THE EFFECT OF HESPERIDIN, CHRYSIN, AND NARINGENIN ON SOMATIC CELL COUNT IN MASTITIS DAIRY COWS AFTER MULTIPLE INTRAMAMMARY ADMINISTRATION*

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Abstract

Hesperidin (HE), chrysin (CH) and naringenin (NA) are flavonoids, being the most important group of polyphenols, and show anti-inflammatory properties which have been demonstrated on various models. Polyphenols have a lot of biological properties, such as antioxidative, antiviral, immunomodulatory and anticancer activities. However, the effect on mastitis has not been yet described. This research aimed to analyse the tolerability of selected polyphenols after multiple intramammary administrations (IMM) as well as to investigate their potential impact on somatic cell count (SCC) in mastitis dairy cows. The study was performed on 12 Polish Holstein Black-and-White cows in their 4th to 6th lactation. Only animals with inflammation in one-quarter of the udder were selected. The selection was based on SCC and clinical assessment. The experiment was performed with multiple intramammary administrations with each of these polyphenols in dairy cows affected with mastitis. Polyphenols were administered at a dose of 30 mg/quarter/day. Milk samples for SCC, blood plasma samples for pharmacokinetics and blood haematology and biochemistry (selected blood parameters were tested) were collected at baseline, treatment period and within the recovery period. Positive effects concerning the SCC in milk of mastitic cows were confirmed for all tested polyphenols.

Key words: dairy cows, inflammation, immunomodulator, mastitis, flavonoids

*Work financed from: WKT/S/63/2021/WET.

Hesperidin (HE), chrysin (CH) and naringenin (NA) are pharmacologically active polyphenols occurring in plants. HE (flavonoid) and NA (flavonon) were isolated from rinds of citrus species (Li and Schluesener, 2017) and *Passifloraceae* spp. is the main CH (flavon) source. The anti-inflammatory action of HE (Parhiz et al., 2015; Ding et al., 2018; Tejada et al., 2018), CH (Hwang et al., 2018) and NA (Pinho-Ribeiro et al., 2016; Oguido et al., 2017; Zhao et al., 2019) has been well described. At present, little is known about the influence of HE on the physiology of the breast or the mammary gland in general. The main direction of HE studies is related to its anti-cancer effect in breast cancer (Lee et al., 2010). The same trend is proposed in the case of NA (Filho et al., 2014; Zhang et al., 2016). The proliferative capacity of NA linked with its affinity to the oestrogen receptor has been investigated and finally assessed as weak (Helle et al., 2014). Similarly, the possibilities of using the CH polyphenol in the treatment of breast cancer are being investigated (Al-Oudat et al., 2019).

Mastitis, which manifests as inflammation of the mammary gland, is currently one of the most widespread diseases affecting dairy cattle, and the costliest medical and economic problem over which there is increasing pressure to avoid the use of antibiotics (Stevens et al., 2016, 2019). The influence of HE, CH and NA on the course of udder inflammation in dairy cows has not been investigated to date. Although the activity of polyphenols is known, their influence on somatic cell count (SCC) levels in dairy cows has not been yet studied. The first studies on the effect of polyphenols on the level of SCC in dairy cows were performed to determine the impact of quercetin only (Burmańczyk et al., 2018). The results of these studies clearly show the high potential role of polyphenols in the treatment of mastitis in dairy cattle. Other studies with other polyphenols have demonstrated such role on mastitis, for example, baicalin enhances the lysozyme-induced bacteriostatic effect during the response to *Staphylococcus aureus* in mice (Gao et al., 2017). Moreover it was shown that in mice low doses of baicalin decreased apoptosis in bovine mammary epithelial cells and increased apoptosis in higher doses *in vitro* (Perruchot et al., 2019). Similar effects were investigated in the case of lipopolysaccharide (LPS)-induced mastitis in a murine model and treatment with other polyphenols, i.e. rutin and baicalein. It has been confirmed that rutin as well as baicalein may have an anti-inflammatory effect in the LPS-induced mastitis model in mice (He et al., 2015).

Based on the findings from studies with quercetin, baicalin, baicalein and rutin, then HE, CH, and NA may be assumed to have the potential to block mastitis progress in dairy cows. Therefore, the aim of this study was to analyse the effect of HE, CH and NA on SCC. To achieve this goal, separate studies were performed consisting of multiple intramammary (IMM) administration of each polyphenol in dairy cows with mastitis.

Material and methods

Dosage and drug preparation

Solutions of HE, CH and NA (Sigma-Aldrich, Poland) in phosphate-buffered saline (PBS) (Biomed-Lublin, Poland) were prepared by sonication (Sonic-2 ultra-

sound bath, POLSONIC Pałczyński, Poland) and shaking for 30 min at 37°C for three separate studies with each polyphenol separately. Final doses were administered via intramammary infusion using a solution of 30 mg of polyphenols in 5 ml of PBS (Biomed-Lublin, Poland). In all the studies, the polyphenols were administered immediately after morning milking at 9:00 a.m. to all quarters of the udder in all animals. Consequently, the 30 mg/quarter/day dose was proposed (120 mg/udder/day). The study design covered three periods: first – the baseline (B1 – B3) where the baseline data for PK, SCC, haematology, and biochemistry were collected (no flavones were administered at this stage); second – treatment (T1 – T6) where the animals were treated with the flavones (in this stage collection of samples for PK, SCC, haematology, and biochemistry were continued); third – recovery (R1 – R5) (in this stage collection of samples for PK, SCC, haematology, and biochemistry was continued) with no flavones administered at this stage (Burmańczuk et al., 2018).

Animals

The animals were handled according to the principles outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Based on SCC and clinical assessment (palpation evaluation of udder), 12 dairy cows with clinical mastitis of one quarter were randomly allocated for each study variant. The animal studies were conducted with the consent of the Ethics Committee (Resolution No. 33/2017, Local Ethical Committee for Animal Experiments in Lublin). SCC > 1,500,000 cells/ml in one of the four quarters was taken as an inclusion criterion. Only cows with one inflamed quarter were included in the study. The study was conducted on 12 Polish Holstein-Friesian Black-and-White cows with average daily milk yield of 33–34 L, weighing ≈ 650 kg each; the cows were between fourth and sixth lactations. The animals were fed with farming feed concentrates and fodder (oats, barley) grain alternated with raw corn, pasture grazing grass silage, green forage, straw, and meadow hay with water access *ad libitum*. The analyses were carried out in a selected farm (Agromarina Sp. z o.o., Poland). Before the drug administration and milk delivery, each udder was disinfected. 0.5 L of milk sample was collected once a day from the inflamed quarter of each cow, immediately before daily morning milking. Milk samples for SCC and blood plasma samples for pharmacokinetics, whole blood samples for haematology and biochemistry (per each cow) were collected at the baseline (B1–B3), in the treatment period (T1–T6), and in the recovery period (R1–R5). Blood samples were collected from the jugular vein into sterile Eppendorf tubes. Milk samples (0.5 L) were collected into sterile tubes and analysed immediately. Samples for the pharmacokinetic analysis in the recovery phase were collected at 4.0, 6.0, 12.0, 24.0, and 36.0 h and 2.0, 3.0, 4.0, 5.0, and 6.0 days after the last dose.

Signs of local or general intolerance or adverse drug reactions related to the IMM administered polyphenols were monitored in each variant. No other medicines were administered during the studies. The animal's health and udder conditions were examined daily by a veterinary clinician.

Somatic cell count, haematology and blood biochemistry analysis

Baseline analysis of haematology, blood biochemistry, and SCC was performed every 24 h in all cows three days before the first dose (B1–B3). Sampling was continued across the treatment and recovery phases every day for each variant. The blood samples were analysed to determine selected blood biochemical parameters, including plasma cholesterol, urea, bilirubin, creatinine, calcium arsenazo III and phosphate. Blood morphotic counts were determined:

WBC – white blood cell volume, LYM – lymphocytes, MID – white blood cells not classified as lymphocytes or granulocytes, GRAN – granulocytes, RBC – red blood cell volume, Hb – haemoglobin, Hct – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, RDW – red cell distribution width, RDW_a – absolute red cell distribution width, Plt – platelets, and MPV – mean platelet volume. SCC analysis was performed using the Bentley BactoCount IBCm analyser (Bentley Instruments Inc.). Haematological and blood biochemistry parameters were determined using an automated haematology analyser – Abacus Junior Vet (Diatron Group, Hungary).

Mass spectrometry analyses

The analytical method for the determination of quercetin in milk described previously was modified and applied for the determination of the CH, HE and NA polyphenols in the milk samples (Gbylik-Sikorska et al., 2019). Ultra-high-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) method was successfully validated and used to determine the pharmacokinetics of these three compounds in cow's milk. HE (purity > 97%), CH (purity > 97%), NA (purity > 95%) and quercetin used as an internal standard (IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were obtained from J.T. Baker (The Netherlands), formic acid was provided by Fluka (USA). All reagents were at least HPLC grade. Water was deionized (>18 MΩ/cm) by the Millipore system (Bedford, MA, USA). The stock solutions of HE, CH, NA and IS were prepared by dissolving in methanol (1 mg/l) and stored at –20°C for 3 months. The working standard solutions of each analyte and IS (10 and 1 µg/l) used as quality control samples were prepared by serial dilution in methanol and stored at 4–8°C for 1 month. The UHPLC-MS/MS system consisted of an ultra-high-performance liquid chromatograph Shimadzu Nexera X2 (Shimadzu, Japan) and a QTRAP® 4500 triple quadrupole mass spectrometer (AB Sciex LLC, Framingham, MA, USA). The Analyst 1.6.3 software controlled the UHPLC-MS/MS system and processed the data.

Sample extraction procedure

Milk (500 µg) was weighed into a 2.0 mL microcentrifuge tube; next, 30 µL of IS solution was added and vortexed for 30 seconds before extraction, followed by adding 950 µL of 0.5% formic acid in acetonitrile. The mixture was then vortexed for 1 min and centrifuged at $140162 \times \text{RFC}$ for 5 min at room temperature. 350 µL of the upper layer was transferred to vials, and a 3-µL aliquot was injected into the UHPLC-MS/MS.

Chromatographic conditions

The chromatographic separation was performed on a ZORBAX SB-C18 column (50 mm × 2.1 mm × 1.8 µm) (Agilent, USA) integrated with a guard column of the same type UHPLC/HPLC. The mobile phase composition was a mixture of 0.5% formic acid (A) and methanol (B). The following gradient elution program was applied: 85% A (0.0–2.0 min), 80% B (2.01–4.0 min), and 85% A (4.01–6.00 min). The flow rate of the mobile phase was 0.45 mL/min and the column temperature was kept at 35°C. The mass spectrometer was operated in the negative electrospray ionization mode. Quantification was performed using the multiple-reaction monitoring mode (MRM). The following transitions were used: CH: m/z 253.0 > 143.0 and 253.0 > 119.0; HE: m/z 301.0 > 164.0 and 301.0 > 284; NA: m/z 271.0 > 151.0 and 271.0 > 119.0, and IS: m/z 301.0 > 151.0. The MS/MS parameter was optimized as follows: Ion Spray voltage: 4500 V, Curtain Gas – 20 psi, ion source gas 1–40 psi, ion source gas 2–50 psi, and a source temperature of 400°C.

Validation procedure

The developed method was fully validated in terms of linearity, precision (repeatability and within-laboratory reproducibility), recovery, and lower limit of quantification (LLOQ). The linearity of the method (determination coefficient, r^2) was validated by two matrix-match calibration curves, which were prepared using blank milk samples spiked with 6 different concentration levels (1, 10, 50, 100, 250, and 500 µg/L) and (500, 1000, 2000, 5000, 7500 and 10 000 µg/L), both calibration curves were above 0.998. Calibration was performed by the least-squares linear regression of the peak area ratios of the analyte to IS. The repeatability was calculated after analysis of 6 plasma samples spiked with analytes at 3 different concentrations (1, 10, and 50 µg/L) on the same day with the same instrument and the same operator. Another two sets of 6 spiked samples were prepared as described above and analyzed on two different days with the same instrument and different operators for determination of reproducibility. The precision of the method, presented as coefficients of variation (CV), was calculated as the ratio of the standard deviation to the mean analyte concentration. The acceptable limits for all analytes were in the range of 2.6% to 4.2% and 7.9% to 12.1% for repeatability and within-laboratory reproducibility, respectively. LLOQ was defined as the lowest concentration standard in the calibration curve that was analyzed with accuracy of $\pm 15\%$ and precision $\leq 15\%$. The LLOQ for each analyte was 1 µg/L. The extraction recovery experiment was carried out by analyzing samples spiked at the same concentration levels as in the case of precision. The mean extraction recovery of the compounds was in the range of 82.0 ± 4.5 to $107.0 \pm 3.6\%$. To estimate whether the matrix influenced the peak area significantly, the matrix effect (ME) was investigated by comparing matrix-matched standards with standards in the solvent (mobile phase) at a corresponding concentration of 1 µg/L; the ME value between 90 to 110% indicated no significant effect of matrix.

Analysis of pharmacokinetics and pharmacodynamics

Multiple-dose pharmacokinetic parameters were calculated in Phoenix 64 Win-Nonlin 8.1 (Certara L.P.) using noncompartmental modelling. The following param-

eters were calculated based on concentrations with the use of the built-in equations and algorithms: $t_{1/2\text{kel}}$ – half-life in the elimination phase (calculated after the last dose); $\text{AUC}_{0-\text{inf}}$ – area under the concentration-time curve measured from zero to infinity; $\text{AUC}_{0-\text{last dose}}$ – area under the concentration-time curve measured from zero to the time of the last dose; AUC_{tau} – partial area under the concentration-time curve from the dosing time to the dosing time plus time to the next dose; C_{avg} – average concentration measured between zero to the time of the last dose; Swing – the degree of fluctuation over one dosing interval at the steady state; Fluctuation% – peak to trough fluctuation within a complete dosing interval at the steady state; and Accumulation index – the magnitude of accumulation.

The number of animals responding positively to the one-week therapy was also determined. The arithmetic mean of SCC was taken as the starting point for the parameterisation of the responder (R) or non-responder (NR) analysis (Snapinn and Jiang, 2007; The Food and Drug Administration, 2019). Appropriate criteria were defined to verify R to the administration of the respective polyphenols. The animal was classified as group R if $\text{SCC}_\text{B}/\text{SCC}_\text{T} > 1$ (responding in the treatment phase) or $\text{SCC}_\text{B}/\text{SCC}_\text{R} > 1$ (responding in the recovery phase), where: SCC_B – the arithmetic mean of SCC over three consecutive days before polyphenol administration (days -2, -1, day 0); SCC_T – the arithmetic mean of SCC at the treatment stage; SCC_R – the arithmetic mean of SCC at the recovery stage. The animal was classified as NR if $\text{SCC}_\text{B}/\text{SCC}_\text{T} < 1$ (non-responding in the treatment phase) or $\text{SCC}_\text{B}/\text{SCC}_\text{R} < 1$ (non-responding in the recovery phase).

Statistical analysis

The analysis of raw data was conducted with GraphPad Prism® v. 6.01 (GraphPad Software Inc.). The Mann-Whitney test was used for comparison of SCC raw data in subsequent groups. The control group was the SCC arithmetic mean for the baseline (B1 – B3) and then the SCC arithmetic mean value on a particular day of the study. Pharmacokinetic data were compared by one-way ANOVA with Dunn's multiple comparison test. Differences between the analysed groups with a P-value lower than ≤ 0.05 were considered statistically significant.

Results

The SCC values are shown in Figures 1, 2, and 3. The number of responders varied in both the treatment and recovery phases in the HE, CH, and NA variants (Table 1). In the treatment phase, the highest percent of R was in the CH variant (41.67%) and the lowest in the NA variant (25.0%), while in the recovery phase the CH variant has the lowest percent of R (33.33%) compared to the HE (50.00%) or NA variant (41.67%).

The baseline, treatment, recovery, arithmetic mean, standard deviation ($\pm$) and RSD% were $1.76 \times 10^6 \pm 0.83 \times 10^6$, 47.31%, $1.34 \times 10^6 \pm 0.76 \times 10^6$, 56.73%, $0.90 \times 10^6 \pm 0.72 \times 10^6$ and 79.62%, respectively, for HE. The same parameters were $1.92 \times 10^6 \pm 0.77 \times 10^6$, 40.52%, $1.68 \times 10^6 \pm 0.75 \times 10^6$, 45.15%, $0.95 \times 10^6 \pm 0.76 \times 10^6$ and 80.64%, respectively, for CH and $2.00 \times 10^6 \pm 0.84 \times 10^6$, 42.12%, $1.54 \times 10^6 \pm 1.11 \times 10^6$, 72.12%, $1.00 \times 10^6 \pm 0.74 \times 10^6$ and 74.69%, respectively, for NA.

B1–B3 – baseline analysed on 3 subsequent days before the first dose; T1–T6 – treatment phase (120 mg/udder/day); R1–R5 – recovery phase; shadow line – the widest range between the 10 to 90% percentiles of the baseline; line in shadow and white boxes – median value; borders of boxes – 50% percentiles; whiskers – range from the 10 to 90% percentiles;
 ** – $P \leq 0.01$, *** – $P \leq 0.001$ arithmetic mean of the baseline vs. subsequent day.

Figure 1. Daily changes in the somatic cells count (SCC) in the milk of cows after multiple intramammary infusion of hesperidin at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

B1–B3 – baseline analysed on 3 subsequent days before the first dose; T1–T6 – treatment phase (120 mg/udder/day); R1–R5 – recovery phase; shadow line – the widest range between the 10 to 90% percentiles of the baseline; line in shadow and white boxes – median value; borders of boxes – 50% percentiles; whiskers – range from the 10 to 90% percentiles;
 ** – $P \leq 0.01$, *** – $P \leq 0.001$ arithmetic mean of the baseline vs. subsequent day.

Figure 2. Daily changes in the somatic cells count (SCC) in the milk of cows after multiple intramammary infusion of chrysin at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

B1–B3 – baseline analysed on 3 subsequent days before the first dose; T1–T6 – treatment phase (120 mg/udder/day); R1–R5 – recovery phase; shadow line – the widest range between the 10 to 90% percentiles of the baseline; line in shadow and white boxes – median value; borders of boxes – 50% percentiles; whiskers – range from the 10 to 90% percentiles; ** – $P \leq 0.01$, *** – $P \leq 0.001$ arithmetic mean of the baseline vs. subsequent day.

Figure 3. Daily changes in the somatic cells count (SCC) in the milk of cows after multiple intramammary infusion of naringenin at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

In the case of all three polyphenols, the greatest decrease in SCC in animals classified as R was recorded in the recovery phase. An approximately 2-fold decrease was noted in the recovery phase (arithmetic mean) compared to the baseline, and was significant in the HE, CH and NA variants ($P < 0.05$).

Non-responder data. The level of SCC in the treatment and recovery phases, arithmetic mean, standard deviation ($\pm$) and RSD% in the NR group were $2.55 \times 10^6 \pm 1.58 \times 10^6$, 61.95%, $4.11 \times 10^6 \pm 2.09 \times 10^6$, and 50.99% for HE. The same parameters were $3.66 \times 10^6 \pm 1.58 \times 10^6$, 43.05%, $2.57 \times 10^6 \pm 1.61 \times 10^6$ and 62.56% for CH, and $2.94 \times 10^6 \pm 1.54 \times 10^6$, 52.22%, $3.82 \times 10^6 \pm 1.94 \times 10^6$ and 50.88% for NA.

In the case of all three substances, the increase in SCC in the NR animals in the treatment phase was significant in comparison to the baseline (HE, $P < 0.001$; CH, $P < 0.001$; NA, $P < 0.05$). Additionally, the increase was also significant in the recovery phase only in the case of NA in the NR group ($P < 0.001$).

The blood haematology and biochemistry data are presented in Table 1. In the case of HE and NA, significant differences were found in bilirubin, RDW and MCV (treatment and recovery), phosphorus and WBC (treatment), and LYM (%) (recovery) which were higher than in the baseline. In the case of NA, a significant difference from the baseline was noticeable only in the calcium levels. No significant differences were found in the remaining parameters.

Table 1. Blood hematology and biochemistry parameters after intra-mammary hesperidin, chrysin, and naringenin infusion at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

Parameters	Hesperidin			Chrysin			Naringenin		
	baseline	treatment	recovery	baseline	treatment	recovery	baseline	treatment	recovery
1	2	3	4	5	6	7	8	9	10
Cholesterol (mg/dL)	152.027; 3.93	146.962; 4.855	137.146; 5.665 **	140.848; 20.793	144.19; 26.752	133.255; 10.857	170.085; 17.805	167.261; 10.204	126.42; 4.299 ****
Bilirubin (mg/dL)	0.418; 0.279	2.917; 0.734 ****	2.606; 0.295 ****	2.898; 2.071	2.852; 1.115	3.142; 0.671	0.545; 0.408	3.401; 1.09 ***	2.635; 0.38 **
Urea (mg/dL)	19.926; 1.08	21.778; 2.455	19.937; 1.918	17.261; 3.107	18.938; 0.546	19.125; 1.735	22.008; 0.142	21.059; 1.458	21.059; 2.098
Creatinin (mg/dL)	1.063; 0.034	1.503; 0.67	1.204; 0.048	1.081; 0.135	1.553; 0.764	1.253; 0.166	1.046; 0.049	1.233; 0.063 ***	1.249; 0.05 ***
Phosphate (mg/dL)	5.568; 0.326	7.526; 0.531 **	6.709; 0.932	6.318; 1.35	8.127; 1.177	7.674; 1.285	5.777; 0.339	7.414; 0.767 *	7.796; 0.678 **
Calcium Arsenazo III (mg/dL)	9.357; 0.205	10.031; 0.346	9.833; 0.442	8.519; 0.728	10.376; 0.488 **	9.026; 0.613	9.476; 0.131	11.758; 3.348	10.147; 0.303
WBC ($10^9/L$)	13.638; 0.827	25.132; 5.404 ***	18.398; 1.249	18.56; 5.982	19.711; 3.213	21.075; 1.754	15.303; 1.624	19.991; 2.727 *	17.61; 1.953
LYM $\times 10^9/L$	6.482; 1.336	9.107; 2.185	7.558; 2.026	9.101; 2.809	10.067; 2.001	11.135; 1.864	4.786; 0.091	7.955; 1.036 ***	7.871; 0.764 ***
MID $\times 10^9/L$	2.477; 0.585	3.721; 0.749	3.517; 0.642	3.299; 1.499	3.089; 0.769	3.458; 0.755	2.189; 0.62	3.795; 0.63 **	4.214; 0.475 ***
GRAN $\times 10^9/L$	7.178; 1.021	8.902; 0.78	8.292; 0.957	7.682; 1.61	8.722; 1.246	9.019; 1.47	7.236; 0.394	7.45; 0.361	10.126; 0.866 ****
LYM (%)	33.914; 0.826	44.683; 1.647 ***	44.411; 3.448 ***	39.375; 6.496	43.55; 4.617	45.444; 3.88	35.544; 4.243	40.581; 3.346	43.673; 2.482 **

Table 1 – contd.

1	2	3	4	5	6	7	8	9	10
MID (%)	15.694; 1.603	17.48; 1.87	17.101; 1.059	16.456; 0.969	14.267; 1.272	15.31; 1.661	14.633; 1.12	16.359; 1.683	16.669; 1.419
GRAN (%)	48.363; 4.344	35.678; 2.619 ***	36.976; 2.513 ***	42.357; 4.644	39.991; 3.488	37.981; 3.204	47.669; 2.706	38.75; 3.916 **	34.265; 3.39 ***
RBC $\times 10^{12}/L$	4.975; 0.417	4.483; 0.077 *	4.576; 0.217	4.695; 0.44	4.784; 0.452	4.722; 0.307	5.191; 0.679	4.864; 0.336	4.659; 0.23
Hct (%)	20.648; 0.883	22.07; 0.751	21.761; 0.805	20.813; 3.032	21.761; 1.288	23.29; 1.318	20.853; 1.634	21.354; 1.214	21.957; 0.601
Hb (g/dL)	10.292; 0.58	9.876; 0.707	9.733; 0.267	9.424; 0.659	9.514; 0.301	9.946; 0.431	11.408; 0.738	9.395; 0.167 *****	9.6; 0.26 *****
MCV (fL)	42.44; 2.448	47.722; 1.179 **	47.372; 1.443 **	44.486; 3.762	48.248; 3.136	49.031; 2.017	40.017; 1.338	46.768; 1.909 ***	46.887; 1.56 *****
MCH (pg)	20.291; 0.447	20.216; 0.504	20.462; 0.29	20.519; 0.226	20.954; 0.476	21.1; 0.308	20.008; 0.619	20.505; 0.314	20.217; 0.292
MCHC (g/dL)	48.462; 2.006	43.735; 0.899 ***	43.053; 0.831 *****	47.011; 3.756	44.117; 2.269	43.249; 1.224	47.008; 3.723	43.831; 1.723	42.246; 1.711 *
RDW (%)	19.025; 0.64	19.864; 0.427	19.887; 0.73	19.214; 0.633	18.893; 0.303	18.625; 0.234	19.131; 0.24	19.628; 1.312	19.893; 0.9
RDWa (fL)	26.817; 1.317	31.938; 0.925 ***	31.059; 1.416 ***	28.946; 2.562	30.672; 1.717	30.69; 0.992	25.986; 1.07	30.668; 1.44 **	31.87; 1.804 ***
Plt $\times 10^9/L$	210.444; 5.098	369.643; 43.527 **	315.94; 56.123 *	366.132; 120.36	331.981; 59.445	291.363; 33.482	329.356; 10.596	452.354; 83.918	374.933; 72.247
MPV (fL)	10.676; 0.364	10.795; 0.505	10.973; 0.366	10.826; 0.784	11.257; 0.443	11.137; 0.416	10.885; 0.587	10.852; 0.192	10.693; 0.194

WBC – white blood cell volume; LYM – lymphocytes; MID – white blood cells not classified as lymphocytes or granulocytes; GRAN – granulocytes; RBC – red blood cell volume; Hb – hemoglobin; Hct – hematocrit; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; RDW – red cell distribution width; RDWa – absolute red cell distribution width; Plt – platelets; MPV – mean platelet volume; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; statistical significance calculated in comparison to the baseline of a specific substance.

Table 2. Pharmacodynamics (%) and pharmacokinetic ($\bar{x} \pm SD$) parameters of hesperidin (HE), chrysin (CH) and naringenin (NA) after multiple intramammary infusion at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

	Pharmacodynamic parameters % per group					
	HE		CH		NA	
R/NR group	R	NR	R	NR	R	NR
Treatment phase	33.33	66.67	41.67	58.33	25.00	75
Recovery phase	50.00	50.00	33.33	66.67	41.67	58.33
SSC trend						
Treatment phase	↓	↑***	↓	↑***	↓	↑*
Recovery phase	↓*	↑	↓*	↑	↓*	↑***
Pharmacokinetic parameters						
$t_{1/2\text{el}}$ (h)	9.926±2.233		7.985±1.232 ***NA		12.226±1.85	
$AUC_{0-\text{inf}}$ (h×ng/g)	36822.32±6153.74 **NA; ****CH		436527.23±43222.36 **NA		144752.357±37990.81	
$AUC_{0-\text{last dose}}$ (h×ng/g)	16366.51±2716.63 *NA; ****CH		327403.49±30330.92 *NA		138870.07±37327.77	
AUC_{tau} (h×ng/g)	1835.63±690.28 *NA; **CH		18662.57±4684.35 ****NA		657.48± 338.14	
C_{avg} (ng/g)	76.485±28.76 *NA; **CH		777.607±195.18 ***NA		27.395±14.09	
Swing	13.356±8.53 ****NA		294.27± 193.05 ***NA		22.45±15.22	
Fluctuation%	161.02±25.28 ****NA		198.25±0.74 ***NA		158.514± 53.93	
Accumulation index	1.122±0.097 **NA		2.59±1.589 ****NA		1±0	

HE – hesperidin; CH – chrysin; NA – naringenin; R – responders; NR – non-responders; SCC – somatic cell count.

↓ – decreasing trend (decreasing value of the arithmetic mean of SCC); ↑ – increasing trend (increasing value of the arithmetic mean of SCC); $t_{1/2\text{el}}$ – half-life in the elimination phase (calculated after the last dose); $AUC_{0-\text{inf}}$ – area under the concentration-time curve measured from zero to infinity; $AUC_{0-\text{last dose}}$ – area under the concentration-time curve measured from zero to the time of the last dose; AUC_{tau} – partial area under the concentration-time curve from the dosing time to the dosing time plus time to the next dose; C_{avg} – average concentration measured between zero to the time of the last dose; Swing – the degree of fluctuation over one dosing interval at the steady state; Fluctuation% – peak to trough fluctuation within a complete dosing interval at the steady state; Accumulation index – the magnitude of accumulation; asterisks indicate: * – $P \leq 0.05$; ** – $P \leq 0.01$; *** – $P \leq 0.001$; **** – $P \leq 0.0001$.

The pharmacokinetic profiles are shown in Figures 4, 5 and 6. The pharmacokinetic parameters are presented in Table 2. After the multiple administration of the polyphenols, the last concentrations were observed on the fifth day after the last dose was administered. On day 6, no HE, CH or NA residues were found in the milk of the experimental cows (the lower limit of quantitation was 1 ng/ml). Validation parameters of the chromatographic method were shown in Table 3.

Table 3. Validation parameters of the chromatographic method

Analyte	Repeatability* (CV, %)	Within-lab reproducibility* (CV, %)	LLOQ (μg/L)	Recovery* (%)	ME (%)
Chrysin	2.6±1.2	9.4±3.2	1.0	107.0±3.6	94.6
Hesperidin	4.2±2.7	12.1±4.1	1.0	82.0±4.5	112
Naringenin	2.8±1.6	7.9±3.3	1.0	94.0±3.7	92.7

*Coefficient of variation of 3 validation levels; LLOQ – lower limit of quantitation; ME – matrix effect.

Black solid line – arithmetic mean representing the general pharmacokinetic profile; dotted lines – pharmacokinetic profiles of each animal.

Figure 4. Pharmacokinetic profiles of hesperidin after multiple intramammary infusion of polyphenol at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

Black solid line – arithmetic mean representing the general pharmacokinetic profile; dotted lines – pharmacokinetic profiles of each animal.

Figure 5. Pharmacokinetic profiles of chrysin after multiple intramammary infusion of polyphenol at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

Black solid line – arithmetic mean representing the general pharmacokinetic profile; dotted lines – pharmacokinetic profiles of each animal.

Figure 6. Pharmacokinetic profiles of naringenin after multiple intramammary infusion of polyphenol at 120 mg/udder/day every 24 h for 6 days in dairy cows with mastitis

Discussion

Many studies indicate a significant potential role of polyphenolic compounds in the treatment of mammary gland diseases (Gao et al., 2017; Burmańczuk et al., 2018; Perruchot et al., 2019; Zhao et al., 2019). In the present study, three different polyphenols, i.e. HE, CH and NA were investigated in terms of their potential use in mastitis treatment. Tracking the effects of polyphenols after intramammary administration is difficult due to the low number of observations made *in vivo* to date (Burmańczuk et al., 2018), and the extreme complexity of the mechanism of action of polyphenols on the inflammation and generally on the immune system (Zierau et al., 2002; Lee et al., 2015; Hwang et al., 2018; Tejada et al., 2018; Ren et al., 2019; Zhao et al., 2019). This mechanism usually involves many factors at the same time, i.e. regulation of gene transcription, inhibition of the activity of intracellular signalling molecules, and interaction with specific receptors, e.g. oestrogen receptors (Zierau et al., 2002). On the other hand, mastitis is a disease that may have a highly variable course. HE is a known factor suppressing the epithelial-to-mesenchymal transition, thus inhibiting inflammation. Such a mechanism has been proved in idiopathic pulmonary fibrosis (Ren et al., 2019). The pharmacological effect of HE treatment is dose-dependent, as in the case of baicalin (Perruchot et al., 2019). This is probably why the therapeutic effect of the HE treatment was visible only in the recovery phase in many animals.

A significant decrease in SCC was found only in the R group in the recovery phase ($P < 0.05$). In the same variant, a significant increase in SCC was noted in the treatment phase in the NR group ($P < 0.001$). This may mean that the therapeutic administration of HE would require a different dosing schedule. The present study shows that, with the proposed experimental design and dosing, a delayed therapeutic effect was observed in the R group, with no significant increase in SCC in the animals in the treatment phase. In turn, a delayed therapeutic effect was also observed in the NR group with no significant increase in SCC in the recovery phase. As a result, almost two weeks after starting the HE therapy without the use of any additional drugs, the animals in the NR group were stabilised (no significant baseline vs. recovery differences) or the SCC decreased significantly in the R group.

CH has anti-inflammatory, antiproliferative and several other pharmacological properties related to inhibition of nitric oxide, cytokines, chemokines and growth factors. Moreover, it inhibits calcium release (Lee et al., 2015). This specific effect is in line with the observations from the current study. Blood calcium levels increased significantly in the treatment phase only in the case of CH. The administration of chrysin resulted in a different location of the therapeutic effect from that in the case of HE and NA. In the CH variant, the largest numbers of animals were included in the R group in the treatment phase, not in the recovery phase as in the case of HE and NA. This means that the effect of CH was not as delayed as in the R groups of HE and NA. Therefore, it can be hypothesised that the CH dosing regimen was much better fitted to its mechanism of action, although this thesis would require separate studies and verification. However, as in the case of HE, a significant decrease in SCC was observed in the R group only in the recovery phase ($P < 0.05$). Furthermore, as in the case of HE, it was accompanied by a significant increase in SCC in the NR group, but only in the treatment phase ($P < 0.001$). Both in the case of HE and CH, it can be concluded that the effect of both polyphenols stabilised the number of SCC (recovery phase, NR) or caused a significant decrease in SCC (recovery phase, R). This pattern was common for both polyphenols.

NA exhibited estrogenic activity in a specific concentration range (Zierau et al., 2002). It has been shown that cows with mastitis have low levels of 17β -estradiol; moreover, the 17β -estradiol levels are associated with neutrophil migration (Medina-Estrada et al., 2016). Consequently, the interaction with the oestrogen receptor is involved in the anti-inflammatory action of NA. In turn, 17β -estradiol may enhance apoptosis in bovine mammary epithelial cells (Yart et al., 2013). A delayed therapeutic effect of NA administration was observed, as in the case of HE, in the recovery phase of the R group. As in the HE variant, a higher percentage of animals were classified as R in the recovery phase. Delayed effects after administration of 17β -estradiol related to mammary tight junctions have been observed as well (Agenäs et al., 2019). Therefore, it can be expected that also other optimised NA dosing regimens will have a delayed effect.

Subclinical mastitis is accompanied by changes in blood biochemical and morphological parameters. The results of the research conducted in mastitis are not always consistent. An important element influencing formulated conclusions is the degree of disease development as well as its aetiology. However, several observations

made in the present study are consistent with studies reported previously. In the current study, the WBC number increased in the cows with mastitis (Zaki et al., 2010; Garba et al., 2019); the WBC numbers were elevated in the HE and NA variants. The increase in WBC was significant between the baseline and the treatment but not in the recovery phase. The return of the WBC values to the baseline after the HE and NA therapy may indirectly indicate the therapeutic effect of the polyphenols. This hypothesis may be supported by the fact that the animals did not receive any other drugs during the treatment and recovery phases, yet their condition in the recovery was stable. In another study, higher calcium levels in mastitis were shown, whose effect is explained by reduced calcium excretion with milk in mastitis (Sarvesha et al., 2017).

In the case of all analysed substances, the calcium levels increased in the treatment phase and decreased in the recovery. Unfortunately, this effect was statistically significant only in the case of CH and only for the increase in the treatment phase, but the general scheme was similar to that of WBC. Another study confirmed that Hb decreased in mastitis (Zaki et al., 2010). The same trend was observed in the HE and NA variants. In the case of both polyphenols, the Hb levels decreased with time from the baseline through the treatment to the recovery phase. Only the Hb decrease in NA was statistically significant (in the treatment and recovery phases, $P < 0.001$). Increased levels of bilirubin were observed in other studies of endometritis and mastitis (Bertoni et al., 1994; Cui et al., 2019). In the current research, increased bilirubin levels were determined in the HE and NA variants in both the treatment and recovery phases. These results were statistically significant.

The pharmacokinetic parameters indicate that the tested polyphenols are quickly withdrawn from this compartment after multiple intramammary administration. The analyses indicate that these substances are eliminated to very low levels (lower than 1 mg/ml) within five days from the last administration. This characteristic of polyphenol elimination is highly advantageous due to the very low levels of residues of these substances in milk intended for consumption. It should be noted that the amount of the polyphenols tested in the current study consumed by humans daily with fruit, juices, etc. are hundreds of times higher (European Food Safety Authority, 2017).

Conclusions

To summarise the studies, it can be concluded that all the polyphenols tested exerted positive effects on the number of SCC in the milk of cows with mastitis. The results of the present study also give hope that the interaction of HE, CH and NA with other intramammary drugs used in the treatment of mastitis may be beneficial. The current results indicate the relevance of the simultaneous use of polyphenols with antibiotics during the treatment of mastitis, and this hypothesis is supported by the results of other studies (Wu et al., 2013; Mikłasińska-Majdanik et al., 2018). The research shows that determination of the influence of polyphenols on the course of mastitis is very difficult, which is mainly related to the very complex mechanism of action of polyphenols. These are pharmacologically active substances; however, it is difficult to regard their mechanism of action as selective. Polyphenols trigger

a massive cascade of immune and endocrine responses. The consequences of this cascade overlap each other and lead to a pharmacodynamic effect that is still difficult to measure in a way that is adaptable to everyday veterinary practice. The results presented in the current study indicate the need to optimise the dosage of polyphenols administered via the intramammary route. Such optimisation requires consideration of both the dosage and the frequency of polyphenol administration.

Ethics approval

The animal procedures undertaken in this study were approved by the Animal Ethics Committee of Lublin in accordance with Directive 2010/63/EU.

Declaration of interest

None.

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Received: 17 III 2021

Accepted: 29 VII 2021