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Effects of plasma acid on rat uterine tissue *in vitro*

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ABSTRACT

The aim of the study was to evaluate the effect of plasma acid on the uterine tissue of laboratory animals *in vitro*.

Materials and methods. Treatment of dimethyl sulfoxide – water solution and water for injections with a spark discharge in air resulted in a decrease in pH, which contributed to generation of plasma acid in the solutions. We incubated uterine tissues *in vitro* in plasma acid at room temperature for 30 minutes. The treated tissues were examined histologically and immunohistochemically.

Results. We showed that plasma acid had pronounced biological activity. Immunohistochemistry was used to show that, depending on the type of a solution, plasma acid altered generation of nitrosative damage products (3-NT) and oxidative DNA damage (8-OHdG) and modulated the number of cells with high proliferative potential (including $CD133^+$ cells) and production of vascular endothelial growth factor (VEGF). These effects contributed to the general cytotoxicity of plasma acid solutions.

Conclusion. During 30-minute exposure *in vitro*, plasma acid prepared from the dimethyl sulfoxide (DMSO) – water mixture exhibits various biological effects in uterine tissue samples obtained from experimental animals. Plasma-treated water exerts cytotoxic effects associated with oxidative DNA damage and promotes induction of pro-angiogenic activity in the uterine tissue. Plasma-treated DMSO does not have a cytotoxic effect. It inhibits cell proliferation, reducing the population of $CD133^+$ cells and VEGF production in the tissue.

Keywords: uterine tissue, plasma acid, cytotoxicity

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Conformity with the principles of ethics. The study was approved by the Bioethics Commission on the Use of Laboratory Animals at the local Ethics Committee and by the local Ethics Committee at V.F. Voino-Yasenetsky Krasnoyarsk State Medical University.

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Эффекты плазменной кислоты на ткани матки крыс *in vitro*

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РЕЗЮМЕ

Целью исследования является оценка влияния плазменной кислоты на ткани матки лабораторных животных *in vitro*.

Материалы и методы. Обработка раствора диметилсульфоксида в воде и воды для инъекций искровым разрядом в атмосфере приводила к снижению pH, что соответствовало формированию в растворах плазменной кислоты. Мы инкубировали ткани матки *in vitro* в плазменной кислоте при комнатной температуре в течение 30 мин. Обработанные ткани исследовались гистологически и иммуногистохимически.

Результаты. Нами показано, что плазменная кислота обладает выраженной биологической активностью. Иммуногистохимическим методом мы зарегистрировали, что, в зависимости от типа раствора, плазменная кислота изменяет в клетках формирование продуктов нитрозативного повреждения белков (3-NT) и окислительного повреждения ДНК (8-OHdG), модулирует количество клеток с высоким пролиферативным потенциалом (в том числе CD133+ клеток), продукцию сосудисто-эндотелиального фактора роста (VEGF), что соответствует суммарному цитотоксическому эффекту вида раствора плазменной кислоты.

Заключение. Плазменная кислота, приготовленная на основе воды и диметилсульфоксида, проявляет различные биологические эффекты в образцах ткани матки экспериментальных животных при 30-минутной экспозиции *in vitro*. Вода, обработанная плазмой, реализует цитотоксический потенциал, связанный с окислительным повреждением ДНК, а также способствует индукции проангиогенной активности в ткани. Диметилсульфоксид, обработанный плазмой, не вызывает цитотоксического действия, подавляет пролиферацию клеток, снижая популяцию CD133+ клеток, а также продукцию VEGF в ткани.

Ключевые слова: ткани матки, плазменная кислота, цитотоксичность

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Соответствие принципам этики. Исследование одобрено представителями биоэтической комиссии по работе с лабораторными животными при локальном этическом комитете и локальным этическим комитетом КрасГМУ.

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INTRODUCTION

In recent years, there has been an increasing interest in the use of gas discharge plasma when performing surgical interventions in gynecology. Argon plasma for coagulation and ablation is the leading technological solution in this area [1, 2]. The impact of cold atmospheric plasma at the final stage of reconstructive and plastic uterus operations (applied directly to the suture area) enhances repair of the myometrium and, consequently, formation of a consistent scar [3]. Interaction of a plasma jet with air entrained by a laminar flow results in an increased concentration of active particles, many of which, having good solubility, increase the concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the tissue [4].

For standard coagulation and ablation, it is impossible to exclude the accumulation of ROS and RNS in tissues and the formation of the so-called plasma acid, as well as a significant decrease in pH (in case of prolonged exposure to plasma) [5, 6]. A significant increase in the RNS concentration in plasma devices for medical applications is achieved by replacing argon with air, which is used in the Plazon system [7] or kINPen MED® [8]. The results of assessing the possibility of using the Plazon unit during surgeries on the uterus and its appendages were discussed in the literature [3, 9–13]. However, the molecular and cellular mechanisms in uterine tissues induced by plasma have not been studied. The effect of plasma acid on the modification of proteins and lipids was described [6], but no data on its effects on cells of the reproductive system, including the uterus, are available in the literature. This prevents from a reasonable, scientifically grounded understanding of the safety and effectiveness of the use of plasma acid in clinical practice.

The aim of the study was to evaluate the effect of plasma acid on the uterus of laboratory animals *in vitro*.

MATERIALS AND METHODS

The study was carried out on female Wistar rats aged 3 months (n = 16), weighing 180–200 g. The selection of female rats was carried out at the end of proestrus and the beginning of estrus (by examining the vaginal opening and evaluating vaginal smears). Then surgery was performed, and after 1 day, the uterus was removed from the euthanized animals. Uterus samples after exposure to plasma acid were fixed in a 4% buffered paraformaldehyde solution and embedded into paraffin blocks for further sectioning.

Plasma acid was prepared by irradiating 5 ml of water for injections or 50% dimethyl sulfoxide (DMSO) – water solution with a nanosecond-pulsed spark discharge in a 50 ml leak-proof container. A spark discharge with a discharge gap length of 20 mm was created by a nanosecond high-voltage pulse generator with a pulse energy of 0.2 J and a voltage of 40 kV. When pH = 2 was reached, the treatment was stopped. The prepared solution was used to incubate freshly isolated uterine tissues of laboratory rats for 30 minutes.

The expression of DAPI, VEGF, CD133, 8-hydroxy-2-deoxyguanosine, 3-nitrotyrosine, and Ki67 in tissues was assessed according to standard protocols for direct and indirect immunohistochemistry (immunofluorescent variant). Microphotography of 10 different fields was performed at x175 magnification using the ZOE™ Fluorescent Cell Imager (Bio-Rad, USA). Digital images were processed using the ImageJ software. Each field was divided into nine frames, empty fields outside the tissue sample were rejected after manual sorting. Then the plugin [14] in the ImageJ software was used for automatic recognition and counting of labels.

We evaluated the median expression and the interquartile range of the number of labels in one frame Me (Q_1; Q_3). The method of non-parametric statistics was used for statistical processing of the obtained data: comparison of several independent samples (the Kruskal – Wallis test) with the subsequent use of a module for pairwise comparison of medians among the samples using the Statsoft Statistica 12.0 package.

RESULTS

Data on the expression of the markers used are shown in the diagrams (Figure). They demonstrate median values with the interquartile range in the control group and two experimental groups exposed to plasma acid (plasma-activated DMSO, plasma-activated water). We used the method of non-parametric statistics (the Kruskal – Wallis test).

The analysis of the number of 4',6-diamidino-2-phenylindole (DAPI)-stained ($DAPI^+$) cells was used to assess the effect of plasma acid on cell survival in a tissue sample in the tested solutions. At the same time, a smaller number of $DAPI^+$ cells in the tissue sample should be regarded as the result of death, exfoliation, and destruction of damaged cells upon exposure to plasma acid in the tested solutions. We

found that when using plasma-activated water, the number of DAPI+ cells was significantly smaller n_{DAPI+}=311 (233;368) compared with the control group n_{DAPI+}=350 (284; 435) (z = 7.638; p < 0.001), thereby suggesting the cytotoxic effect of the solution. In the tissue samples incubated in a plasma-activated DMSO solution, the median number of cells n_{DAPI+}= 351 (267; 414) did not significantly differ from the control group (z = 1.443; p = 0.44), so the cytotoxic effect of this solution should be considered unproven.

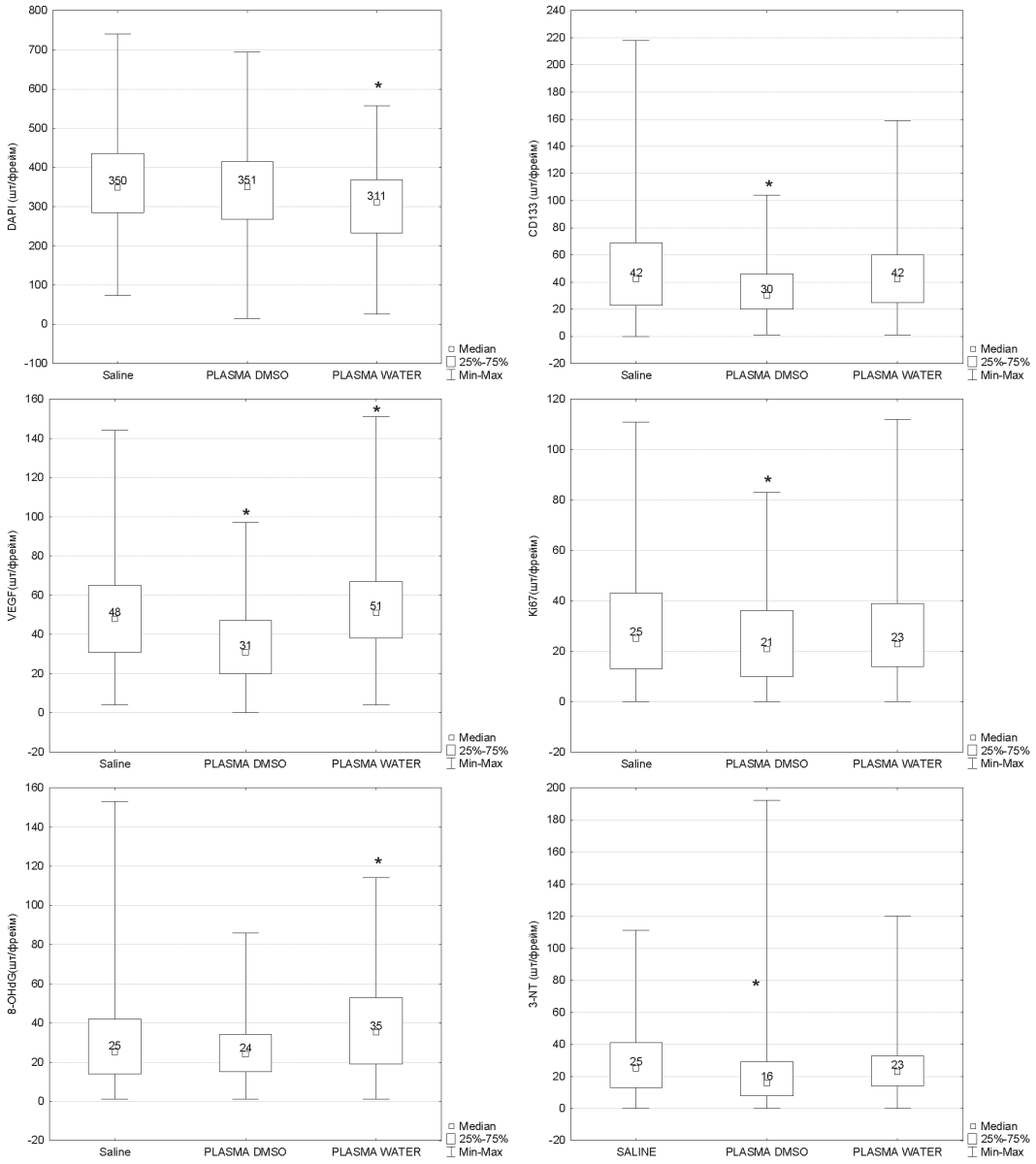

Figure. Results of the analysis of DAPI+ cell number, expression of VEGF, Ki67, CD133, 3-NT, and 8-OHdG during incubation of rat uterine tissue samples in solutions treated with spark discharge plasma, *Me* (Q_1; Q_3): * experimental groups with p < 0.05 compared with the control group; plasma-activated DMSO – 50% aqueous solution, plasma-activated water – water for injections compared with the control (saline)

Expression of 8-hydroxy-2-deoxyguanosine (8-OHdG) as a marker of oxidative DNA damage under the effect of plasma-activated water was significantly higher $n_{8\text{-OHdG}}$ = 35 (19; 53) (z = 5.98; p < 0.001) compared with the control group $n_{8\text{-OHdG}}$ = 25 (14; 42). In the group of samples incubated in plasma-activated DMSO solution, the expression level of $n_{8\text{-OHdG}}$=24 (15; 34) did not significantly differ from the control group (z = 2.16; p = 0.09). In the tissue samples incubated in the plasma-activated DMSO solution, tissue expression of 3-nitrotyrosine (3-NT) as a marker of nitrosative protein damage was significantly lower

$n_{3\text{-NT}}$ = 16 (8; 29) (z = 8.35; p < 0.001) compared with the control group $n_{3\text{-NT}}$ = 25 (13; 41), while plasma-activated water did not have such an effect $n_{3\text{-NT}}$ = 23 (14; 33) (z = 1.64; p = 0.29). It is worth noting that these effects of DMSO were accompanied by a lower proliferative potential of cells, as it could be concluded from the expression of Ki67 protein, n_{Ki67}= 21(10; 36) (z = 3.47; p = 0.0015) compared with the control group n_{Ki67}= 25 (13; 43). The cytotoxic effect of plasma-activated water was not associated with significant changes in cell proliferation n_{Ki67} = 23 (14; 39) (z = 1.18; p = 0.71).

To what extent do the revealed effects correspond to the response of cells with pro-angiogenic potential in the tissue? When fragments of the uterine wall were treated with the plasma-activated DMSO, there was a significantly smaller median number of cells expressing CD133, n_{CD133} = 30 (20; 46) (z = 5.78; p < 0.001) compared with the control group, n_{CD133} = 42 (23; 68). In the group of samples treated with plasma-activated water, n_{CD133} = 42 (25; 60), no significant differences were found (z = 1.30; p = 0.57). CD133 is a marker of stem and progenitor cells in the tissue, whereas VEGF expression characterizes the pro-angiogenic potential of the tissue and may also reflect the degree of hypoxic cell damage. We found that the median value of VEGF expression in the endometrium and uterine stroma was lower under the effect of plasma-activated DMSO, n_{VEGF} = 31 (20; 47) (z = 8.13, p < 0.001), or higher under the effect of plasma-activated water, n_{VEGF} = 51 (38; 67) (z = 3.36, p = 0.003) compared with the control group, n_{VEGF} = 48 (31; 65).

DISCUSSION

We found that plasma-activated DMSO did not have a pronounced cytotoxic effect and did not induce oxidative DNA damage or nitrosative damage to cellular proteins, but reduced the proliferation of cells (including $CD133^+$ cells) and suppressed VEGF expression, thereby affecting proliferative and pro-angiogenic activities. On the contrary, plasma-activated water demonstrated a pronounced cytotoxic effect due to the induction of oxidative DNA damage and VEGF expression. In general, this indicates that the cytotoxic effect of plasma-activated water was more determined by the action of ROS, but not by RNS. Interestingly, plasma-activated DMSO prevented the development of physiologically mediated nitrosative damage to cellular proteins. These differences in the effects of solutions were probably due to their physical and chemical properties that determined the ability to cross a cell membrane and / or trap ROS / RNS in the solution.

CD133 (prominin) is a stem cell marker present in various tissues, but it can also be detected in adult differentiated cells. It is believed that its expression is high in the endometrium [15; 16]. It is known that a decrease in CD133 expression is a consequence of an increase in the activity of the mTOR protein which, in turn, is activated by oxidative stress [17]. However, the observed decrease in the number of CD133+ cells under the effect of plasma-activated DMSO cannot be explained by oxidative stress (with no signs of oxidative DNA damage or general cytotoxicity). Presumably, it is associated with a decrease in VEGF expression. It is known that $CD133^+$ cells are very sensitive to the effects of this growth factor in various tissues, and CD133 can control the release of VEGF [18]. Hypoxia [19] and oxidative stress [20] are known to be powerful triggers of VEGF expression. Increased expression of VEGF was recorded in endometriosis [21]. In addition to stimulating angiogenesis, VEGF activates the mitochondrial biogenesis through the mTOR-dependent pathways as a response to insufficient mitochondrial activity due to oxygen deficiency, damage to mitochondria, or an increased demand for cells to produce ATP [22]. Thus, the increase in VEGF expression in cells under the effect of plasma-activated water might be associated with the development of mitochondrial dysfunction, oxidative stress, and hypoxia in the tissue. Indeed, plasma-activated water caused a cytotoxic effect accompanied by the formation of 8-OHdG, so an increase in VEGF expression is expected.

The two tested solutions had different effects on cell proliferation. Plasma-activated water had no significant effect, while plasma-activated DMSO inhibited Ki67 expression in tissue samples. On the one hand, this effect could be associated with the fact that actively proliferating cells predominantly die under the effect of plasma-activated DMSO. However, taking into consideration that DMSO generally showed no cytotoxic effect, we may assume that a decrease in the number of $Ki67^+$ cells in the tissue was associated with a decrease in the number of $CD133^+$ cells and suppression of VEGF expression.

CONCLUSION

Plasma-activated water and plasma-activated DMSO exhibited various biological effects in the uterine tissue samples of experimental animals at

30-minute exposure *in vitro*. Plasma-activated water demonstrated cytotoxicity associated with oxidative DNA damage and promoted pro-angiogenic activity in the tissue. Plasma-activated DMSO did not exert cytotoxic effects, but inhibited cell proliferation, reducing the number of $CD133^+$ cells and production of VEGF in the tissue. Generally, plasma acid solutions can be considered as promising agents for local use in gynecologic pathology.

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Authors contribution

Salmin V.V., Makarenko T.A., Salmina A.B. – conception and design, analysis and interpretation of data, substantiation of the manuscript and critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Perevertov T.A., Muradyan G.A., Gudkova E.S., Epova A.S., Kutyakov V.A., Lychkovskaya E.V., Chekisheva T.N., Semichev E.V., Malinovskaya N.A., Medvedeva N.N. – carrying out of the experiments.

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