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The antioxidant effect of the novel bee-product based intramammary formulation Apimast® in dairy cattle

Jelena Šuran[1]*, Jasna Aladrović[2], Blanka Beer Ljubić[3], Josipa Vlainić[4], Marija Mamić[5], Božo Radić[6], Goran Bačić[7], Nino Mačešić[7], Miroslav Benić[8], Antun Kostelić[9], Frane Božić[1], Hrvoje Pavasović[1], and Lada Radin[2]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[2]Department of Physiology and Radiobiology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[3]Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[4]Division of Molecular Medicine, Institute Ruđer Bošković, Zagreb, Croatia

[5]Clinic for Surgery, Orthopaedics and Ophthalmology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[6]Hedera Ltd., Split, Croatia

[7]Clinic for Reproduction and Obstetrics, Faculty of Veterinary Medicine, University of Zagreb, Croatia

[8]Department for Bacteriology and Parasitology, Croatian Veterinary Institue, Zagreb, Croatia

[9]Department of Animal Science, Faculty of Agriculture, University of Zagreb, Croatia

ŠURAN, J., J. ALADROVIĆ, B. BEER LJUBIĆ, J. VLAINIĆ, M. MAMIĆ, B. RADIĆ, G. BAČIĆ, N. MAČEŠIĆ, M. BENIĆ, A. KOSTELIĆ, F. BOŽIĆ, H. PAVASOVIĆ, L. RADIN: The antioxidant effect of the novel bee-product based intramammary formulation Apimast® in dairy cattle. Vet. arhiv 90, 225-233, 2020.

ABSTRACT

The aim of this study was to determine the antioxidant effect of the novel bee-product based intramammary formulation Apimast® on the oxidative status of dairy cows. Apimast® was administered intramammarily three times at 12 h intervals to 10 dairy cows on each of the three dairy farms. The oxidative status markers and some metabolic parameters were determined in the milk (GSH-Px, d-ROM, SOD, BAP, TAS) and in the serum (SOD, GSH-Px, NEFA, BHB, glucose). The results showed that the effect of Apimast® was mostly local, regardless of the concentration used (3% and 1%) as there was an increase in the concentration of GSH-Px and TAS in the milk, but not in the blood. It is also of a short-term nature since the values of these parameters were significantly lower 7 days after application. The expected connection between the increase of ROM in milk samples from the microbiologically positive quarters was not found. The results of this study confirm the local antioxidant effect of Apimast® in the udder, with implications for non-antibiotic subclinical mastitis treatment.

Key words: mastitis; oxidative stress; Apimast®; propolis; dairy cows

*Corresponding author:
Assist. Prof. Jelena Šuran, DVM, PhD, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone: +385 2390 118; Fax: +385 2390 159; E-mail: jelena.suran@vef.hr

Introduction

With the development of the modern dairy industry and the ever-increasing demands of the market over the last forty years, selection of cows for high milk production leads to the doubling of milk production per cow (OLTENACU and ALGERS, 2005). Mastitis, inflammation of the mammary gland, is the most common health problem of dairy cows, with special importance in high-yield dairy herds. The financial cost of mastitis can be attributed to production losses, extensive treatment costs and negative impacts on animal welfare, among others (NIELSEN et al., 2010). The aetiology of mastitis is complex and is considered to be a result of the interaction between the hosts, microorganisms, the environment, and farming (BAČIĆ, 2009). Established clinical mastitis therapy implies the use of antibiotics, whereby the choice of therapy is adjusted to the course of the inflammation and the cause. On the other hand, subclinical mastitis, considered to be a major problem on large, high-yield dairy farms due to reduced milk production and the high number of somatic cells (reduced milk quality), is not treated since the cost of discarded milk outweighs the benefits of antimicrobial therapy (SANDGREN, 2008). Also, due to the increasing problem of antibiotic resistance, there is a trend (and regulatory pressure) towards minimizing antibiotic use in veterinary medicine, including dairy production, so new, non-antibiotic or antibiotic alternatives for mastitis treatment are being studied (PYÖRÄLÄ, 2002; VARELLA COELHO et al., 2007; WU, 2007).

Apimast® is a novel bee-product based intramammary formulation, developed for mastitis treatment and prevention. The product has not been authorized as a veterinary medicinal product (VMP), but is being studied extensively as the first cost-effective intramammary antibiotic alternative (ŠURAN et al., 2015; 2016). The main active components of the product are polyphenols from standardized poplar type propolis non-alcoholic extract. Propolis is a resinous substance that bees collect, process and use as the building material in the beehive. It contains 50% balsam and resin, 30% beeswax, 5% pollen, 10% essential oils and 5% other organic components. The last two include low molecular weight phenols that are considered to have biologically active properties, namely flavonoids, phenolic acids and esters, aromatic aldehydes and terpenoids (BANSKOTA et al., 2001; SILVA-CARVALHO et al., 2015). For medical purposes, propolis has been used for years - in ancient times the Egyptians used to balm and mummify the bodies of the dead, and in the records of the Greeks and Romans its use is described in the treatment of some skin diseases and for disinfection of wounds, for protection against infection, pain relief and reduction of swelling (CRANE, 1999). The biological effects of propolis are related to its antimicrobial, anti-inflammatory, and recently, antioxidant properties (BANSKOTA et al., 2001; ISLA et al., 2001; SILVA-CARVALHO at al., 2015) and due to its great potential role as free radical scavenger (PASCUAL et al., 1994). Active components from propolis may be effective in mastitis treatment (FIORDALISI et al., 2016), not just due to their antimicrobial activity, but also due to their antioxidant effects, since one of the contributing factors to subclinical infection aetiology and lower udder immune response is considered to be oxidative stress (BOUWSTRA et al., 2010).

On the basis of previous findings we treated animals with Apimast® - as a replacement for antibiotic treatment of subclinically present mastitis. The aim of this study was to investigate the effect of Apimast® intramammary administration on the antioxidant/oxidation markers (glutathione peroxidase (GSH-Px), ROM (reactive oxygen metabolites), SOD (superoxide dismutase), biological antioxidant potential (BAP), TAS (total antioxidant status), non-esterified fatty acids (NEFA) and beta hydroxybutyrate (BHB) in dairy cows, in order to elucidate some of the possible mechanisms of its action during mastitis.

Materials and methods

Animals and protocol. The research was conducted on three large dairy farms with the Holstein breed; Krndija (Farm 1), Grube (Farm 2) and SNK milk (Farm 3). The study included 10 dairy cows from each farm diagnosed with subclinical mastitis (in at least one udder quarter)

while ten healthy animals served as the control. The animals were kept on deep bedding, and fed with a standard feed (Table 1).

Table 1. Mean chemical composition (g/100g of DM unless otherwise stated) of basal diet fed to experimental cows

Chemical composition	
Crude protein	19.9
Crude fat	3.0
Starch	24.8
Crude fibre	13.9
aNDF	34.0
NEL, MJ/kg	6.9
Calcium	1.2
Phosphorus	0.52
Sodium	0.31
Magnesium	0.76
Fe, mg/kg	494.0
Zinc, mg/kg	87.8
Manganese, mg/kg	91.2
Copper, mg/kg	17.5
Vitamin A, IU/kg	6700.0
Vitamin D, IU/kg	636.0
Vitamin E, IU/kg	18.7

Subclinical mastitis was screened before and after each propolis application by measuring the number of somatic cells (SCC) in the milk using a DeLaval (Tetra Laval, Sweden) device by optical somatic cell counting, by analysing milk yield data collected on the farm and using microbiological screening of milk samples in an accredited laboratory (Croatian Veterinary Institute, Zagreb) according to the recommendations described in the Laboratory Handbook on Bovine Mastitis (National Mastitis Council, 1999).

Apimast® was administered three times at 12 h intervals, in all four quarters: after the morning and evening milking, and the next day after the morning milking.

Sample collection. Milk was sampled into labelled sterile plastic tubes from each quarter of dairy gland in a specific manner. Namely, after squirting the first milk stream, the tip of the teat was thoroughly disinfected with 70% alcohol. Samples were taken before the first application, 12h after the first application, 12h after the second propolis application, and on the 7th day after the first propolis application. Milk samples were kept at 4 °C (microbiological screening) or at -20 °C (determination of oxidants and antioxidants) for a maximum of 24 hours. Milk samples collected from healthy animals were prepared and analysed as bulk (pooled). In order to determine the concentration of d-ROM, BAP, SOD, GSH-Px and TAS milk samples were prepared by a modified method based on BERMEJO et al. (1997) and EZAKI et al. (2008). In brief, milk samples were centrifuged in two phases. In the first stage, the milk was centrifuged for 15 minutes at 1600 rpm and, after separation of the cellular elements, the supernatant was centrifuged for 45 minutes at 10,000 rpm at 4 °C. Following the described procedure, a sample of fat-removed milk was used to measure oxidative stress parameters.

Blood samples were taken before the first application and 48h after the first application of Apimast® into anticoagulant-free antisera tubes (Venosafe Biochemical Gel Tubes) (with the permission of the Ethics Committee of the Veterinary Faculty of the University of Zagreb, class: 640-01 / 15-179, No. 251-61-01 / 139-15-3

dated 22 April 2015). The control group samples were taken at the same time intervals. Blood samples were centrifuged at 3000 pm for 15 minutes, after which the serum was separated into several tubes and stored at -20 °C until analysis.

Apimast® formulations. Apimast® 1% and 3% formulations were used in the study, prepared by SME Hedera d.o.o. according to an innovative protected procedure (patent No. P20190325) of non-alcoholic extraction from poplar type propolis. The raw material was collected from bee-keepers from Croatia, grinded, homogenized and mechanically purified prior to extraction.

Biochemical tests. The GSH-Px activity was assessed using a commercial kit (Ransel, Randox Laboratories, Ireland). Briefly, GSH-Px activity was assessed indirectly at the absorbance wavelength of 340 nm for 5 minutes of NADPH oxidation to $NADP^+$. The assay mixture consisted of 50 mM phosphate buffer with 0.4 mM EDTA and 1 mM sodium azide (pH = 7.0), 0.12 mM NADPH, 3.2 units of GR, 1 mM glutathione, and 0.0007% (w/w) H_2O_2. One unit of GPx activity catalyses the oxidation by H_2O_2 of 1.0 μmol of reduced glutathione to oxidized glutathione per minute at pH = 7.0 and 25 °C. GSH-Px activity was calculated by using the molar extinction coefficient for NADPH disappearance (ε = 6220 dm^3 mol^{-1} cm^{-1}). Serum and milk SOD activity was evaluated by the spectrophotometric method using the commercially available RANSOD kit (RANDOX Laboratories, UK). In this assay, xanthine and xanthine oxidase were used to generate superoxide anion radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride quantitatively to form a red formazan dye. SOD inhibits the reaction by converting the superoxide radical to oxygen. Homogenized liver tissues, *Fasciola* spp. parasites and standard solutions were used for the assay of SOD. Absorbance was measured at 505 nm (Olympus AU640, Japan) for 30s after the addition of xanthine oxidase as the start reagent, and 3 minutes after reaction as duplicate samples. NEFA, BHB, and glucose concentrations were also measured with commercially available Randox reagents on an Olympus AU640 (Olympus, Japan). The d-ROMs test (Diacron International, Italy) was carried out to assess the concentration of reactive oxygen metabolites in the milk samples. The method was carried out by monitoring at 505 nm the absorbance of solutions obtained by dissolving the milk samples (10 μL) in 1 mL of acetate buffer (pH 4.8) and adding 10 μL of a 3.7×10^{-1} M solution of the chromogen (DEPPD) (the absorbance values being converted to arbitrary Carr units) (DOTAN et al., 2004). According to the manufacturer's specifications, the d-ROMs test readings are classified as follows: 250-300 Carr units: normal range; 300-320 Carr units: borderline condition; more than 320 Carr units: increasing oxidative stress condition. A control serum (320 Carr units) supplied with the kit was used for calibration of the test.

Quantification of TAS, as the measure of non-quantitative antioxidant function in the milk samples, was carried out using ABTS (2,20-azinodi-[3-ethylbenzothiazoline sulphonate]) formation kinetics. The commercially available colorimetric TAS assay (Randox Laboratories, UK) is based on the suppression of the absorbance of ABTS cation radicals by antioxidants in the test sample, when ABTS is incubated with a radical generating system (metmyoglobin + H_2O_2). A reaction mixture containing 100 μM H_2O_2, metmyoglobin and 610 μM ABTS was incubated with 5 μM trolox at 37 °C. Namely, the method is calibrated with the vitamin E analogue, known as trolox equivalent, and the results are expressed in mmol/L. The spectra were recorded in time scan mode until the reaction reached a plateau.

BAP was assessed using a test kit from Diacron (Grosseto, Italy). This test provides a global measurement of many antioxidants, including uric acid, ascorbic acid, proteins, α-tocopherol, and bilirubin. Briefly, 10 μL sample is added to a solution of ferric chloride and thiocyanate derivate. The assay is based on the ability of a coloured thiocyanate-derived substrate, which contains bounded Fe^{3+} ions, to decolorize when Fe^{3+} ions are reduced to Fe^{2+}. The absorbance is measured at 505 nm. The level of change is used to calculate the ability of the sample to reduce ferric ions. The results are expressed as μEq ferric ions reducing antioxidants per L of sample (BENZIE and STRAIN, 1996).

Statistical analysis. The results were categorized on the basis of the bacteriological status of the quarter: to a negative or a bacteriologically positive group. Results were analysed using GraphPad 8 and presented as mean ± standard deviation (SD). The distribution normality was checked by the Shapiro-Wilks test. The significance of the differences in repeat measurements was verified by Kruskall-Wallis's variation analysis and rank-level test, and the difference between the groups was analysed using the Mann-Whitney U test. The correlation between the observed indicators was tested by Spearman's correlation coefficient. Statistical significance was set at $P \leq 0.05$.

Results

The values of antioxidant enzymes (SOD, GSH-PX), reactive oxygen metabolites (ROMs) and total antioxidant capacity (TAS, BAP) measured in cow's milk on Farms 1 and 2 after intramammary administration of 3% Apimast® are shown in Table 2, while the values measured after 1% Apimast® administration are presented in Table 3. The values of biochemical and antioxidant ratios are shown in Table 4. The activity of the GSH-Px enzyme and the concentration of the total antioxidant potential (TAS) was statistically significantly increased after the 1st and 2nd 3% Apimast® administration compared to the initial value, while there was no change between the initial concentration of TAS and GSH-Px and its level on the 7th day after Apimast® administration. Other indicators did not change over time within the same farm.

A high positive correlation between oxidative status in the milk of positive ($P<0.05$; $r = 0.58$) and negative ($P<0.05$; $r = 0.51$) quarters was found for GSH-Px and TAS activity, while for other indicators no correlation was found. There was also a statistically significant low ($P<0.05$; $r = 0.33$) correlation between the activity of GSH-Px and SOD in the milk and blood of all samples in total.

Metabolic status indicators NEFA (up to 0.14 ± 0.09 mmol/L) and BHB (up to 0.35 ± 0.07 mmol/L) were below the cut-off limit for dairy cattle throughout the study period (Table 4). The glucose values were within the reference range. There was a statistically significant ($P<0.05$; $r = 0.65$) correlation between serum free fatty acid concentration and d-ROM in the milk of the dairy cattle.

Table 2. Mean ± SD values of oxidative stress markers on Farm 1 and 2, in bacteriologically negative quarters and positive quarters, at different time points before (0) or 12 h, 24 h and 7 days after 3% Apimast® intramammary administration

	Negative quarters				Positive quarters			
	0 h (control)	12 h	24 h	7 days	0 h (control)	12 h	24 h	7 days
GSH-Px (U/L)	275.4 ± 33.4	320.7 ± 25.8*	379.7 ± 78*	279.2 ± 17.4	355.5 ± 133	410.3 ± 107.2	415.8 ± 126.1	369.1 ± 115.6
BAP (mmol/L)	6.3 ± 0.4	6.3 ± 0.4	6.2 ± 0.7	6.3 ± 0.4	5.9 ± 0.9	5.5 ± 1.4	6 ± 0.9	5.9 ± 0.9
dROM (U Carr)	40.6 ± 24.9	74.4 ± 19.2*	55.4 ± 27	43.5 ± 23.3	47.1 ± 20.8	57 ± 9.5	64.7 ± 11.7*	46.7 ± 29.3
SOD (U/L)	2 ± 0.6	2.2 ± 0.8	2 ± 0.7	2.1 ± 0.6	1.6 ± 0.7	2.1 ± 1.3	2 ± 0.6	2.3 ± 1.2
TAS (mmol/L)	0.7 ± 0.3	1.3 ± 0.4*	1.6 ± 0.4*	0.9 ± 0.2	0.9 ± 0.5	1.9 ± 0.8*	1.8 ± 0.8*	1.4 ± 0.6*

* - significantly different from control (0 h) ($P<0.05$)

Table 3. Mean ± SD values of oxidative stress markers on Farm 3, in bacteriologically negative quarters and positive quarters, at different time points before (0) or 12 h, 24 h and 7 days after 1% Apimast® intramammary administration

	Negative quarters				Positive quarters			
	0	12 h	24 h	7 days	0	12 h	24 h	7 days
GSH-Px (U/L)	272 ± 16	319 ± 71	356 ± 69*	303 ± 84	326 ± 110	317 ± 89	367 ± 121	284 ± 24
BAP (mmol/L)	6.2 ± 0.4	6.3 ± 0.2	6.3 ± 0.3	6.5 ± 0.4	6.3 ± 0.4	5.7 ± 1.2	5.1 ± 1.3	6.2 ± 0.3
dROM (U Carr)	573 ± 275	728 ± 209	779 ± 58*	526 ± 30	599 ± 227	540 ± 153	605 ± 30	533 ± 138
SOD (U/L)	1.9 ± 0.8	2 ± 0.8	2 ± 0.6	1.9 ± 0.7	1.9 ± 0.7	1.5 ± 0.5	1.7 ± 1.4	1.8 ± 0.9
GSH-Px/ SOD	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.5	0.2 ± 0.1

* - significantly different from control (0h) (P<0.05)

Table 4. Biochemical parameters and antioxidant enzymes in serum of cows before and after 3% and 1% Apimast® intramammary administration in positive or negative quarters

	3% Apimast®				1% Apimast®			
	Negative		Positive		Negative		Positive	
	0 h (control)	48 h	0 h (control)	48 h	0 h (control)	48 h	0 h (control)	48 h
Glucose (mmol/L)	3.43 ± 0.38	3.34 ± 0.26	3.17 ± 0.17	3.56 ± 0.29	2.68 ± 0.42	2.83 ± 0.28	2.97 ± 0.6	3.12 ± 0.7
GSH-Px (mmol/L)	619.74 ± 68.77	608.62 ± 104.69	651.66 ± 20.98	627.17 ± 134.21	541.62 ± 56.7	558.8 ± 32.9	615.27 ± 30.8	556.05 ± 76.3
NEFA (mmol/L)	0.11 ± 0.05	0.11 ± 0.03	0.05 ± 0.01	0.11 ± 0.06*	0.13 ± 0.04	0.14 ± 0.09	0.08 ± 0.02	0.09 ± 0.04
SOD (U/L)	0.23 ± 0.15	0.16 ± 0.02	0.29 ± 0.26	0.28 ± 0.15	0.11 ± 0.07	0.29 ± 0.26*	0.08 ± 0.09	0.08 ± 0.03
BHB (mmol/L)	0.30 ± 0.09	0.35 ± 0.07	0.21 ± 0.08	0.32 ± 0.12*	0.27 ± 0.20	0.21 ± 0.08	0.16 ± 0.03	0.2 ± 0.06*

* - significantly different from control (0h) (P<0.05)

Discussion

There is undoubtedly a recognized role of oxidative stress in the pathogenesis of mastitis in ruminants (BOUWSTRA et al., 2010; ĐURIČIĆ et al., 2017; KLECZKOWSKI et al., 2017; LYKKESFELDT and SVEDSEN, 2007). Oxidative stress is defined as the shift of equilibrium in cellular oxidative-reduction reactions with enhancement of reactive oxygen species (ROS), resulting in unbalanced free radical formation, and finally in cell and tissue damage (CELI, 2011). Physiologically, free radicals are constantly formed in a cell as normal products of its metabolism, and in low concentrations are extremely important in multiple physiological processes (DRÖGE, 2002), such as phagocytosis, arachidonic acid metabolism, ovulation and fertilization (SINGH et al., 2004). However, when free radical formation is excessive, oxidative stress plays a significant role in the aetiology of various diseases, one of them being mastitis (ATAKISI et al., 2010; BOUWSTRA et al., 2010; ĐURIČIĆ et al., 2017; KLECZKOWSKI et al., 2017). The search for reliable indicators of this

imbalance between pro-oxidants and antioxidants is an important on-going issue. While measurement of antioxidant enzymes SOD and GSH-Px and TAS is a "classical" method, new methods, such as a d-ROM and BAP determination which measure the total amount of antioxidants or substances capable of reducing iron in fero- and feri- form (BENZIE and STRAIN, 1996), are still not in common use. Given the relatively low spread of these tests in herd health research, reference ranges for these indicators in biological ruminant fluids do not exist. The normal range of d-ROM plasma values in humans is between 250 and 300 U Carr. However, in our study, values were 10 times lower than in humans (39.13 ± 20.73 U Carr to 77.32 ± 10.22 U Carr), which corresponds to the findings of PASQUINI et al. (2008) in dogs, where they are between 56.4 - 91.4 U Carr, and in sheep (PICCIONE et al., 2006). In milk samples positively related to mastitis, we found no significant change in d-ROM concentrations in the milk. 3% Apimast® intramammary administration increased d-ROM levels in milk in negative and positive quarters, and 1% Apimast increased these levels in only the negative quarters. This possibly led to increased activation of some of the antioxidant mechanisms such as GSH-Px, also visible in Table 2 and 3. The positive correlation found between the NEFA and BHB concentrations in the blood and the d-ROM in milk suggests an increase in total oxidation (free radicals, but also non-radical oxygen compounds, such as H_2O_2, hypochlorous acid, etc.) as a response to fatty acid mobilization and potential lipid peroxidation (BERNABUCCI et al., 2005) in highly productive dairy animals.

Repeated measurements showed that BAP values after Apimast® administration over time remained at approximately the same levels, while TAS values increased significantly in all quartile groups after the 1st and 2nd 3% Apimast® administration (regardless the presence of pathogenic microorganisms). No positive correlation between these two parameters was found in this study, which can be attributed to their different sensitivity in measuring the concentration/activity of compounds with antioxidant properties. Specifically, the BAP method is more appropriate for evaluating the efficacy of low molecular weight anitoxidation molecules (such as urinary or ascorbic acid). One of the few studies in which BAP was measured in milk in humans (EZAKI et al., 2008) has an average value of 3.85 ± 1.35 mmol/L, which is half the level found in this study (Table 2).

The effect of 3% Apimast® on the oxidative status of the udder was the same, regardless of the initial microbiological status of the quarter. After the Apimast® administration, the concentration of TAS significantly increased in all animals and in all quarters (Table 2), due to the statistically significant increase in the activity of the GSH-Px enzyme, with which a positive correlation was found ($P<0.05$; $r = 0.58$ in positive regions, $P<0.05$, $r = 0.51$ in negative) after the first and second Apimast® administration in all samples (Table 2).

Although the antioxidant and antibacterial effects of phenolic acids and poplar propolis are known, and many studies of its *in vitro* efficacy have been performed (BANSKOTA et al., 2001; ISLA et al., 2001; SHIMIZU et al., 2004), a smaller number of papers have demonstrated its efficacy *in vivo* (SILVA-CARVALHO et al., 2015). It is interesting that propolis samples taken at different geographic longitudes and latitudes around the globe have similar biological properties, regardless of differences in their composition (BANSKOTA et al., 2001). Preparations made from bee products that can be found on the market are obtained by various extraction methods that significantly contribute to the loss of active substances from primary raw materials, which consequently reduce their antioxidant and antimicrobial efficacy. The innovative technological concept for polyphenol extraction from poplar type propolis (patent No. P20190325) used in this research enables the preservation of the maximum activity of defined and standardized substances, and reproducible biological effects, compared to all the existing bee product formulations on the market, and certainly with respect to the antibiotics that are commonly used in farming (ŠURAN et al., 2016). Besides its antimicrobial efficacy, Apimast® has a positive effect on the antioxidant defence mechanisms of the udder, and may be regarded as non-antibiotic mastitis therapy as well.

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SAŽETAK

Cilj istraživanja bio je odrediti učinak nove intramamarne formulacije na bazi pčelinjih proizvoda, Apimasta®, na oksidacijski status muznih krava. Apimast® je apliciran u tri navrata s razmacima od 12 sati u 10 krava na tri različite farme. Izmjereni su markeri oksidacijskog statusa kao i određeni metabolički pokazatelji u mlijeku (GSH-Px, d-ROM, SOD, BAP, TAS) i serumu (SOD, GSH-Px, NEFA, BHB, glukoza). Učinak Apimasta® bio je uglavnom lokalan, bez obzira na primijenjenu koncentraciju (1 i 3 %), jer porast koncentracija GSH-Px i TAS-a u mlijeku nije pratio porast u serumu. Promjene svih mjerenih pokazatelja bile su kratkotrajne i nakon sedam dana vraćale su se na približne vrijednosti početnih koncentracija. U bakteriološki pozitivnim četvrtima vimena nije zabilježen porast ROM-a. Ovo istraživanje potvrđuje lokalni antioksidacijski učinak Apimasta® u vimenu, što je relevantno s aspekta neantibiotskog liječenja supkliničkog mastitisa.

Ključne riječi: mastitis; oksidacijski stres; Apimast®; propolis; muzne krave