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Evaluation of the effect of excess and deficiency of serum hydrogen sulfide on the condition of the vaginal wall of intact rats

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Various pathological conditions can be characterized not only by a decrease or increase in basal levels of hydrogen sulfide in the serum, but also the levels of hydrogen sulfide can modulate the course of the pathological process. The impact of serum hydrogen sulfide on the condition of the intact vaginal wall of rats was evaluated in this study. The aim of the study was to evaluate the effect of excess and deficiency of serum hydrogen sulfide on the condition of the vaginal wall of intact rats. The study was performed on 75 female Wistar rats under 1 year of age and weighing 160.0 to 200.0 grams. All animals were divided into 6 groups: control (intact rats); experimental 1 (H_2S excess); experimental 2 (H_2S deficiency); experimental 3 (intravaginal administration of suppositories with clindamycin); experimental 4 (H_2S excess + suppositories with clindamycin); experimental 5 (H_2S deficiency + suppositories with clindamycin). The levels of serum hydrogen sulfide were studied, as well as microscopic examination of the structure of the vaginal wall and determination of the levels of $TNF-\alpha$ and $IL-1\beta$ in tissue homogenate were performed. In experimental groups 3, 4 and 5 all studies were performed in dynamics - 10 minutes, 4, 8 and 24 hours after a single intravaginal administration of clindamycin phosphate. The data were processed using the statistical software package SPSS 20.0 for Windows. Under conditions of both hydrogen sulfide deficiency and excess, no statistically significant changes in $TNF-\alpha$ and $IL-1\beta$ levels in the vaginal wall of intact rats were observed. Also, no changes in the histological structure of the wall were found. Similar data were demonstrated in experimental groups 3, 4 and 5. This picture is explained by the fact that hydrogen sulfide affects various parts of the inflammatory process, while reducing the production of inflammatory mediators. In intact tissues, in the absence of an inflammatory process, there is no point of application of hydrogen sulfide, and therefore no significant changes are observed. Thus, both excess and deficiency of serum hydrogen sulfide do not affect the condition of the vaginal wall of intact rats.

Keywords: hydrogen sulfide, vaginal wall, $TNF-\alpha$, $IL-1\beta$, clindamycin phosphate, rats.

Introduction

Despite the constant progress in clinical microbiology and pharmacology of antibacterial drugs, inflammatory diseases of the lower genital tract continue to occupy a leading place in the structure of obstetric and gynecological pathology. The most significant of these is bacterial vaginosis [1, 15].

This pathology is a serious health problem for women of reproductive age, their children and partners, as bacterial vaginosis is associated with adverse effects on reproductive health, such as pelvic inflammatory disease, miscarriage, premature birth, and can lead to increased risk of human immunodeficiency virus [7, 22, 23].

The social and practical significance of this pathology encourages the search for new mechanisms of control and modulation of the inflammatory process in the lower genital tract. One such modulator of the inflammatory process is hydrogen sulfide.

Since 1777, when the Swedish chemist Carl Wilhelm Scheele first synthesized hydrogen sulfide (H_2S), our ideas about this compound have undergone significant transformation. Hydrogen sulfide, which from the beginning was considered only as a highly toxic exogenous product of protein breakdown [13], is now recognized as one of the important endogenous factors in maintaining homeostasis.

Today, this compound belongs to the family of gas transmitters, which also includes nitrogen monoxide (NO) and carbon monoxide (CO). Hydrogen sulfide is involved in the regulation of vascular tone, neuromodulation, cytoprotection, inflammation, apoptosis and other processes [6, 18, 21].

For many years, various studies have indicated the role of hydrogen sulfide in the inflammatory process. Reactive forms of oxygen from activated neutrophils can oxidize H₂S to form sulfite, which further enhances the regulation of leukocyte adhesion and neutrophil function by activating beta-integrin Mac-1 (CD11b/CD18) and Ca²⁺/calmodulin dependent protein kinase, respectively [3, 4]. In addition, hydrogen sulfide has been shown to induce short-term granulocyte survival by inhibiting the cleavage of caspase-3 and mitogen-activated protein kinase p38 (MAPK) and thus contributing to the bactericidal activity of neutrophils [24].

It is proved that not only various pathological conditions can be characterized by a decrease or increase in the basal content of H₂S in blood plasma, but also the level of hydrogen sulfide itself can modulate the course of the pathological process [29].

At present, the scientific literature has virtually no information on the peculiarities of gynecological inflammatory diseases in conditions of excess and deficiency of serum hydrogen sulfide.

However, the study of the peculiarities of the inflammatory process in conditions of excess and deficiency of hydrogen sulfide, requires a preliminary study of the influence of similar factors on intact tissues.

That is why, in our opinion, it is important to study the condition of the intact wall of the vagina in conditions of excess and deficiency of serum hydrogen sulfide.

The aim of the study was to evaluate the effect of excess and deficiency of serum hydrogen sulfide on the condition of the vaginal wall of intact rats.

Materials and methods

The experimental study was performed on the basis of a research laboratory of preclinical study of pharmacological substances of National Pirogov Memorial Medical University, Vinnytsya.

All experiments were performed in accordance with the "Regulations on the use of animals in biomedical experiments" with the permission of the Bioethics Committee and in accordance with the provisions of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 "On the protection of animals used for scientific purposes".

The study included 75 female Wistar rats under 1 year of age and weighing 160.0 to 200.0 grams (180.5±12.6 grams).

All rats were randomly divided into 6 groups:

control group (n=5) - intact animals;

experimental group 1 (n=5) - rats with excess hydrogen sulfide;

experimental group 2 (n=5) - rats with hydrogen sulfide

deficiency;

experimental group 3 (n=20) - rats, which were intravaginally administered clindamycin phosphate in the form of suppositories;

experimental group 4 (n=20) - rats with excess hydrogen sulfide, which were intravaginally administered clindamycin phosphate in the form of suppositories;

experimental group 5 (n=20) - rats with hydrogen sulfide deficiency, which were intravaginally administered clindamycin phosphate in the form of suppositories.

Groups with intravaginal administration of clindamycin phosphate in the form of suppositories were included in the study, as this antibiotic is the drug of choice and an integral part of the comprehensive treatment of inflammatory diseases of the vagina [14, 27]. Although clindamycin phosphate does not have a local irritant or anti-inflammatory effect [19], its effect on the vaginal wall in conditions of excess or deficiency of hydrogen sulfide requires careful study.

To synchronize estrous cycles, all test animals were injected subcutaneously with 17 α -hydroxy-6 α -methylprogesterone (Pfizer Inc., USA), diluted in Ringer's solution lactate at a dose of 12 mg per test animal 7 and 3 days before the study date [5].

The dose of 12 mg per experimental rat was determined by recalculating the dose of the drug for mice. The dose was recalculated according to the method proposed by Nair A. B. and Jacob S. [20].

The synchronization of estrous cycles was checked immediately before the start of a set of manipulations to study the condition of the vaginal wall, by microscopy of smears from the vagina of experimental animals according to the criteria described by Fu X. Y. and co-authors [9].

Excess hydrogen sulfide in animals was created by intraperitoneal administration of donor H₂S - sodium hydrosulfide (Sigma-Aldrich, USA) at a dose of 1.5 mg/kg on 0.1 M phosphate buffer (pH 7.4), as a freshly prepared aqueous solution at the rate of 0.1 ml per 100 g weight rat, once a day for 5 days immediately preceding the date of the study of the vaginal wall [28].

Hydrogen sulfide deficiency was created by the introduction of a specific inhibitor of cystathionine gamma-lyase - D, L-propargylglycine (Sigma-Aldrich, USA) at a dose of 50 mg/kg as a freshly prepared 5 % aqueous solution at the rate of 0.1 ml per 100 g of rat weight once a day for 5 days immediately preceding the date of the study of the condition of the vaginal wall [28].

Clindamycin phosphate (Pfizer Inc., USA) was administered to rats intravaginally as micro-suppositories. The dose of the drug according to the conversion tables was 1.5 mg [20]. Given that the suppository contains 100 mg of active substance and weighs 2.5 grams, and based on the fact that the active substance is evenly distributed in the suppository, to provide an equivalent dose (1.5 mg of

In each group and at each time point, the condition of the vaginal wall was studied in 5 rats.

To assess the condition of the vaginal wall, a

microscopic examination of its structure was performed and the levels of TNF- α and IL-1 β in the tissue homogenate were determined.

The study of the condition of the vaginal wall in experimental groups 2, 4 and 6 was performed in the dynamics - 10 minutes, 4, 8 and 24 hours after a single intravaginal administration of clindamycin phosphate, according to the method of cervical toxicity and inflammation of local intravaginal dosage forms described by Catalone B. J. and co-authors [5] and adapted by us for research on laboratory rats.

Blood sampling to determine the level of hydrogen sulfide was performed by percutaneous puncture of the heart under ketamine anesthesia at the rate of 0.22 ml of ketamine per 100 grams of body weight of the experimental animal. The content of hydrogen sulfide in blood serum was determined by spectrophotometric method in the reaction between sulfide anion and para-phenylenediamine hydrochloride in an acidic environment in the presence of iron ions (III) [28].

After blood collection, rats were removed from the experiment by translocation of the cervical vertebrae.

The vagina was removed and washed from the remnants of blood in saline, followed by careful drying of the tissues with a sterile napkin. The removed organ was cut in the longitudinal direction into 4 equal parts. Three parts were fixed in 10% neutral formalin solution and sent for microscopic examination. The latter fragment was homogenized by adding 1 ml of phosphate buffer in an automatic tissue disaggregator Medimax (CTSV, Italy) using disposable Medicon cartridges (Becton Dickinson, USA) with pyramidal blades of 35 μ m. The tissue homogenate was then filtered through Filcon filters (Becton Dickinson, USA) with a pore diameter of 30 μ m.

The levels of TNF- α and IL-1 β in the vaginal tissue homogenate filtrate were investigated by enzyme-linked immunosorbent assay using the Rat TNF- α ELISA kit and Rat IL-1 β ELISA Kit (CUSABIO, China).

After fixing the preparation of the vagina in a 10 % solution of neutral formalin for 3 days, the preparations were prepared according to standard methods. Paraffin sections 5-7 μ m thick were stained with hematoxylin and eosin. Microscopy and photographing of histological specimens were performed using a light microscope OLIMPUS BX 41 at magnifications of 40, 100, 200, 400 and

1000. Microscopy assessed the condition of all layers of the body. Images were obtained and processed, and morphometry was performed using the program "Quick PHOTO MICRO 2.3".

The obtained data were processed using the statistical software package SPSS 20.0 for Windows.

Results

In all groups and at all times of the study, no behavioral changes were observed in experimental animals. All rats maintained normal motor activity. Consumption of feed and water met the standards for this species.

Evaluation of the synchronization of estrous cycles showed the presence of the same changes in the microscopic examination of vaginal swabs. The smears showed a small number of cells, with a predominance of small epitheliocytes with nuclei and almost complete absence of neutrophils, large epitheliocytes with nuclei and non-nuclear keratinized epitheliocytes. According to the criteria described by Fu X. Y. and co-authors [9], this picture was identified by us as characteristic of proestrus.

Serum hydrogen sulfide levels in groups of experimental animals are shown in Figure 1.

The level of serum hydrogen sulfide in the control group was 75.60 ± 5.05 μ mol/L. In experimental group 1, where an excess of hydrogen sulfide was artificially created, this indicator significantly ($p < 0.01$) increased by 16.3 % compared with the control group. In experimental group 3 (hydrogen sulfide deficiency) there was a significant ($p < 0.01$) decrease in serum H₂S by 22.4 %.

The serum hydrogen sulfide values in animals of experimental groups 3, 4 and 5 did not differ significantly ($p > 0.05$) from similar indicators in the control group and experimental groups 1 and 2, respectively.

Macroscopic evaluation of vaginal preparations revealed no changes. The mucosa of the organ in all samples had a normal color, hemorrhage, visible damage, ulcers or punctate erosions were not observed.

Microscopic examination of the vaginal wall in rats of all groups showed a typical pattern characteristic of the proestrus phase (Fig. 2). The integrity of the vaginal wall is not violated. The mucous membrane is represented by a multilayered epithelium with cuboid and ovoid cells. Epithelial degeneration and desquamation are virtually absent. Single keratinized epitheliocytes are observed.

Fig. 1. Serum hydrogen sulfide levels in groups of experimental animals.

Fig. 2. Typical morphological picture of the vaginal wall of experimental rats in the proestrus phase.

Table 1. TNF- α and IL-1 β levels in the vaginal wall of intact rats, as well as in conditions of excess and deficiency of hydrogen sulfide.

Group	TNF- α , pg/mL	IL-1 β , pg/mL
Control	45.25 $\pm$ 5.68	218.5 $\pm$ 20.3
Experimental group 1	44.94 $\pm$ 5.16	215.4 $\pm$ 21.6
Experimental group 2	47.12 $\pm$ 5.75	223.6 $\pm$ 18.0

Fig. 3. Dynamics of changes in TNF- α levels in the vaginal wall.

Fig. 4. Dynamics of changes in IL-1 β levels in the vaginal wall.

Mitotic figures are observed. Polymorphic structures are present in some places.

As can be seen from Table 1, the numerical values of both TNF- α and IL-1 β are slightly reduced under conditions of excess hydrogen sulfide in the body of experimental rats and increase under conditions of its deficiency. However, it should be noted that these level fluctuations did not exceed

4 % relative to those in intact rats and did not differ significantly from them, as well as from each other in the statistical analysis ($p > 0.05$).

The data obtained by analyzing the dynamics of changes in the levels of TNF- α and IL-1 β in the vaginal wall using the intravaginal form of clindamycin phosphate are graphically depicted in the diagrams (Fig. 3, 4).

As can be seen from Figures 3 and 4, in all groups there were slight fluctuations in the studied indicators. However, it should be noted that no significant statistical differences between the indicators of different groups at each time point were demonstrated ($p > 0.05$). In addition, there was no clear tendency to change over time in each of the groups ($p > 0.05$).

Discussion

At present, one of the most probable and well-founded mechanisms of protective effect of hydrogen sulfide in the inflammatory process is the blockade of the signaling pathway of mitogen-activated protein kinase p38 (MAPK) [25, 30]. Regulation of MAPK activity mediates inflammation and/or oxidation, thus exacerbating tissue damage. Hydrogen sulfide, by inhibiting the activation of the MAPK signaling pathway, indirectly protects tissues from inflammation. The expression of MAPK and ERK 1/2 (kinases regulated by extracellular signals) was reduced with the use of hydrogen sulfide in a model of inflammation and hypoxic damage in cell cultures [16].

In addition to protein kinases, nuclear factor kappa-B (NF- κ B) and nuclear factor 2 associated with erythroid 2 (Nrf2) play an active role in the development of the inflammatory process [2]. NF- κ B is responsible for the transcription of many genes involved in inflammation and is activated in many acute and chronic inflammatory diseases, such as sepsis, inflammatory bowel disease, arthritis, asthma. Nrf2 belongs to a family of proteins that regulate endogenous antioxidant protection and promotes the transcription of a set of detoxifying genes that encode proteins (such as enzymes, drug transporters, antiapoptotic proteins, and proteasomes) involved in the regulation of physiological and pathophysiological cellular responses to oxidants and xenobiotics.

F. Benedetti and co-authors [2] demonstrated that hydrogen sulfide not only inhibits NF- κ B activation and nuclear translocation by reducing the transcription of proinflammatory genes, but also enhances Nrf2 function by activating a cascade of enzymes such as hemoxygenase-1 (HO-1) superoxide dismutase-1 (SOD1).

The main substrates for endogenous hydrogen sulfide in tissues are sulfur-containing amino acids - L-cysteine and L-homocysteine, its main enzymes-producers are pyridoxal phosphate-dependent enzymes cystathionine- β -synthase, cystathionine- γ -lyase, as well as cysteine aminotransferase [26].

The main reactions that ensure the formation of hydrogen sulfide in animal and human tissues include

[26]: desulfurization of L-cysteine to pyruvate with cystathionine- γ -lyase; condensation of L-homocysteine with L-cysteine and desulfurization of L-cysteine to L-serine with the participation of cystathionine- β -synthase; transamination of L-cysteine with α -ketoglutarate with the participation of cysteine aminotransferase with the formation of 3-mercaptopyruvate, from which further H₂S is released with the participation of 3-mercaptopyruvate sulfurtransferase.

The introduction of sodium hydrosulfide as a donor of hydrogen sulfide and propargylglycine as a selective inhibitor of cystathionine- γ -lyase synthesis (key enzyme-producer of H₂S) allows to significantly change the levels of serum hydrogen sulfide and modulate a wide range of physiological, and pathophysiological processes [6, 12, 18, 21].

The methods used in our study [28] allowed to create a statistically significant excess and deficiency of hydrogen sulfide in experimental rats. In conditions of both deficiency and excess of hydrogen sulfide, statistically significant changes in the levels of TNF- α and IL-1 β in the vaginal wall of intact rats could not be achieved. Regardless of the level of serum hydrogen sulfide, we did not observe any changes in the histological structure of the vaginal wall.

This picture is explained by the fact that hydrogen sulfide affects various parts of the inflammatory process, while reducing the production of inflammatory mediators. In intact tissues, in the absence of an inflammatory process, there is no point of application of hydrogen sulfide, and therefore no significant changes are observed.

The absence of pro- and/or anti-inflammatory effect in the modulation of serum hydrogen sulfide levels in rats without signs of inflammation has been repeatedly

described in the scientific literature. Thus, other authors have demonstrated the absence of morphological changes and changes in the levels of inflammatory mediators (TNF- α and/or IL-1 β) in the study of the hippocampus, kidneys, heart, lungs [10, 11, 17, 25, 30].

In addition, both in conditions of deficiency and in conditions of excess hydrogen sulfide, no statistically significant differences in the levels of the studied mediators of inflammation were demonstrated with topical application of clindamycin phosphate in the form of suppositories. The absence of changes in the vaginal wall in these groups of experimental rats was also not demonstrated in the morphological study.

The lack of dynamics of markers of inflammation and morphological changes of the vaginal wall, in our opinion, can be explained by the gentle effect of clindamycin phosphate on the vaginal mucosa and the lack of local irritant effect [8, 19].

The effect of excess and deficiency of hydrogen sulfide on the condition of the vaginal wall in the local inflammatory process requires further careful research.

Conclusions

1. Both excess and deficiency of hydrogen sulfide do not affect the condition of the vaginal wall in intact rats, as evidenced by the absence of changes in local levels of TNF- α and IL-1 β , as well as microscopic changes in morphological examination.

2. Regardless of the background level of hydrogen sulfide, clindamycin phosphate when administered intravaginally in the form of suppositories does not affect the condition of the vaginal wall of intact rats.

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