



CODEN [USA]: IAJPBB

ISSN: 2349-7750

INDO AMERICAN JOURNAL OF PHARMACEUTICAL SCIENCES

<http://doi.org/10.5281/zenodo.3752815>

Available online at: <http://www.iajps.com>

Research Article

STUDY OF THE ENDOMETRIAL INNATE IMMUNE RESPONSE TO TLR9 AGONIST THERAPY.

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Article Received: February 2020

Accepted: March 2020

Published: April 2020

Abstract:

The morphological structure of the endometrium is a very important link in the formation of a healthy pregnancy, and the innate immune response in the uterine mucosa is important for establishing the microenvironment during pregnancy. The cells of innate immunity penetrate the decidual tissue and accumulate at the attachment sites of the trophoblast. The main such cells that may be mediated are toll-like receptors. Currently, there is growing evidence that the innate immune response is activated by uteroplacental interaction, i.e. direct involvement of the endometrium as a structural component. Toll-like receptors are transmembrane proteins that can evolutionarily recognize bacteria, viruses, fungi and parasites. The study of immunity parameters with the use of TLR9 agonists (Derinat) in women with chronic endometritis associated with infertility in women of reproductive age. The drug belongs to the pharmacotherapeutic group: immunomodulatory agents, regenerant, reparant, according to published data, derinat has the properties of an agonist toll-like receptor agonist. The result of the study showed that the use of derinate improved reproductive function in women whose IVF was previously ineffective.

Key words: Chronic endometritis, failed IVF attempts, TOLL-like receptors, innate immunity, TLR9 agonist.

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Please cite this article in press Kosykh E.V et al, *Study Of The Endometrial Innate Immune Response To TLR9 Agonist Therapy.*, Indo Am. J. P. Sci, 2020; 07(04).

INTRODUCTION:

The internal mucous membrane of the uterine body - the endometrium - is the leading morphological component of the antenatal period of fetal development, the role of which is to create the necessary conditions from the moment of blastocyst implantation in the uterine cavity to the normal formation of the placenta, indirectly affecting the full delivery of oxygen and nutrients to the embryo [3.11 , 28]. The importance of the endometrium during pregnancy is also determined by its morphological structure, which is a complex and multicomponent system, the structure of which includes 2 types of epithelium (integumentary and glandular), fibroblastic stromal component and numerous blood vessels [2,10,18,24,27] . And if the diversity of the epithelial layer, consisting of many secretory and ciliated cells, is explained by the dominant function of the endometrium, then the variety of cellular forms of the stroma (from low-grade to mature fibroblasts, macrophages, T and B lymphocytes and mast cells) indicates not only direct immune function mucous membrane of the uterus, but also a high degree of severity of the organ's reparative potential [4,14,23,35,38]. The modern idea of immunomodulating processes gives particular importance to the innate immune system, which is the first line of defense that provides an immediate response against invading pathogens, due to its ability to directly distinguish between foreign proteins [16,43]. Thus, the innate immune response in the uterine mucosa is fundamental for establishing the acquired microenvironment during pregnancy, elimination of infectious agents (bacteria, viruses), and maintaining tolerance to a non-infectious agent (own antigens, placenta, and fetus) [32]. Currently, there is growing evidence that the innate immune response is activated by uteroplacental interaction, i.e. direct involvement of the endometrium as a structural component [6,8,25,34,36,39,40]. It has been established that cells of the innate immune response, such as natural killers, macrophages and dendritic cells penetrate the decidual tissue and accumulate around the attachment site of the trophoblast [7,9,12,13,15]. In addition to increasing their numbers, these cells acquire an activated phenotype during pregnancy [1,17,20,22].

One of the evidence of the above processes is the analysis of the expression of a group of cell receptors of the innate immune response, known as the family of PRRs (pathogen-recognizing receptors), capable of recognizing and binding to sequences of pathogen-associated molecular patterns - PAMPs [5,21,26,37]. In addition, non-immune cells, such as epithelial cells, also express PRRs, which allows them to respond to PAMPs and provide an inflammatory response to pathogen invasion [19,41]. The main family of drug-affected

PPRs is Toll-like receptors (TLRs). Toll-like receptors are transmembrane proteins with extracellular domains rich in leucine, repeating recognition motifs that are evolutionarily conserved and recognize PAMPs of bacteria, viruses, fungi and parasites.

To date, eleven TLRs (from TLR1 to TLR11) have been identified in mammals [29,33]. Each receptor is specific: for example, TLR4 is crucial for body cells in responding to gram-negative bacteria lipopolysaccharides; TLR2 has the widest species-specificity, recognizing lipopolysaccharides of gram-positive bacteria, peptidoglycans (PDG), lipoprotein acids and zymosan fungi; the list of ligands for TLR2 will probably expand due to its hetero-dimerization with other TLRs, so that TLR1 / 2 heterodimers recognizing lipoproteins differ from those of TLR2 / 6 heterodimers. TLR3 and TLR7 appear to play an important role in the immune response to viral invasion. TLR3 is known to bind double-stranded viral PHK, while TLR7 and TLR8 interact with single-stranded PHK [30]. TLR9 activator in the cell is recognition of the unmethylated nucleotide pair of cytosine-guanine bacterial DNA (CpG motif). Additionally, the ability of TLRs to respond to other pathogen patterns (stimuli) such as reactive oxygen species, proteins released from dead or dying cells, surfactants, fibrinogen, decomposition products of the extracellular matrix, for example, fibronectin fragments, hyaluronic acid, oligosaccharides, neurotoxin, of eosinophilic origin, heat shock proteins [31]. As a rule, TLR activation leads to the production of cytokines and antimicrobial factors through common intracellular signaling pathways. After ligand recognition, TLR uses an intracellular protein — a signal adapter, myeloid differentiation factor 88 (MyD 88), which leads to a subsequent cascade kinase reaction that activates the NFκB pathway (Kappa nuclear factor), resulting in the activation of an inflammatory response [42].

Considering that TLRs are widely represented (in particular, type 9 TLRs) in the endometrial structures not only by immune cells (macrophages, T and B lymphocytes), but also not immune (trophoblast cells, decidual cells and amniotic epithelium cells), the question arises of the role of these cells and their effect on the regulation of local and systemic innate immune responses during pregnancy, as well as the possibility of drug correction by TLR agonists in clinical gynecological practice.

PURPOSE OF THE STUDY:

The study of changes in the parameters of the innate immune response in the clinical use of the drug - a TLR9 agonist in the treatment of chronic

endometritis associated with infertility in women of reproductive age.

Research objectives:

1. Relief of clinical symptoms, if any (pains in the lower abdomen of a pulling and aching nature, pain, enlargement and hardening of the uterus, positive dynamics by ultrasound: increased uniformity of the endometrial echostructure, endometrial growth in the 2nd phase of the menstrual cycle);
2. Effective treatment of sexually transmitted infections;
3. Reducing the number of repeated intrauterine interventions;
4. Restoration of reproductive function
5. Assessment of TLR9 expression in the speaker during pathological study of the endometrium in patients.

MATERIALS AND RESEARCH METHODS:

The clinical part of the study was carried out on the basis of the Department of Obstetrics and Gynecology N 1 "Voronezh State Medical University named after Burdenko" of the Ministry of Health of the Russian Federation. Laboratory studies were carried out on the basis of the clinical diagnostic laboratory of the Voronezh Regional Clinical Hospital No. 1. Pathomorphological studies were carried out in the department of morphological analyzes of the State Institution "Voronezh Regional Pathological Bureau. The study was initiated by the holder of the registration certificate of the drug Derinat® (000 PharmPack, Russia) and corresponded to the structure of the post-registration study of efficacy in the uniformity of group samples, age of patients, gender, disease frequency over the study period, and background pathology.

The study included 60 patients aged 20 to 41 years, which met the criteria of active reproductive age with an established diagnosis: habitual miscarriage, chronic endometritis. The criteria for exclusion of patients from the study included: individual intolerance to the applied therapy; the presence of diseases requiring the appointment of antibacterial, antiviral and immunomodulating therapy and / or chronic diseases of the genitourinary system; participation in any clinical trials during the previous month and / or patient refusal take part in

the study. All patients included in the study received treatment in the gynecological departments (No. 1, N2 and N4) of the Perinatal Center "Voronezh Regional Clinical Hospital N1". During the study, 9 visits of patients were carried out (table 1). The observation period was 6 months. The research groups are presented as follows: group 1 (main) - 30 patients with habitual miscarriage and chronic endometritis in aged 21 to 41 years who underwent standard antibacterial treatment in combination with the drug derinat® solution for intramuscular injection of 15 mg / ml (vial), manufacturer 000 "Immunolex", Russia) 5 ml (75 mg) 1 time per a day after 48 hours for 10 days, 10 injections per course; group 2 (control) - 30 patients with habitual miscarriage and chronic endometritis aged 21 to 41 years of age who underwent standard antibacterial treatment without the complex use of immunomodulating therapy. Antibacterial drugs are used according to the results of bacteriological studies according to the approved treatment regimens for STDs (KP approved by the Expert Commission, protocol No. 10 of July 4, 2014).

The morphological research method was confocal immunohistochemical analysis of expression of TLR9 using monoclonal antibodies (Anti-TLR9 antibody). An endometrial biopsy was performed on all women using vacuum aspiration of the endometrium with a Pentrafast manual vacuum aspirator. The processing of tissue samples in the study was carried out through the AGT 11-FMI-I 4 system and the BenchMark HT immunostainer. Unmasking was carried out on a PTLINK device, En Vision FIDEX buffer Target Retrieval Solution high pH buffer (50x), heating time 20 minutes ($t = 95^{\circ} \text{C}$). Visualization system - Histofine (M according to catalog 414152F), polymer incubation time 20 minutes ($t = 22-24^{\circ} \text{C}$). Chromogen Daco (catalog number K802 (SM803) / 20044820), incubation (time / temperature) - 10 minutes / 22-24 $^{\circ} \text{C}$, without amplification. Sections of the biopsy material were prepared using a Slide 4004M microtome. Morphometry of the preparations was carried out on a Olympus microscope series BX2WI, a hardware-software complex for biological research with a documenting system based on a direct research microscope ZEISS Axio Imager.A2.

Table 1: The algorithm of medical visits in the study

Stage of study	Period of study	Description of the algorithm of actions
1 st visit	1st month observations	screening before to study (day 0) held examination: history a history of chronic endometritis, abortion; intrauterine invasive procedures (curettage of the uterus, administration or VMC extraction); infertility; habitual miscarriage, duration of the disease, information about concomitant diseases and concomitant therapy), evaluation of general indicators (heart rate, blood pressure, body temperature), bimanual study
2 nd visit		Ultrasound of the pelvic organs for 5-7 days of the menstrual cycle
3 rd visit		On the 9-10th menstrual cycle, endometrial biopsy (endometrial vacuum aspiration with Pentcraft manual vacuum aspirator) followed by microbiological (Femoflor) and immunohistochemical (syndecan-1) analysis of the uterine cavity composition, confocal analysis of TLR9 expression
4 th visit		Ultrasound of the pelvic organs on the 22-24 day of the menstrual cycle
5 th visit	2 nd month observations	the use of therapy according to a group of patients from the 1st day of the menstrual cycle
6 th visit	3 rd month observations	Ultrasound of the pelvic organs on the 5-7 day of the menstrual cycle
7 th visit		On the 9-10 th days of menstrual cycle, endometrial biopsy (endometrial vacuum aspiration with Pentcraft manual vacuum aspirator) followed by microbiological (Femoflor) and immunohistochemical (syndecan-1) analysis of the uterine cavity composition, confocal analysis of TLR9 expression
8 th visit		Ultrasound of the pelvic organs on the 22-24 day of the menstrual cycle
9 th visit	4-6 th months observations	control visit during pregnancy (on the 150 ± 30th day of the study)

Evaluation of the effectiveness of therapy based on: restoration of reproductive function (pregnancy, safe pregnancy), endometrial growth according to ultrasound. When interpreting the results, standard variational methods were used. statistics, one-way analysis of variance and pair correlation analysis. Statistical processing of the research results was carried out using packages programs Bxcel 2010, Statistica 10.0, EREV Sot Windows using parametric or non-parametric criteria (Student t-test, as well as Mann-Whitney and Wilcoxon criteria, respectively). The differences were considered statistically significant at $p < 0.05$.

Characterization of the studying drug

The study evaluated the clinical efficacy of Derinat® (an intramuscular solution of 15 mg / ml, vial) in the treatment of chemotherapy associated with infertility in women of reproductive age. The drug belongs to the pharmacotherapeutic group: immunomodulatory agents, regenerant, reparant. According to published data, Derinat® has the properties of an agonist of toll-like receptor agonist 9 (Toll-like receptors 9, TLR 9, CO 289) [11]. The immunomodulating effect is due to the interaction of the active substance of the drug

(cytosine guanine (CG)) with pathogen-recognizing receptors (TLR 9) on immunocompetent cells, which leads to the subsequent activation of a number of immune mechanisms. First of all, stimulation of toll-like receptors in dendritic cells increases their ability to influence the differentiation of T-helpers, towards the formation of type 2 T-helpers (Th2). Under the influence of Th 2, differentiation of B-lymphocytes into plasma cells secreting IgG2, IgG4, IgM occurs. A stimulated derivate through TLR 9 epithelial the cells enhance the secretion of sIgA, which performs both the barrier function and the opsonin function for interaction with the cellular link of the local immune response: macrophages and NK. Thus, stimulation of TLR of 9 macrophages, as well as their IFN γ , leads to activation of three levels of macrophage response and an increase in the reparative potential of cell structures.

RESULTS:

As a result of the studies, it was found that in 100% of cases, as in the first so in the second group of the study, the leading clinical symptom of CE was "unsuccessful attempts of IVF" (table 2).

Table 2: The ratio of clinical symptoms of CE in the study before treatment

Clinical symptom of CE	Group 1 n(%)	Group 2 n(%)
Menstrual irregularities	(80%)	(93%)
Infertility (tube-ovarian causes)	(40%)	(44%)
Infertility (hormonal cases)	(30%)	(24%)
A history of inflammatory diseases of the reproductive system	(65%)	(58%)
Failed IVF attempts	30(100%)	30(100%)

It is also worth noting that none of the patients had a monosymptomatic course of CE. As a rule, polysymptomatic was formed from a combination of 3 or more symptoms such as failed IVF attempts, infertility with tubular-ovarian causes, menstrual irregularities - in 29 (48%) patients of both groups. Or unsuccessful IVF attempts, infertility with hormonal causes, menstrual irregularities - in 21 (35%) women. Inflammatory diseases of the reproductive system were closely associated with infertility (tube-ovarian causes), menstrual irregularities and failed IVF attempts - 19 (32%) women. Immunohistochemical analysis of biopsy material following changes expression of TLR9 in the tissues of patients (table 3).

Table 3: Manifestation of TLR9 Expression in Biopsy Material of Patients in a Study

Controle group		Main group		The average dynamics of changes (before treatment - after treatment, %)
Before treatment (average number of cells / bulk density%)	After treatment (average number of cells / bulk density%)	Before treatment (average number of cells / bulk density%)	After treatment (average number of cells / bulk density%)	
3,3/2,6	3,9/2,9*	3,3/2,4	4,5*/3,6*	73,3/67,6

Note: * - $p < 0.001$

The average number of TLL + cells (expression of TLR9) in the control group of patients amounted to 3.3 units with a bulk density of 2.6%. After the standard treatment of chronic endometritis in the biopsy material of these same patients, the level of TOL + cells (TLR9 expression) increased by 15%, but these changes were unreliable.

Bulk cell density significantly ($p < 0.001$) increased by 12% (from 2.6 to 2.9). The following changes were observed in the group of patients who, together with the standard treatment of chronic endometritis, were supplemented with the course application of Derinat®: the mean number of TOL + cells (TLR 9 expression) increased significantly ($p < 0.001$) after treatment by 36.3% (from 3.3 up to 4.5), and the bulk density of these structures - from 2.4 to 3.6. Thus, an immunohistochemical study showed the efficacy of Derinat® in patients with chronic endometritis: TLR9 expression of immunocompetent cells increased, the clinical picture changed (in 25% of “hopeless” cases, the patients became pregnant).

The obtained results indirectly confirmed the role of the innate immune response in the formation of treatment effects in patients with chronic endometritis.

CONCLUSION:

The results of the study indicate the feasibility of inclusion in the complex therapy of chronic endometritis of the Derinat® preparation, which provides a long-term positive effect (an increase of 36.3% in expression and TOL + cells), restores the main links of immunity (increase in bulk density by 43%) - all this allows to improve the reproductive function of patients who previously IVF was ineffective.

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