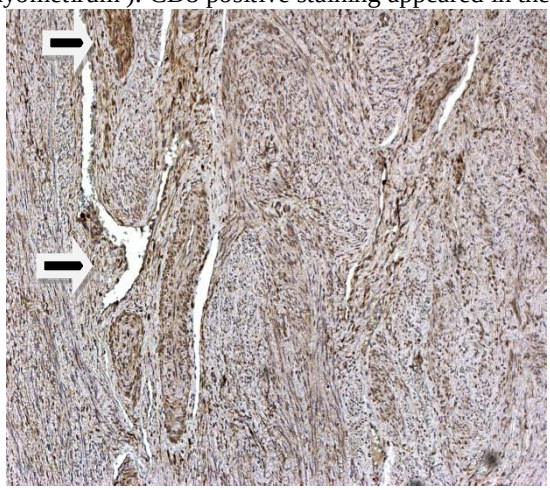


(A1) CD8 +

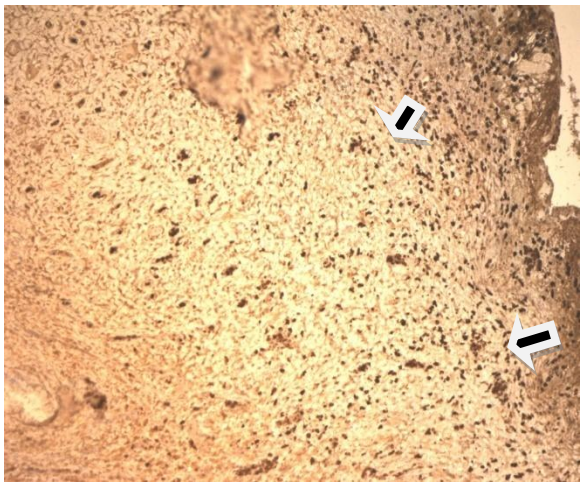


(A2) CD8+++

Figure 1: CD8 infiltrating cells were detected in the uterus biopsy of patient with infectious vaginitis and non-infectious vaginitis disease (A1 with score +1 for non-infectious vaginitis being present in scattered form in uterus(myometirum) .and A2 with Score +3 for infectious vaginitis being present in a group in Endometirum And myometirum). CD8 positive staining appeared in the cell membrane **.**

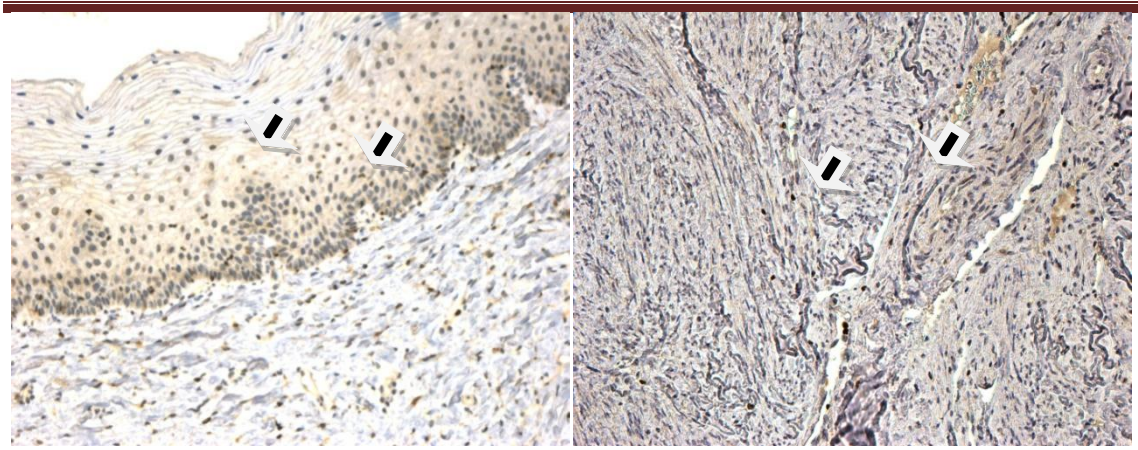


(A1) Perforin+



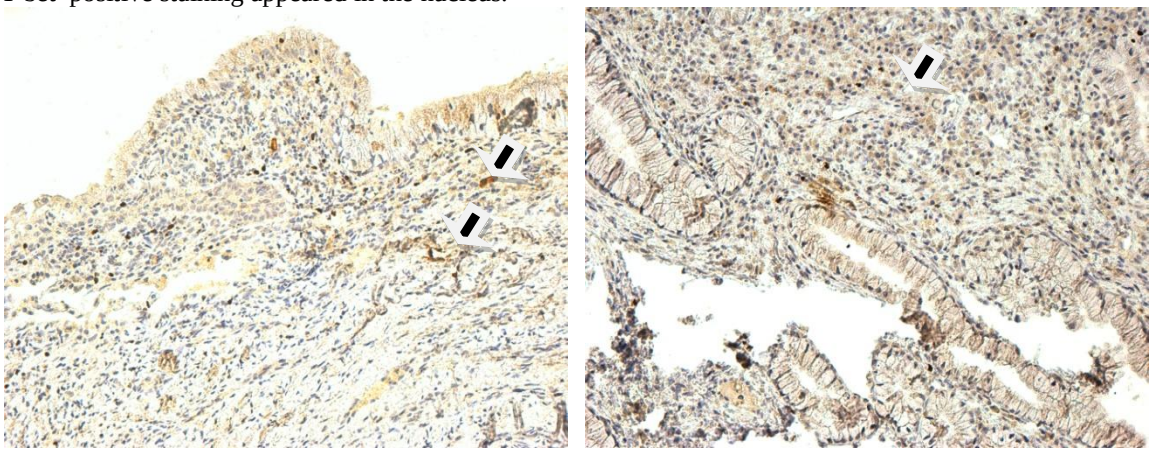
(A2) perforin +++

Figure 2: Perforin infiltrating cells were detected in the uterus biopsy of patient with infectious vaginitis and non-infectious vaginitis disease (A1 with score +1 for non-infectious vaginitis being present in scattered form in myometirum uterus and A2 with Score +3 for infectious vaginitis being present in a group in endometirum And myometirum). Perforin positive staining appeared in the cytoplasm.



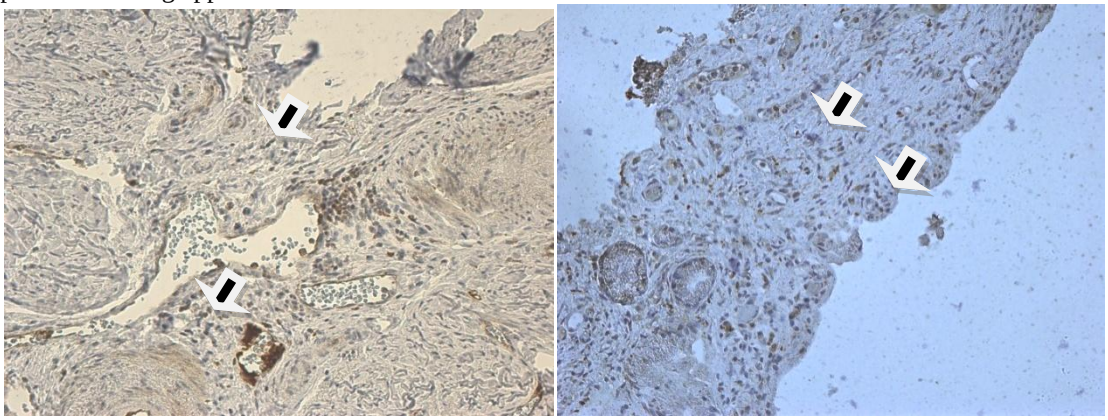
Tbet++(A1) **Tbet +(A2)**

Figure 3: T bet infiltrating cells were detected in the uterus biopsy of patient with infectious vaginitis and non-infectious vaginitis disease (A1) with score ++ for infectious vaginitis being present in scattered form in Endomaterum and Myomaterum, And (A2) with Score + for non-infectious vaginitis being present in Endomaterum. T bet positive staining appeared in the nucleus.



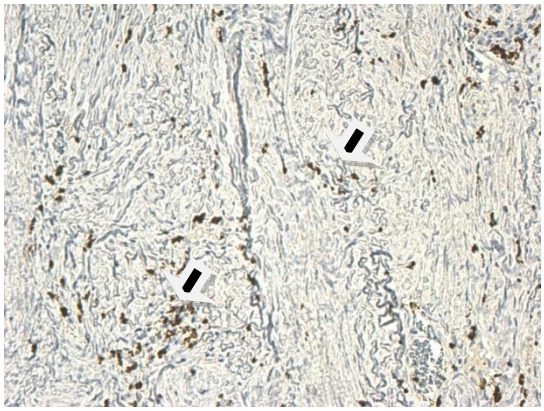
Foxp3++ (A1) Foxp3++(A2)

Figure 4: Foxp3 infiltrating cells were detected in the uterus biopsy of patient with infectious vaginitis and non-infectious vaginitis disease (A1) with score ++ for non-infectious vaginitis being present in scattered form in Endometrial and myomatrial And (A2) with Score ++ for infectious vaginitis being present in Endometrial . Foxp3 positive staining appeared in the nucleus.

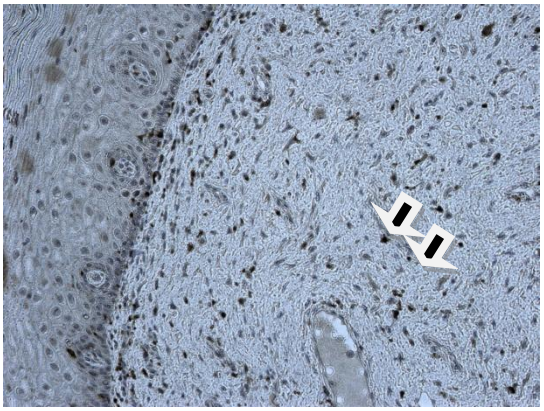


Gata +(A1) Gata+++(A2)

Figure 5: Gata 3 infiltrating cells were detected in the uterus biopsy of patient with infectious vaginitis and non-infectious vaginitis disease (A1) with score +1 for non-infectious vaginitis being present in scattered form in Endometrial and (A2) with Score +3 for infectious vaginitis being present in a group in Endometrial). Gata3 positive staining appeared in the nucleus.

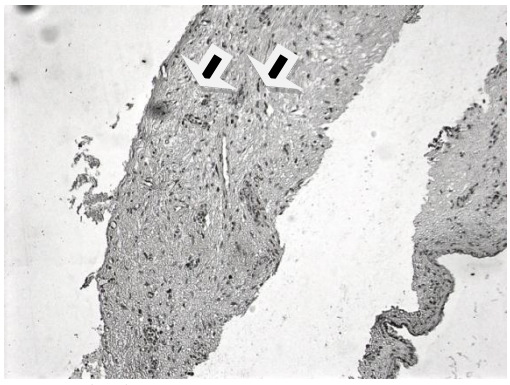


CD68 Ve++ (A1)

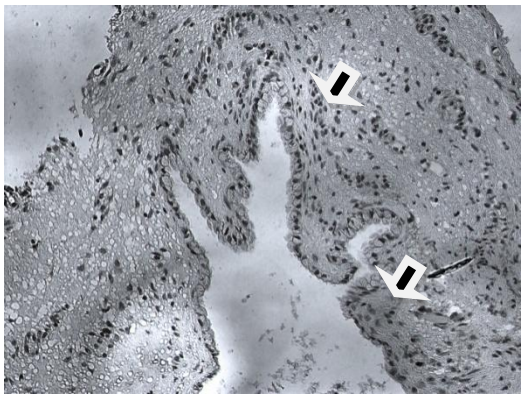


CD68 Ve++ (A2)

Figure 6: CD68 infiltrating cells were detected in the uterus biopsy of patient with infectious vaginitis and non-infectious vaginitis disease (A1) with score ++ for infectious vaginitis being present in scattered form in Endometrial and myomatrial And (A2) with Score ++ for non-infectious vaginitis being present in Endometrial). CD68 positive staining appeared in the Cytoplasm.



TH17+ (A1)



TH17+++(A2)

Figure 7: Th17 infiltrating cells were detected in the uterus biopsy of patient with infectious vaginitis and non-infectious vaginitis disease (A1) with score + for infectious vaginitis being present in scattered form in Endometrial and myomatrial And (A2) with Score +++ for non-infectious vaginitis being present in Endometrial).Th17 positive cells expressed staining in the cytoplasm

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