Influence of ozonated flaxseed oil on microorganisms, endometrium and mammary gland in cows

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Abstract. The search for alternative drugs for treating inflammatory diseases of bacterial etiology in high yielding cows that do not cause antibiotic resistance and do not reduce the quality of the obtained products is an urgent task for veterinary obstetrics. As one of this remedy, the authors have tested ozonated flaxseed oil (OFO), obtained by bubbling flaxseed oil with an ozon-oxygen mixture for four hours by means of ceramic spray with the ozone concentration being 30 mg / liter. During the bacteriological studies, it was found out that a two-hour incubation of the drug with a bacterial culture inhibits the growth of Staphylococcus aureus and Escherichia coli at a dilution of 10^8 microbial bodies (m.b.) in ml, bacteria of the genus Citrobacter at a concentration of 10^6 m.b. and Streptococcus agalactiae at the dilution of 10^7 m.b. in ml. The therapeutic efficiency of OFO was evaluated on the basis of using it for the cows of the Kholmogory Holstein breed, with the signs of postpartum septic catarrhal endometritis. For this purpose, 2 groups of animals were formed on the basis of analogues. For treating the cows of the experimental group the OFO has been used, and the cows of the control group were treated with the antibiotic containing the drug tylosinocar. For 5 months of observation, all animals were fertilized in the control group, and in the group where OFO was used this number was 93.3 %. Moreover, in the experimental group 47 % of the cows were fertilized after the first insemination, which is 27 % more in comparison with the control group. The duration from calving to fertilization in the group where OFO was used averaged 104 days, which is 21 days less ($P < 0.05$) compared to the control group. Studies have shown that the use of OFO did not lead to the inhibitors emergence in milk in the experimental group of cows.

1 Introduction

The impaired reproductive function in cows is currently the main problem for the further development of dairy farming [1–3]. One of the causes leading to infertility and reducing the rate of herd reproduction is postpartum complications, among which endometritis is diagnosed most often. [4–6]. As a rule, acute inflammation of the endometrium results from the contamination of the uterine cavity by opportunistic microflora (bacteria, fungi, etc.) [7, 8]. For treating the inflammatory diseases of the reproductive tract, antibiotics of various groups are usually used, which leads to the emergence of resistant strains of microorganisms and causes culling of products during the treatment period. Therefore, the search for alternative agents competing with the use of well-known etiotropic drugs that do not cause antibiotic resistance and do not reduce the quality of the obtained products is an urgent task for veterinary obstetrics. Developing the methods of ozon therapy based on vegetable oils [9, 10] plays a special role in this field of research.

The purpose of the research was to study the antimicrobial characteristics and efficiency of using the ozonated flaxseed oil in postpartum purulent - catarrhal endometritis in cows.

2 Materials and methods

Experimental and clinical studies were conducted in 2016, 2017, 2018. And they have been conducted this year. To enrich seed oil with ozone, we have used the certified medical ozone generator “A-s-GOKSf-5-02-OZON” produced by JSC “Electric Machine-Building Plant named after LEPSE”, the city of Kirov. Ozone was obtained from chemically pure oxygen. Raw materials in the volume of 400.0 ml were bubbled with the ozone-oxygen mixture by means of a ceramic atomizer, with the ozone concentration being 30 mg / liter at an output during 4 hours.

At the first stage, the bactericidal properties of ozone-treated oils were determined on museum strains of Staphylococcus aureus ATCC 25923 No. 201189 and Escherichia coli ATCC 25922 No. 240533. Then to study the antibacterial characteristics of OFO we used the microorganisms most often isolated from exudate from cows with mastitis and endometritis: Staphylococcus aureus, Citrobacter spp., Escherichia coli, Streptococcus agalactiae. The evaluation was carried out according to the method developed by Platonov V.A. et al., which was modified by us [8]. To do this, according to the turbidity standard, the bacteria

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suspension was created using the physiological saline with the concentration of 1×10^9 m.b. / ml and also we prepared a series of ten-fold dilutions from 10^9 to 10^2. The bacteria suspension was mixed 1: 1 with the set 50 % emulsion of an ozonated preparation.

The emulsion was obtained by mixing 10.0 ml of oil with 10.0 ml of physiological solution of sodium chloride and adding 0.2 ml of polysorbate 80 emulsifier. The bacteria suspension mixed with the obtained emulsion was incubated for 30 minutes or 2 hours at room temperature, shaking in a shuttle machine, then 0.1 ml of the mixture was plated on meat infusion agar (MIA) and incubated in an incubator at a temperature of 38 ^{0}C. The result was taken into account after 24, 48 and 72 hours by visual counting of colonies. The samples with native oil served as control ones.

To study the influence of intrauterine administration of OLM on subsequent fertilization, three groups of cows have been formed, and the groups included animals on 45–75 days of lactation, that had a yellow body on the ovaries and without clinical reproductive pathology. In cows of all groups, sexual cyclicity was pre-induced by double injection of estrofan in a dose of 3.0 ml intramuscularly with the interval of 14 days.

On the 5th day after the first injection of prostaglandin, the cows of the first group underwent uterine sanitation with the use of OLM at a dose of 30.0 ml (experiment, n = 30), the cows of the second group underwent uterine sanitation with the use of Endometramag K (positive control, n = 25), in the cows of the third groups the reproductive organs were not treated with pharmacological agents (negative control, n = 25).

After the second injection of prostaglandin, with the manifestation of phenomena of the excitement stages of the sexual cycle the cows were artificially inseminated with thawed sperm in a cervical manner with the rectal fixation of the uterine cervix. Immediately after the insemination, all the animals were injected with surfagon in a dose of 5.0 ml.

The therapeutic efficiency of using OFO was studied on cows of the Kholmogory Holstein breed whose productivity is from 4800 to 7200 kg of milk per year. For this purpose, 30 animals of the second and third lactation, with the signs of postpartum purulent-catarrhal endometritis, were selected according to the principle of analogues.

The animals were divided into 2 groups: the experimental group (n = 15), for treating of which we used the ozonated preparation, and the control group (n = 15), in which we used the antibiotic containing the drug tylosinocar. Etiotropic drugs, preheated in a water bath to the temperature of 35-40 ^{0}C, were administered intrauterine using a polystyrene pipette. The volume of the administered drug depended on the uterus size (from 150 to 50 ml), and the interval between administrations was 48 or 72 hours.

The use of antimicrobial agents was carried out before clinical recovery. On the 1st and 3rd day of treatment, the cows of the both groups were injected with the 2 % solution of Sinestrol at a dose of 4.0 ml, and the vitamin-containing preparation of tetrahydrovit at a dose of 10.0 ml. on the 1st and 7th day of treatment.

From the cows included in the experiment, the milk was received in 12, 24, 48, 72 hours after the intrauterine administration of the preparations and it was examined for the presence of inhibitory substances using the Delvotest ® SP-NT test system.

The animals were artificially inseminated in the spontaneous stage of sexual arousal by the cervical method with the rectal fixation of the uterine cervix. The presence of pregnancy was defined on the 30...35th day after the insemination using the ultrasonic scanner Easi-Scan 3.84 (BCF Technology, UK). The animals were observed during five months, the number of fertilized cows was taken into account, including the first insemination, the multiplicity of insemination and the period from calving to fertilization.

In order to study the influence on the mammary gland, the lactating cows of the first group (n = 3) were intracisternally administered with the physiological solution of sodium chloride in the volume of 10.0 ml, heated to 40 °C in the front right quarters after milking (control), the cows of the second group (n = 3) were instilled with OFO (experience). The secretion obtained from the experimental quarters of the udder was investigated before the administration of the preparations and after 12, 24, 36, 48, 72, 96 and 120 hours for maintaining the somatic cells (SC) using the Somatos-Mini device.

The obtained data were verified by the method of variation statistics, the reliability of differences between the compared indicators was proved by using the Student criterion. The digital material was processed using the program “Micro Office 2007”, and the arithmetic mean (M) and standard error (m) were calculated. The differences in the arithmetic mean were considered statistically significant in case of $P \leq 0.05$.

3 Results and discussion

At the first stage of the experimental work, the comparative evaluation of the antibacterial characteristics of various seed oil was carried out after bubbling the ozone – oxygen mixture for 4 hours. The research results are shown in table 1.

As the research results have shown (table 1), while shaking the mixture for 2 hours, the antimicrobial characteristics of ozonated flaxseed, olive and corn oils had the same values and inhibited the growth of museum cultures at their maximum concentration in the dilution corresponding to 108 m. b. / ml.

When shaking the ozonated oils with the suspension of museum cultures for 30 minutes, the highest antimicrobial activity was shown by OFO, which inhibited the growth of Staphylococcus aureus at the concentration of 104 m. b./ml and E. coli at the concentration of 105 m. b/ml. The data obtained can be explained by the peculiarity of the fatty acid composition of flaxseed oil, namely by the presence of a large number of unsaturated bonds, as a result of interaction

with which ozone forms ozonides with antimicrobial effects.

Table 1. Antibacterial characteristics of seed oil treated with ozone in relation to museum strains of bacteria depending on the exposure period

Ozonated oil	The maximum concentration of microbial cells in one ml, at which culture growth is not observed after the exposure with oil			
	Exposure for 120 minutes		Exposure for 30 minutes	
	Staph. aureus	E. coli	Staph. aureus	E. coli
Flaxseed oil	10^8	10^8	10^4	10^5
Olive oil	10^8	10^8	10^3	10^3
Corn oil	10^8	10^8	10^2	10^2
Sun flower oil	10^7	10^7	Growth in all the dilutions	Growth in all the dilutions
Oil of mustard	10^5	10^7	10^2	10^3
Control (native oils)	Growth in all the dilutions	Growth in all the dilutions	Growth in all the dilutions	Growth in all the dilutions

Table 2. Antimicrobial characteristics of ozonated flaxseed oil in relation to the causative agents of mastitis and endometritis in cows

The maximum concentration of microbial cells in one ml, at which culture growth is not observed after 30 minutes of exposure with oil				
Flaxseed oil	Staph. aureus	Citrobacter spp.	E. coli	Str. agalactiae
Ozonated	10^4	10^3	10^4	10^3
Non-ozonated	Growth in all the dilutions			
The maximum concentration of microbial cells in one ml, at which culture growth is not observed after 2 hours of exposure with oil				
Ozonated	10^8	10^6	10^8	10^7
Non-ozonated	Growth in all the dilutions			

Subsequently, the antagonistic properties of OFO in relation to field strains have been studied. According to the data obtained, OFO showed the most pronounced inhibitory effect against Staphylococcus aureus and Escherichia coli (table 2), which were predominantly isolated from the uterus secretions of the cows with endometritis. Thus, the studied agent inhibited the growth of the above-mentioned microorganisms during the 30-minute incubation in the dilution corresponding to 10^4 m. b. in ml and during the incubation for 120 minutes 10^8 m. b. in ml of the suspension. Bacteria of the genus Citrobacter and Streptococcus agalactiae obtained from the secretion of the mammary gland of cows with mastitis turned out to be more resistant to the tested substance. OFO inhibited their growth at the 30-minute incubation in the dilution corresponding to 10^3 m. b. in ml. The two-hour incubation of the drug inhibited the growth of bacteria of the genus Citrobacter at the concentration of 10^6 m. b. in ml and Streptococcus agalactiae 10^7 m. b. in ml.

Thus, the data obtained indicate the possibility of using OFO as an etiotropic drug for treating the cows with endometritis.

Taking into account the fact that the number of pregnant cows during the first artificial insemination after calving often does not exceed 40 %, we have decided to study the effect of ozonated flaxseed oil during uterine application on the increased results of their fertilization. The results of studying the influence of endometrial treatment with ozonated flaxseed oil on cows` rate of fertilization are shown in Table 3.

Table 3. Influence of uterine sanitation with various drugs on the subsequent cows` rate of fertilization

Indicator	Without sanitation	Endometr amag K	OFO
Number of animals	25	25	30
The number of fertilized animals, in total (%)	16 (64.0 %)	21 (84.0 %)	24 (80.0 %)
The number of fertilized animals from the first time (%)	9 (36.0 %)	13 (52.0 %)	19 (63.3 %)
The rate of fertilization	1.5±0.16	1.4±0.15	1.4±0.16
The average number of days from induction to productive insemination	17.3±4.9	16.0±3.2	16.3±5.2

As the research results show (table 3), over the three months of the experiment, the rate of fertilization of animals without treatment of the uterine mucosa with pharmacological etiotropic drugs was 64 %, with the result of the first artificial insemination being no more than 36 %. The use of Endometromag K for this purpose ensured the fertility of 84 % of animals, including 52 % of animals after the single insemination. The proximate sanitation of the uterus with ozonated flaxseed oil guaranteed pregnancy in 63 % of the cows, and additionally 16.7 % of the cows became pregnant during the next 2 months. The rate of fertilization in animals with uterine sanitation was 1.4.

The next stage was devoted to performing the comparative evaluation of the efficiency of using OFO and the drug based on the antibiotic of the macrolide group. The research results are shown in table 4.

According to the data obtained (table 4), in the treatment of cows with postpartum endometritis, in the experimental group the average quantity of the spent drug was 614 ml with the frequency of drug administration being 5.5 times, which is 47 ml and 0.4 times more than the same indicator in the control group. For 5 months of observation, in the control group all animals were fertilized, and in the group where OFO was used this indicator made up 93.3 %. At the same time, after the first insemination, 47 % of the cows were fertilized in the experimental group, which is 27 % more in comparison with the animals which were insufflated with tylosinocar. On the average, in the group of cows

that were treated with the ozonated agent one insemination required 1.8 inseminations, which is less by 0.5 compared to the group where the antibiotic was administered to the cows. In the group of cows treated with OFO the duration from calving to fertilization averaged 104 days, which is 21 days less (P <0.05) in comparison with the control group.

Table 4. Efficiency of using OFO and tylosinocar in the treatment of postpartum endometritis in cows

Indicator	Control group (tylosinocar)	Experimental group (OFO)
Number of cows	15	15
The used preparation, ml	567±32	614±28
Frequency of drug administration	5.1±0,3	5.5±0.3
The number of fertilized cows, total (%)	15 (100 %)	14 (93.3 %)
After the 1-st insemination, in particular	3 (20 %)	7 (47 %)
The fertilization coefficient	2.3±0.3	1.8±0.2
The period from calving to fertilization, in days	125.6±7.6	104.4±6.9*

* *P* <0.05 in relation to the control group

During the milk research, it was found out that intrauterine administration of OFO was not accompanied by the inhibitors emergence in the experimental group of cows, while the use of tylosinocar caused a positive reaction to antibiotics within 48 hours after its use.

The clinical trial of the influence of ozonated flaxseed oil on the amount of irritation in relation to the mammary gland was performed on cows during lactation.

The results of studying the irritating effect of ozonated flaxseed oil on the mammary gland of lactation cows are shown in table 5.

From the data of table 5 it can be seen that already after 12 hours from the moment of administration of the ozonated flaxseed oil there was the sharp increase in the number of somatic cells in the udder secretion, which lasted more than 72 hours. After 96 hours from the start of the experiment, there was the decrease in the number of somatic cells relative to their number at the time of the previous study, but it was still 4.3 times higher than their initial value.

At the end of the experiment, the number of somatic cells in the milk from the quarters of mammary glands in the experimental animals continued to decrease and it differed by 1.7 times from the initial number. The pattern in the dynamics of somatic cells after intracisternal administration of the physiological salt solution was identical with respect to ozonated flaxseed oil.

The difference was that during the experiment, the number of somatic cells in the udder secretion of control animals returned to the original one after 72 hours. Therefore, ozonated flaxseed oil in a dose of 10.0 ml when administered intracisternally has a prolonged period of irritation on the udder tissue, which excludes its use in case of mastitis.

Table 5. Change in the content of somatic cells (in thousand) in one ml of the cows` udder secretion after the administration of the studied drugs (n = 3)

Time of research	Ozonated flaxseed oil	Physiological salt solution
Before the administration	166.7±24.0	186.6±13.3
In 12 hours	More than 1500	986.7±256.7*
In 24 hours	More than 1500	600.0±34.6*
In 36 hours	More than 1500	416.7±92.7
In 48 hours	More than 1500	286.7±40.9*
In 73 hours	900.0±40.1*	190.0±15.0
In 96 hours	720.6±64.2*	190.0±10.0
In 120 hours	286.7±15.9*	178.3±16.9

P<0.001…0.05 in relation to the level before the administration

4 Conclusion

Thus, the highest bactericidal activity was shown by OFO. This preparation has the clear antimicrobial effect against Staphylococcus aureus and Escherichia coli, suppressing the growth of these microorganisms during two-hour incubation at the concentration of 10^8 m.b. in ml of the suspension. The single intrauterine administration of OFO before insemination promotes pregnancy in 63.3 % of cows when the decrease in the rate of fertilization. The use of OFO for cows with postpartum endometritis is accompanied by the recovery of 93.3 % of animals, with the frequency of insemination being 1.8, and the average period from calving to fertilization being 104.4 days. The use of OFO as an etiotropic agent does not cause the inhibitors emergence in milk. When administered intracisternally only once, OFO leads to the irritation on the cows` udder tissue for 120 hours, and thus it can`t be used for treating mastitis in the lactation period.

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