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The application of EDTA-Tris and chlorhexidine in the treatment of endometritis as a replacement for antibiotic therapy in cows

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Abstract: The treatment of reproductive disorders is based on the use of the available antibiotics or antiseptic agents. Prolonged or inadequate antibiotic treatment contributes to the development of antimicrobial resistances, while the use of antiseptics, particularly in high concentrations, leads to endometrial lesions and prolonging of the service period. The present study included monitoring of: 1) reproductive disorders of cows; 2) cytological, morphological, and bacteriological laboratory analyses; 3) susceptibility testing of isolated bacterial and reference strains to the EDTA-Tris (ethylene diamine tetraacetic acid-hydroxymethyl aminomethane) and chlorhexidine (CHX); and 4) an estimate of the time before onset of the new sexual cycle. The aim of the research was to determine whether the combination of EDTA-Tris and CHX in concentrations that are not harmful to the endometrium may result in the satisfactory treatment of sick animals and provide optimal service periods of reproductively active animals. Research results of in vitro and in vivo analysis showed that by using the combination of EDTA-Tris and CHX bactericidal effects were achieved at lower concentrations in comparison with the concentrations that had the same effect in the absence of EDTA-Tris. The application of CHX in a concentration of 0.05% in the treatment of endometritis 10 min after infusion of EDTA-Tris solution had a positive effect on both the involution of the uterus and acutization of chronic inflammatory processes, and it was also economically advantageous when compared to the cost of treatment and the possible presence of residues in the products.

Key words: EDTA-Tris, chlorhexidine, microorganisms, endometritis, cow

1. Introduction

Modern animal production is often faced with the need to use antimicrobial drugs. Unfortunately, due to the widespread presence of antibiotics in different segments of production, there is a decreased efficiency of their action or complete resistance of microorganisms to certain antibiotics. The treatment of reproductive disorders depends on the causal factors that lead to them.

In relation to the etiopathogenesis, medical disorders of the genital tract of cattle can be extragenital and genital. Extragenital bovine infertility is more common and usually is manifested as a functional disorder of the genitals (1). It is caused by bioecological factors such as irregular nutrition and housing; high production; adverse bioclimatic factors and poor adaptation; metabolic diseases; general organic, infectious, and parasitic diseases; neurohormonal disorders; immunological sources; and other unknown factors. Genital sterility is manifested by pathomorphological changes of the genitals caused by hereditary anomalies or infections often induced by gram-positive and gram-negative bacteria (2).

Knowledge of the structure and function of the bacterial cell wall plays a very important role in the selection of therapy since it defines the sensitivity to certain antimicrobial drugs, disinfectants, and antiseptics. Gram-negative bacteria are resistant to a number of noxae depending on the functioning of the permeability of the external cell membrane, which prevents the penetration of large hydrophobic molecules (3). Since many agents harmful to bacteria, including antibiotics, are compounds that have hydrophobic groups, it is very difficult for them to pass through the cell membrane.

The molecular basis of resistance is related to the lipopolysaccharide structure of the external membrane of the bacterial cell wall. Lipopolysaccharide binds cations due to the multiple negative charges of lipid A, which is its component. A mutual link in the lipopolysaccharide structure is realized with the help of electrostatic forces

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that are achieved thanks to the molecules Ca^{2+} and Mg^{2+} located on the external bacterial membrane (3).

Any substance that can disrupt the structure and permeability of cell walls increases the susceptibility of bacteria to different biostatic and biocidal agents (4). Today, numerous compounds are recognized that have poor bactericidal effects or influences on the permeability of the external cell wall membrane. Ethylene diamine tetraacetic acid (EDTA) and hydroxymethyl aminomethane (Tris) have a synergistic effect on the cell wall of bacteria as they lead to the chelation of Ca^{2+} and Mg^{2+}, i.e. to the removal of these ions from the cell membrane, which maintain the structure of lipopolysaccharides and limit their permeability to some molecules (3). In addition to these substances, in the treatment of endometritis, antiseptic chlorhexidine (CHX) can be used, which has a bacteriostatic and to a lesser extent a bactericidal effect (5). The mechanism of action of the antiseptic will depend upon the interaction with the surface of the cell, penetration of the cell, and direct impact on the target molecule (4). The target site of action of CHX as an antiseptic is the cytoplasmic cell membrane (inner membrane) (4).

The present study included the monitoring of reproductive disorders in cows and the possibility of an alternative approach to the treatment of endometritis without the use of antibiotics. The aim of the research was to determine whether the combination of EDTA-Tris and CHX at concentrations that are not harmful to the endometrium may result in satisfactory treatment of sick animals and provide optimal service periods of reproductively active animals.

2. Materials and methods

The study included monitoring of: 1) reproductive disorders of cows, 2) cytological-morphological and bacteriological laboratory analysis, 3) susceptibility testing of isolated bacterial and reference strains to EDTA-Tris and CHX, and 4) an estimate of the time before onset of a new sexual cycle.

The study of the susceptibility of microorganisms to antibiotics, EDTA-Tris, and CHX was conducted in parallel on strains isolated from the field and reference strains representing the control group (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922). The impact of EDTA-Tris on the possibility of changing the sensitivity of selected strains, individually and in combination with CHX, was analyzed.

Analysis of growth control of field and reference strains in EDTA-Tris was done as follows: a suspension of inoculum density of 0.5 McFarland was made in a test tube, to which the same amount of a nutrient broth medium liquid (trypticase soy broth) and EDTA-Tris were added. The growth of bacteria was examined for 3 days. In order to determine the inhibitory effect of CHX on field and reference strains, different concentrations of chlorhexidine gluconate (mL) were added to a prepared suspension (trypticase soy broth) with the number of bacteria at 0.5 McFarland density. Finally, the effect of CHX in the presence of EDTA-Tris on tested strains was estimated by adding different concentrations of CHX in prepared suspensions (suspensions of tested bacteria, EDTA-Tris, and nutrition broth). After 2 h of incubation at room temperature, the viability of tested bacteria was checked by seeding on blood agar and incubating for 24 h on 37 °C.

Clinical trials were conducted on dairy farms, and laboratory tests were carried out in the laboratories of the Scientific Veterinary Institute "Novi Sad" in Novi Sad, Serbia, where the cytomorphological and microbiological analyses were performed. The trial that included animals was carried out in two stages. The first stage involved the study of the action/activity of EDTA-Tris-CHX during puerperium (up to 42 days after calving) including 193 reproductive cycles over a 2-year period of time, in which 65 cows were treated (93 cycles), while in the control group 75 cows were included (100 cycles), where the involution process was mostly undisturbed and smooth and possible treatments were indicated after the end of the period of involution, before the start of insemination. As a part of the diagnostics, laboratory analysis was performed by cytological examination.

The second stage involved the study of the effect of EDTA-Tris-CHX on cows with 3 or more failed inseminations with different degrees of inflammation of the uterus. In this study, 4 groups of animals (trials and controls) with different clinical findings were formed as follows: I – endometritis with very hard uterus walls, with uterus within the pelvis (E1); II – endometritis with very hard uterus walls, with uterus exceeding the edges of pelvis (E2); III – endometritis with soft uterus walls, with uterus within the pelvis (Bo1; Lat. *bene obducta*, well recovered); and IV – endometritis with soft uterus walls with uterus exceeding the edges of pelvis (Bo2).

The analysis included a total of 80 animals with 20 cows per group (trials and controls). Animals that served as trial subjects were treated with EDTA-Tris-CHX. For control animals, Lugol solution preparations were used to treat hard endometritis in groups I and II (in a concentration of 2%, 100 mL for E1 and 150 mL for E2), while for groups III and IV antibiotics (ceftiofur hydrochloride 50 mg) were selected and used based on the sensitivity of the isolated bacteria causing endometritis. The effects of treatment were analyzed in two consecutive cycles of insemination to determine the final reproductive status (pregnant or not pregnant).

For preparation of solutions in the laboratory, EDTA-Tris solution was prepared as recommended by Reading et al. (6). The following components were used: 1) EDTA (Titriplex III; ethylenedinitrilotetraacetic acid disodium salt dihydrate, 2.1 g/L, manufacturer: Merck KGaA, Darmstadt, Germany; $CC_{10}H_{14}N_2Na_2O_8{\times}2H_2O$; molar weight: 372.24 g/mol; CAS #: 6381-92-6); 2) Tris (6.5 g of Trizma base; 2-amino-2-(hydroxymethyl)-1,3-propanediol, THAM, manufacturer: Sigma, Ref. Number T1503; CAS number 77-86-1; linear formula $NH_2C(CH_2OH)_3$; molecular weight 121.14); 3) chlorhexidine gluconate (chlorhexidine digluconate, 5.0 g, in the form of a 20% solution of chlorhexidine digluconate, manufacturer: Bosnalijek); 4) HCL aq. (Centrohem, Stara Pazova, quality p.a. > 36.5%).

For greater clarity, the results obtained in these trials are presented in tables. The following descriptive statistical parameters were calculated: mean, standard deviation, and variation interval.

Statistical significances were calculated using Student's test (t-test for independent samples). $P < 0.05$ was considered significant. These analyses were carried out using the PC software package Statistica 8 (Stat Soft, Inc., Tula, OK, USA).

3. Results

Table 1 presents test data of the impact of EDTA-Tris on isolated and reference strains over 72 h.

The analysis showed that after 24 h of incubation of the reference strains in the nutrient/culture medium, there was no growth in suspensions with *E. coli* and *S. aureus*, while in the suspension with *P. aeruginosa* slightly turbid medium (-/+) was observed in the test tube. Over the next 48 h growth of all strains was observed, except for reference strain *S. aureus*.

Results of investigation of the effect of different concentrations of CHX on the growth and multiplication of isolated and reference strains in the presence of nutrient/culture medium are shown in Table 2.

The study of the inhibitory influence of chlorhexidine gluconate on the reference strains and the strains isolated from the field showed that, depending on the investigated strain, the limit of the bacterial growth began with a concentration of 0.05%; that is, this concentration no longer had -cidal/static action.

Results of in vitro trials of the bactericidal effects of CHX subsequent to exposure of bacterial strains to EDTA-Tris are presented in Table 3.

Table 1. Results of the growth of reference strains and clinical isolates in a medium with EDTA-Tris.

Hours	Bacterial growth in the presence of EDTA-Tris					
	E. coli ATCC 25922	*E. coli*	*S. aureus* ATCC 25923	*S. aureus*	*P. aeruginosa* ATCC 27853	*P. aeruginosa*
24 h	-	+	-	-/+	-/+	+
48 h	-/+	+	-	+	+	+
72 h	+	+	-	+	+	+

- no growth; -/+ weak growth; + growth.

Table 2. Results of the inhibitory activity of different concentrations of CHX on bacteria.

Concentration of chlorhexidine gluconate	Isolates' growth in the presence of chlorhexidine reference strains and field isolates					
	E. coli ATCC 25922	*E. coli*	*S. aureus* ATCC 25923	*S. aureus*	*P. aeruginosa* ATCC 27853	*P. aeruginosa*
0.5%	-	-	-	-	-	-
0.1%	-	-	-	-	-	-
0.05%	+	-	+	-	-	+
0.01%	+	-	+	+	+	+
0.005%	+	-	/	/	/	/
0.001%	+	+	/	/	/	/

+ bacterial growth; - no bacterial growth; / not analyzed.

Table 3. The inhibitory activity of varying concentrations of CHX on the bacteria in the presence of EDTA-Tris.

Concentration of chlorhexidine gluconate	Isolates' growth in the presence of EDTA-Tris and chlorhexidine reference strains, field isolates					
	E. coli ATCC 25922	*E. coli*	*S. aureus* ATCC 25923	*S. aureus*	*P. aeruginosa* ATCC 27853	*P. aeruginosa*
0.5%	/	/	/	/	-	-
0.1%	-	/	/	/	-	-
0.05%	-	/	-	-	-	-
0.01%	-	-	-	-	-	-
0.005%	-	-	/	/	/	/
0.001%	/	-	/	/	/	/

+ bacterial growth; - no bacterial growth; / not analyzed.

The results presented in Table 3 show that concentrations of CHX that had no inhibitory activity/action became inhibitory to the studied strains after exposure of microorganisms to EDTA-Tris. Increase of sensitivity to CHX has been recorded in all studied bacteria, so the concentrations of CHX in which the bacteria were grown in the absence of EDTA-Tris were changed subsequent to exposure to these substances.

As shown in Table 4, in the experimental group there were 80/93 (86.02%) pregnant cows compared to 77/100 (77.00%) in the control group, with increased consumption of doses (3.59 ± 2.47 compared to 3.00 ± 1.95), and the service period was extended in pregnant cows (191.5 ± 140.54 compared to 152.94 ± 100.70 days of service period). The percentage of pregnant animals and service period did not differ significantly, suggesting that problematic animals after treatment can return to the regular reproductive procedure (sexual cycle synchronization and insemination).

Treatments with CHX resulted in better contractility and faster involution of the uterus after calving. In chronic endometritis and in cows with service period exceeding 150 days, this therapy achieved an acute exacerbation of the process until the uterus was cured. The duration of service period was considerably shortened ($P < 0.05$) and conception in herds increased ($P < 0.01$), with a significant increase in milk production. The total fertility of the herd increased on 45 farms by 7% to 13%.

The obtained results demonstrate that treatment with EDTA-Tris-CHX at a concentration of 0.05% was 80% successful with two consecutive cycles of insemination for conditions Bo1 and Bo2, while this success was somewhat lower in the findings for E1 and E2 (65%) (Table 5). Treatments using conventional methods recorded similar or lower values, but with a higher consumption of doses per gestation, probably due to slower recovery of the endometrium after treatment.

4. Discussion

According to the currently available literature, there are no data concerning the possibility of treatment of described reproductive disorders in cows with the use of Tris-EDTA-CHX. Therefore, this discussion can only address issues of reproductive disorders and treatment possibilities in a more general way, since there are no topic-specific research results of other authors for comparison.

Problems caused by the presence of antimicrobial resistance are attempted to be solved in different ways. Production of new antimicrobial drugs, creation of efflux pump blockers, production and use of essential plant extracts (7), design of a vaccine for cows in the dry period, and the use of certain disinfectants in small concentrations (8,9) are possible options for addressing the reduced susceptibility of bacteria to existing drugs. The use of CHX or EDTA-Tris, in combination or individually, has been examined in the case of otitis media (10,11), genital infections in mares (12), and treatment of resulting biofilms (13).

Establishment of the optimal therapy is based on precise diagnostics. Subclinical endometritis is the most common problem in practice, and setting an accurate diagnosis requires the use of laboratory methods. Taking biopsies of the womb, making microscopic smears to determine the presence of cells of the immune response, and conducting microbiological analysis of biopsy specimens constitute the comprehensive diagnostic method to verify the health status of animals (2).

The ability of EDTA-Tris through chelation to change the structure of the cell walls of bacteria, increasing their susceptibility to antibiotics and/or antiseptics, allows the combination of those compounds and CHX for therapeutic purposes (10,11). CHX is an antiseptic agent whose mechanism of action depends on interaction with the surface of the cell, penetrating the cell and directly impacting the target molecules (4).

Table 4. The effect of treatment of cows with EDTA-Tris-CHX after calving on the final reproductive status of cows during the trial.

Conception	Pregnant	Not pregnant	No. of AIs		Service period	
	n (%)	n (%)	Pregnant	Not pregnant	Pregnant	Not pregnant
Trial group (no. of cycles: 93)	80 (86.02%)	13 (13.98%)	3.59 ± 2.47	5.62 ± 3.43	191.5 ± 140.54	291.08 ± 138.09
Control group (no. of cycles: 100)	77 (77.00%)	23 (23.00%)	3.00 ± 1.95	5.83 ± 3.86	152.94 ±100.70	281.39 ± 131.83
Total:	157 (81.35%)	36 (18.65%)	-	-	-	-

A t-test of independent samples showed no statistical significance between trial and control group ($P > 0.05$).

Table 5. Reproductive indicators in the treatment of chronic endometritis and the number of cows inseminated.

Clinical finding	E1 and E2 Groups I and II			Bo1 and Bo2 Groups III and IV		
	Pregnant	Not pregnant	No. of doses	Pregnant	Not pregnant	No. of doses
Trial group (N = 20); (EDTA-Tris-CHX 0.05%)	13 (65%)	7 (35%)	1.31 ± 0.48	16 (80%)	4 (20%)	1.56 ± 0.51
Control group (N = 20); (Lugol sol. or ceftiofur 20 mL)	12 (60%)	8 (40%)	1.50 ± 0.52	14 (70%)	6 (30%)	1.67 ± 0.49

E1 and E2 - Endometritis with very hard walls of uteri of different sizes.
Bo1 and Bo2 - Endometritis with soft walls of uteri of different sizes.

The change of cell membrane impermeability is demonstrated under the influence of EDTA-Tris for aminoglycoside antibiotics due to the inability of diffusion of hydrophobic substances through lipopolysaccharide structures of the wall of the microorganism (3). The increase of the sensitivity of *S. aureus* and *P. aeruginosa* to gentamicin after exposure to EDTA-Tris was recorded in the study of Banin et al. (13), while the survey by Abbes et al. (14) showed an increase in the sensitivity of *P. aeruginosa* to different groups of antibiotics (fluoroquinolones, aminoglycosides, beta-lactamase, and polymyxin) after exposure to EDTA-Tris.

The use of EDTA-Tris as a chelator that increases the permeability of the cell walls of bacteria and CHX can be diminished by the presence of protein components in the substrate, or in the environment of the microorganisms. Bactericidal effects depend on the concentration of biocidal substances used, the type of microorganism, and the effect of different environmental characteristics (organic matter, temperature, dilution) (4,11). The experiences of other authors should be considered when applying therapy with EDTA-Tris-CHX.

In our study with 140 Holstein-Friesian cows kept in a loose housing system, a high percentage of chronic inflammatory processes in the uterus was recorded (in about 45% of cows) in the advanced lactation period. According to Gilbert et al. (15), the frequency of endometritis diagnosed 40–60 days after calving is 53%. Frequent endometritis findings are the result of poor hygienic conditions, as well as early or iatrogenic use of corrosive preparations in the prevention of endometritis during the early and middle puerperium, with frequent occurrence of adhesions or abscesses in the cervix and surrounding connective tissues (adnexitis, parametritis, etc.). Studies by other authors suggest that early treatment of the uterus using an infusion of corrosive substances is not recommended (16).

Some studies have shown that the lowest pregnancy rates are achieved by applying Lugol solution, which can be attributed to degenerative changes that interfere with embryo implantation. Furthermore, it takes 59.82 ± 55.37 days to achieve pregnancy, which indicates a slower rehabilitation of the uterus (2). As part of the diagnostic procedure, in the present study, laboratory analysis was performed by cytological examination. In cows with reproductive disorders swab samples were taken from the uterus and the cervix in order to monitor the degree of inflammation, especially in a uterus with thin walls

and undetermined inflammatory signs (subclinical endometritis). This form of endometritis is characterized by intense endometrial infiltration by neutrophilic granulocytes, which can be diagnosed only after cytological examination. Discharge in the uterus is absent or minimal, making diagnosis difficult on clinical examination (15) and reducing subsequent success in insemination (17).

In conclusion, growth analysis of selected microorganisms in EDTA-Tris suspension and nutrient medium showed that the growth and multiplication of the studied bacteria in the first 24 h was limited, and that completely blurred nutrient broths, as an indicator of bacteria multiplication, occurred after 48 or 72 h. In the analysis of the results obtained in the present study, it was found that the inhibitory concentrations of CHX decreased, i.e. subsequent to exposure of the studied microorganisms to EDTA-Tris, the CHX concentrations required to inhibit the growth of bacteria decreased. This allows the use of CHX at concentrations that are not harmful to the endometrium, at least not to the extent to disturb the fertility of cows, allowing the recovery period from endometritis to be shortened. A clinical trial of the application of EDTA-Tris and CHX as a possible therapy in cows with reproductive disorders has shown that, depending on the severity of clinical findings, an increase in pregnancy rates occurs in puerperium as well as in cows with a greater number of inseminations. These results provide encouragement for possibility of reducing the use of antibiotics or their complete replacement, which could help relieve the pressure of the emergence and spread of antimicrobial resistance.

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