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Clinical Research

Significance of the TLR Expression in the Cells of the Female Reproductive Tract of Patients with Chronic Endometritis Treated with Sodium Nucleospermate

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Abstract

Immunocytochemistry was performed on the cells taken from the ecto/endocervix and endometrium. The monoclonal antibodies produced against CD20, CD56 and TLR have been used in the immunocytochemistry. The frequency of the immunocytochemically positive cells was estimated before and after a course of sodium nucleospermate treatment, in women suffering from infertility. The real–time PCR for HPV of high oncogenic risk was also studied. After the course of the treatment, the frequency of CD20, and especially of CD56 positive cells, was found to decrease. The concomitant lowering of the HPV of high oncogenic risk quantity was determined by real–time PCR. Otherwise, the frequency of TLR4 and TLR9 positive cells was found to be elevated. The data presented here proved the assumption that sodium nucleospermate actively induces an innate immune response and eliminated factors inciting endometrial inflammation.

Key words: *TLR, CD20, CD56, endometrium, cervix of uteri, sodium nucleospermate.*

Introduction

The female reproductive system is a system of cavities covered by mucous membranes (in the structure of a particular organ), and are interconnected with each other, forming a part of the mucosal immune system. Like all other mucous membranes, they possess non-specific factors of the immune system, providing a reliable barrier against pathogens, in most cases. Immune protection by the mucous membranes is provided as a result of several humoral and cellular factors. There are two populations of mucosal membrane cells, which are able to produce antigens. These are antigen-presenting cells and cells which implement the transport of an unmodified or processed antigen from the surface of the epithelial layer. Innate immunity is the first line of defense against primary infection and / or recurrent disease. It reveals mechanisms that are characterized by very rapid, almost immediate reactions. Reactions initiated by the innate immune system receptors do not require the proliferation of the T and B cell clones, which are necessary for the development of adaptive immunity reactions. This explains the high speed of innate immune responses and their low specificity.

It is well known that in response to a pathogenic invasion, an immediate response of the innate immune system in mammals is mainly mediated by a family of receptors called TLRs or Toll-like receptors [1-5], which in German translates as “amazing” receptors .These receptors are seen as macrophages, neutrophils, natural killer cells and dendritic cells. They recognize the

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highly conserved model molecules typical for the entire group of microorganisms, the so-called PAMP (pathogen-associated microbial patterns). An example of such structures could be components of the bacterial walls, lipopolysaccharides of Gram-negative bacteria, peptidoglycans and lipoteichoic acids of Gram-positive bacteria, flagellin, and viral nucleic acids.

To date, 11 types of this receptor family have been cloned. At the same time, different types of receptors predominantly connect with different ligands (antigens). However, despite a great variety of receptors in this family, the 1, 2, 4, 5 and 6 types of TLRs were found to bind preferentially to bacterial antigens, whereas the 3, 7, 8 and 9 types of TLRs were noted to preferably bind to the RNA and / or DNA nucleotide sequences. Clinical observations indicate that a variety of viruses including the herpes simplex virus, human papilloma virus, hepatitis B and C, Cytomegalavirus, human immunodeficiency virus, etc. can infect the female genital tract. In light of this, the special role of the receptors is supported by data which reveals the mechanism of the triggering of an immediate immune system response mediated by the Toll-like receptors 3, 7, 8 and 9 types to viral infection. Thus, for example, it is assumed, that TLR3 recognizes the double-stranded RNA. However, it should be noted, that the double-stranded RNA(dc-RNA) can arise from various sources. However, even samples of single-stranded RNA viruses often contain defective particles that primarily contain defective primary double-stranded genomes. Intracellular viral double-stranded RNA (dc-RNA) may be generated in different ways. In the case of single-stranded viruses, the formation of intermediate products of replication like dc-RNA is an obligatory stage in viral production. Whereas, in the case of DNA viruses, the complementary matrix RNA is often produced in such a manner that it is synthesized by the partially overlapping genes, located on the opposite chains of the viral genome (double-stranded). Long viral polycistronic matrix RNA often contains extra stable double-stranded "stems". These observations indicate that all viral infections pass through the stage of double-stranded RNA at the time of viral genome replication. Recently, it was shown that the matrix RNA arising from the dying or dead cells of the host organism activates TLR3, suggesting that the activation of the innate immune response via TLRs may occur under various conditions. It has also been convincingly demonstrated that the secondary structure forming a loop as a "stem" in the matrix RNA is responsible for the TLR activation, where the nucleic acids are the ligands.

It was found that, for instance, that TLR3 detects the double-stranded nucleic acid from Schistosoma mansoni and is very involved in the immune response against parasites. To date, it has been generally found that the DNA and RNA from different sources may trigger an immediate immune response through certain types of Toll-like receptors.

Moreover, the TLRs may represent a target for the so-called targeted therapies. The human papilloma virus, during its evolution has acquired the ability to "escape" from the immunological protection of the human immune system. It is shown that HPV 16 inhibits the expression of the 9th type of TLR in the epitheliocytes and dentritic cells. As a result, therapeutic vaccines are produced with adjuvants that can activate the Toll-like receptors expressed on the surface of the dentritic cells. In light of this, it is of great interest to study the drugs that interact with the TLR. However, various immunomodulators act as TLR modulators. Thus, the degree of TLR expression in the multi-planar and cylindrical epithelium of the cervical and endometrial tissue in bacterial and viral infections before and after the treatment assumes great significance.

Material and Methods

The material taken from the surfaces of the ectocervix and endocervix and the aspirate from the uterine cavity were deposited on a glass slide and immediately subjected to wet fixation using fixative «BioFix».

The drugs thus fixed were stored in a dry, dark place for several weeks before starting the immunocytochemical reaction. Prior to beginning the immunocytochemical reaction, the products were incubated in 50% ethanol for 10 min to remove the fixator. Then, they were washed twice with distilled water and placed in a citrate buffer (pH 6.0) (Diagnostic BioSystem, K 035), in which the desired antigen unmasking was later performed.

To accomplish this, a pressure cooker was filled with the citrate buffer and, on reaching boiling point, the drugs were placed in it. Antigen unmasking was thus performed in 40-45 min. At the end of the unmasking, the pressure cooker was cooled, and after the buffer cooled down, the products were removed. To prevent drying, excess moisture was removed using a filter paper, and the immunocytochemical field was localized with a hydrophobic pen (Elite PAP Pen (Diagnostic BioSystem, K 039)).

Excessive quantities of endogenous peroxidase were blocked for 10 min using a commercial composition «Peroxidase Block», which is a set of the «PolyVue HRP / DAB Detection System» (Diagnostic BioSystem). After incubation, the products were carried out in the washout buffer and the first antibody in the following format were applied: for cell verification, to express the CD20 and NCAM/CD56 antigens, mouse monoclonal antibodies (Ab) - mouse monoclonal anti B cell, CD20 (clone L26) and mouse monoclonal anti CD56 (clone 123C3.D5), respectively, were used.

Both the products were obtained from «Diagnostic BioSystem». For cell verification, to express the TLR4 and TLR9, mouse monoclonal antibodies to human antigens - a clone of a clone and 76V357.1 26S593.2, respectively, were used. Both products were obtained from «Lifespan, Biosciences». Dilutions of all the first antibodies were optimized individually. Incubation of the products with the first antibody was conducted in a moist chamber at 37°C for half an hour.

By the end of the incubation, the products were washed three times using the washout buffer. Then the «Polymer Penetration Enhancer» reagent was applied for 10 min (at room temperature), which was later washed off in the buffer.

For the next stage of the immunocytochemical reaction, a polymer system was used, directed against the first antibodies (Anti-Mouse/Rabbit PolyVue HRP Label from a set «PolyVue HRP / DAB Detection System»). Products were incubated for an hour at room temperature. After washing the incubation products, they were visualized within the optimum time period for the product of immunocytochemical reaction, using chromogen - diaminobenzidine (DAB / plus chromogen). Next, the products were washed thoroughly to remove traces of the chromogen solution with distilled water and staining was done

using hematoxylin (hematoxylin Karratsi).

Bearing in mind, that the drugs obtained from the endometrium and lower genital tract represent cellular material, it is useful to evaluate CD20-and CD56-positive cells, as well as the cells expressing TLR, irrespective of how many of them were found in one field of view or another, and to evaluate the frequency of their occurrence in terms of percentage. It should be noted, that the «Histoscore system» is also unacceptable in this study, as it is based on a study of histological preparations, whereas in the present study we investigated only cellular material.

Documentation of the immunocytochemical studies was performed using an MD125 Leica microscope (Germany) in the laboratory of functional morphology of the central and peripheral nervous systems (lab supervisor, Korzhevsky, MD, DM) in the Institute of Experimental Medicine, RAMS (St. Petersburg).

To estimate the viral load of HPV (human papilloma virus), taken from patients before and after sodium nucleospermate (POLYDAN, Russia) treatment, the "polymerase chain reaction in real time» method, generally accepted in practice across the world, was employed.

Results

As shown by the results obtained, the cells reacting positively to the desired antigens, were stained brown due to the reaction of horseradish peroxidase, which is associated, in the imaging system, with diaminobenzidine chromogen (Fig.1).

Signal distribution (color) in the cells is usually uneven. For example, it is clear, that the cytoplasm compared with the region "above the core" has a more intense brown color.

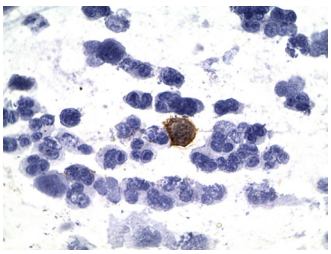

Figure 1

Aspirate from the uterine cavity of patient with chronic endometritis: CD20⁺. Immunocytochemistry. Hematoxylin ×1000

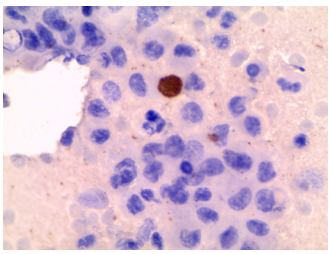

Figure 2

Aspirate from the uterine cavity of patient with chronic endometritis: CD20⁺. Immunocytochemistry. Hematoxylin ×1000

Antigens CD20 and CD56 are glycoproteins expressed on the B-and NK-cell plasma membranes, respectively. In some cases, the cytoplasmic portion "above the core" also gets intensely stained (Fig.2).

To identify the reasons for the variation in the staining intensity with the monoclonal antibodies of the cytological object, we referred to the following scheme. In Figs. 3 and 4, a formalized scheme of spatial staining of the cells in the cytological preparations is given, i.e., an immunocytochemical study which is fundamentally different from the spatial staining of the histological preparations (immunohistochemical study). Based on the spatial location of an object, it is obvious, that in the cytological preparations the plasma membrane, which is not addressed on the glass slide, can fully "present" all the required antigens on its surface, for binding with these monoclonal Ab.

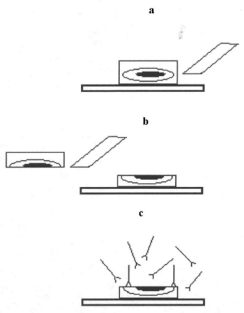

Figure 3

The scheme of histological slide in paraffin block (a), after preparation with microtome (b) followed by immunohistochemistry with monoclonal antibodies (c)

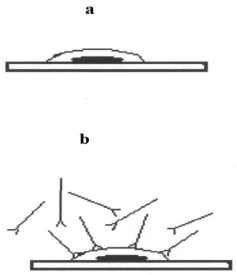

Figure 4

The scheme of smear (liquid cytology) which spread on the surface of microscope slide (a) followed by immunocytochemistry with monoclonal antibodies (b)

Whereas, for the object (cell) in the histological preparation the intensity of tagging depends on how "many times" the plasma membrane is "cut off" (or "left") as a result of the histological processing unit.

The frequency of the cells in the endometrium expressing the CD20 and the CD56 antigen varies significantly, which is

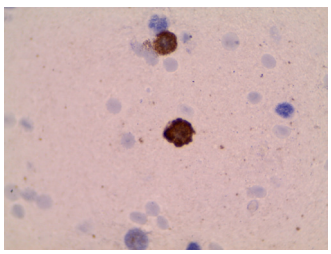

Figure 5

Aspirate from the uterine cavity of patient with chronic endometritis: CD20⁺. Immunocytochemistry. Hematoxylin ×1000

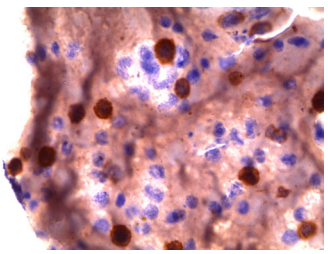

Figure 6

Aspirate from the uterine cavity of patient with chronic endometritis. CD56⁺. Immunocytochemistry. Hematoxylin ×1000

due, apparently, to the varying rates of inflammation (Figs. 5 and 6, respectively). It should be noted, that in the cytological preparations, often, cells may be artificially divided; however, it does not diminish the value of the immunocytochemical study of the mucosal endometrium.

Assessment of the frequency of the cells expressing the desired antigen is more accurate when compared with the mechanical extrapolation of the histological approach, which is reflected in the results presented on the system "in the field of view."

In the last case, it could lead to an "overestimation" of the results of the research on the cells expressing the desired antigen. As seen from Fig. 7 (The scheme of immunocytochemical study;

the cells of gray color are elaborated by immunocytohemistry), if the result is shown in the system "in sight", it can be concluded that there is a high degree of infiltration of the endometrium by cells expressing this antigen in the field, as shown in Fig. 7a.

Therefore, it can be concluded, that in this field of view there are six cells positively labeled by the monoclonal antibodies, compared with the material of the endometrium, as shown in Fig. 7b, (only three cells with a positive signal are observed in the immunocytochemical reaction in the conditional).

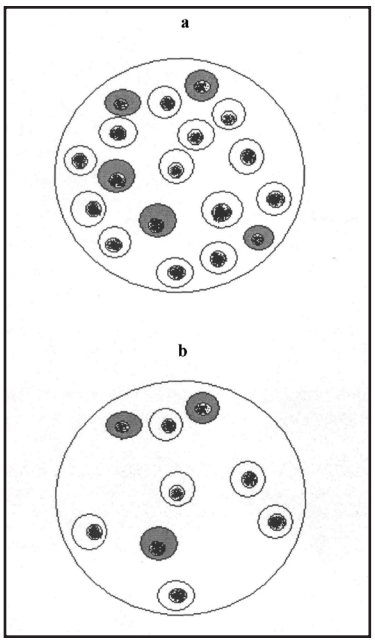

Figure 7

However, if we estimate the frequency of the positive-labeled cells in all the cell populations represented in these fields of view, we find that in the field of view in Fig. 7a, the frequency of the positive-labeled cells is not more than 26%, while in the field of view shown in Fig. 7b, the frequency of positive cells in the conventional immunocytochemical reaction is not less than 33%.

As seen from Fig. 8, on documenting the immunocytochemical study of material from the endometrium using monoclonal antibodies to the antigen CD 56, the NK - cells can form clusters that appear to indicate a high degree of infiltration of the endometrium in chronic endometritis. After treatment with sodium nucleospermate mucosal the infiltration of the endometrial cells, expressing the CD56 antigen decreased (Fig.9). However, it should be noted, to achieve a more accurate estimation of the frequency of the positive-labeled cells with monoclonal antibodies it is necessary to study the largest possible number of fields of view (and a larger amount of cells), because during inflammation, the material received from the endometrium often contains mucus and depleted cell material

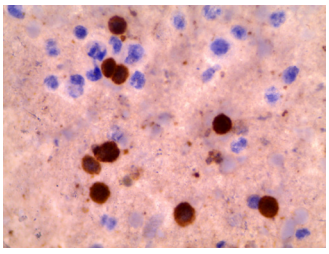

Figure 8

Aspirate from the uterine cavity of patient with chronic endometritis: CD56⁺. Immunocytochemistry. Hematoxylin ×1000

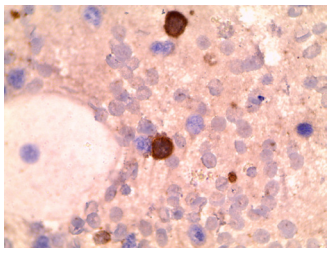

Figure 9

Aspirate from the uterine cavity of patient with chronic endometritis: CD56⁺. Immunocytochemistry. Hematoxylin ×1000

(Fig.10). Immunocytochemistry of the cells expressing TLR4 and TLR9 elucidated the following difference. The both types of TLRs were detected with low frequency (less than 1 %) in the smears taken from the ectocervix of patients with a high HPV viral load (Fig.11). The cells expressing TLR4 were as high as

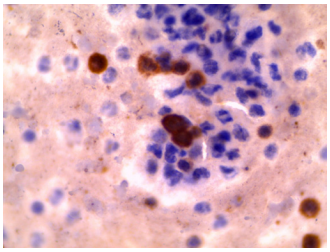

Figure 10

Aspirate from the uterine cavity of patient with chronic endometritis: CD56⁺. Immunocytochemistry. Hematoxylin ×1000

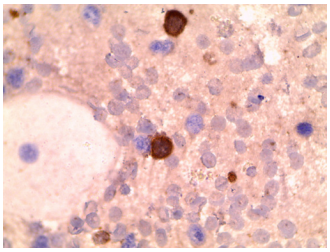

Figure 11

Ectocervix of patient with chronic endometritis(cytological smear): TLR4⁺. Immunocytochemistry. Hematoxylin ×1000

10% in the endocervix of the same patients. The endometrium of patients without chronic endometritis has been documented for high frequency of cells expressing TLR4 (>50 %) (Fig.12).

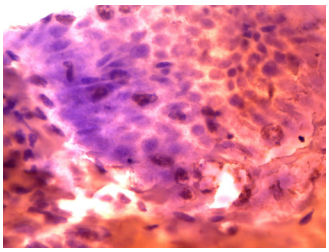

Figure 12

Aspirate from the uterine cavity of patient with chronic endometritis: TLR4⁺. Immunocytochemistry. Hematoxylin ×1000

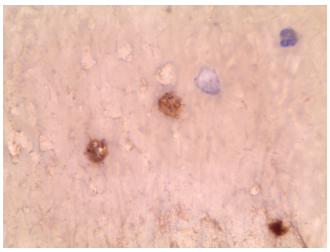

Figure 13

Aspirate from the uterine cavity of patient with chronic endometritis. TLR9⁺. Immunocytochemistry. Hematoxylin ×1000

As seen from Fig. 13, showing the documentation of the immunocytochemical study of the cells expressing TLR9, the signal is diffusely distributed into the cells. As is known, the nucleic acids are ligands for TLR9 which express onto the internal membranes of the cell endosomes [1-7]. Consequently, the monoclonal antibodies for TLR9 penetrate the plasma membrane. In the next step, they enter into the cell and then bind to the TLR expressing onto the internal side of the endosomal membrane (Fig.14).

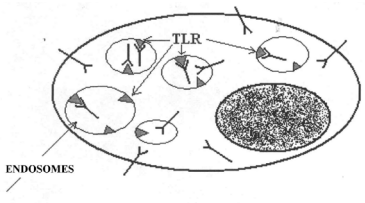

Figure 14

The scheme of immunocytochemistry with monoclonal antibodies raised against TLR9 localized in internal membrane of cell endosomes

Discussion

In the preliminary studies, it was found that the Toll-like receptors are one of the key components of innate immunity. The fact that, thus far, at least 11 types of TLR in mammals have been cloned emphasizes the evolutionary significance of this receptor type in the body's defense (in its internal environment) against a variety of pathogens. The expression of certain types of TLRs in the lower and upper parts of the female reproductive tract, however, was quite different.

Therefore, with a high degree of reliability, it was found, that the regular expression of TLR (both the 1 and 6 types), was registered in the epithelium lining of both the upper and lower portions of the reproductive tract: the fallopian tubes, endometrium, endo- and ectocervix and the vagina, regardless of the phase of the cycle [3, 17-19].

It was interesting to note that the expression of TLR1 was registered on the NK-cells and endothelial cells of the uterus, as well as in the smooth muscle cells of this organ, whereas the expression of TLR6 was revealed not only, for instance, on the NK-cells infiltrating the stroma of the uterus, but in the vagina as well, in the submucosal fibroblasts [18].

A regular expression of the TLR2 was detected in the mucosal and stromal cells, in all sections of the reproductive tract. At the same time, in the endometrial stromal cells, the TLR2 expression was higher when compared with the cells of the single-layer columnar epithelium. Peak expression of TLR2 was registered during the secretory phase of the menstrual cycle [19-21].

One of the key types of the TLR in the defense against microorganisms is undoubtedly the Toll-like receptor type 4. The ligand for TLR4 is lipid A or an endotoxin, which is linked with the oligosaccharide chain and O-antigen. These components together represent a lipopolysaccharide, which is an integral part of the cell wall of gram-negative bacteria (e.g., Helicobacter pylori).

In addition, the well-documented ligands for TLR4 also include the Heat-shock proteins with a molecular mass of 60 Da, glycosphingolipids of protozoa and a number of proteins of viral capsids. But most importantly, the binding of Toll-like receptor type 4 with molecules MD-2 and CD14 leads to the mobilization of factor MyD88.

This, in turn, leads to the phosphorylation of the kinase associated with the receptor for interleukin 1 type, oligomerization of factor 6 associated with the receptor for TNF and degradation of the molecule IkB, following after this molecular cascade [22-23].

At the cellular and tissue level, binding of the TLR4 with the MD-2 and CD14 molecules leads to the activation of the transcription genes encoding the various antiphlogistic cytokines, chemokines and their associated factors, which are also necessary for the further development of immune response.

In the context of the clinical data, it must be stated, that with regard to the microorganisms, the ligands for TLR4 is LPS of N. gonorrhoeae, LPS and heat-shock protein of the C. trachomatis microorganism and the carbohydrate mannan of the C. albicans microorganism [24-27]. It is, therefore, not surprising, that the TLR4, as a receptor, for which the ligands are clinically relevant factors, is expressed by the cells lining both the lower and upper portions of the reproductive tract.

In our study, the expression of TLR4 by the cells lining the endo- and ectocervix was recorded (see results). Also, we found, that in the material taken from the endocervix, the frequency of cells expressing TLR4 was higher than that in the epithelium of the mucous membranes of the ectocervix. However, the highest frequency of cells expressing TLR4 was found in the endometrium. It should be noted that the frequency of cells lining the endometrium were positive in their immunocytochemical reaction for TLR4, although they varied widely in different patients.

Patients were identified having a frequency of at least 80% of cells expressing TLR4 in the endometrial lining; patients with a frequency of less than 15% of cells expressing TLR4 in the endometrial lining were also revealed.

Indeed, several authors, although this is not true of all the researchers, discovered, that the "gradient" of the expression of TLR4 is higher in the cells lining the upper reproductive tract and are at their lowest in the cells lining the vaginal and stromal elements [20].

However, there is some controversy regarding the localization of the cells showing TLR4 expression; however, the intensity of expression of TLR4 may be related to the phase of the menstrual cycle when the material taken from the reproductive tract was studied. To date, very strong evidence has been received confirming that the most pronounced expression of the TLR4 in the endometrium is registered during the secretory phase of the menstrual cycle [19,28].

Our data strongly suggest that, as a rule, in the material of the ectocervix in patients with persistent HPV infection, cells expressing TLR9 were totally absent. In rare cases (10%), in the material of the ectocervix, cells expressing TLR9 were recorded. If, prior to treatment, the frequency of the cells in the endometrium expressing TLR9 did not exceed 3%, then post treatment with sodium nucleospermate the frequency of the positive cells in the immunocytochemical reaction with monoclonal antibodies for Toll-like receptor type 9 increased and accounted for more than 10% (i.e., more than three times).

It is known that the TLR9 binds to the nucleic acids containing CpG-islands (not subjected to methylation) [8,29], which are also found in the genome of the herpes simplex virus [30]. Expression of TLR9 was registered in the cells of the fallopian tubes, endometrium and cervix. It should be noted that the expression of the TLR9 in the endometrium is not limited to the epithelial cells, but is also recorded in the stromal elements [21].

The result of the activation of the TLR9 may be an increase in the number of cytokines, including IL-8, which the experimental data strongly suggest. Thus, the unmethylated CpG oligonucleotides, which are the agonists of Toll-like receptor type 9, induce the production of the IL-8 by the primary cells, isolated from the tissues of the fallopian tubes and cervix [6].

In light of this observation, it is interesting to notice, that the CpG oligonucleotides also inhibit the persistence of the cytomegalovirus and Epstein-Barr virus in the epithelium of the vaginal mucosa. One of the cytokines that inhibits the production of these pathogenic viruses is IFN - beta. The significance of the contamination of the human reproductive system by the Epstein-Barr virus as inflammation and the inability to conceive has not yet been studied and a full research is warranted with regard to the function of the TLR9 in providing an immediate antiviral

immune response. Our data strongly suggest the possibility of a correction of the expression of the TLR cells by the female reproductive system, with the use of sodium nucleospermate.

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