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The effect of transdermal flunixin meglumine on blood cortisol levels in dairy calves after cautery disbudding with local anesthesia

M. Röder,[1] W. Heuwieser,[1]* S. Borchardt,[1] J. L. Plenio,[2] R. Palme,[3] and F. Sutter[1]
[1]Clinic for Animal Reproduction, Faculty of Veterinary Medicine, Freie Universität Berlin, Königsweg 65, 14163 Berlin, Germany
[2]Institute for Veterinary Epidemiology and Biostatistics, Faculty of Veterinary Medicine, Freie Universität Berlin, Königsweg 67, 14163 Berlin, Germany
[3]Unit of Physiology, Pathophysiology, and Experimental Endocrinology, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

ABSTRACT

The objective of this study was to evaluate the effect of the nonsteroidal anti-inflammatory drug transdermal flunixin meglumine (Finadyne Transdermal) on plasma cortisol, average daily weight gain, and standing and lying behavior of calves, when given at the time of disbudding combined with local anesthesia. A sedative was not used to minimize pharmacological interactions. Seventy-one female Holstein Friesian calves aged 13 ± 2 d, with an average weight of 48.9 ± 4.26 kg were enrolled in the study. All calves were randomly assigned to one of 3 treatment groups: (1) control group (CON, n = 27), (2) 1-flunixin group (1-FLU, n = 26) with a single administration of transdermal flunixin meglumine at disbudding, and (3) 2-flunixin group (2-FLU, n = 24) with 2 administrations of transdermal flunixin meglumine, the first treatment at disbudding and the second 6 h after disbudding. Although the CON group received a placebo, 1-FLU and 2-FLU received flunixin meglumine transdermally. To account for plasma cortisol changes due to manipulation and handling of the calves, a sham disbudding procedure was performed one week before disbudding took place. Sham disbudding was conducted by using a cold cautery dehorner applied to each horn bud for 10 s. Disbudding was performed in a similar way by using a hot cautery dehorner. Plasma samples were collected to measure the stress biomarker cortisol at 7 different time points. Body weights were measured 4 times in 2 wk. Standing and lying behavior was assessed via 3-dimensional accelerometer. During sham disbudding and disbudding mean plasma cortisol concentrations were 6.09 ± 2.5 ng/mL and 5.16 ± 2.8 ng/mL, respectively. Treatment tended to have an effect on plasma cortisol concentrations during sham disbudding and had an effect on plasma cortisol concentrations during disbudding. Plasma cortisol concentrations were affected by treatment 2 h after disbudding in comparison to CON group. Furthermore, there was a significant effect on plasma cortisol concentrations 6 h after disbudding in contrast to CON. A return to baseline plasma cortisol levels (initial concentrations) was not achieved in CON during disbudding. There was no statistical difference between average daily weight gain and the treatment procedure. Total lying time was not affected by treatment after disbudding. In conclusion, transdermal flunixin meglumine given at the time of disbudding combined with local anesthesia decreased concentrations of the stress biomarker cortisol, but a second dose 6 h after disbudding had no further effect on plasma cortisol levels.
Key words: disbudding, dairy calves, cortisol, flunixin meglumine, transdermal

INTRODUCTION

Disbudding dairy calves before 8 wk of age is a common practice in the dairy industry (Winder et al., 2016). There are 2 main reasons why dairy calves are being disbudded: easier handling of cattle and reduced risk of injuries in staff and cattle. In about 81% of dairy herds in the European Union (**EU**; Cozzi et al., 2015) and 94% in the United States (USDA, 2009), disbudding of dairy calves is conducted. In the United States, 32% of farmers use a hot-iron for disbudding procedure (FARM v. 4.0; https://nationaldairyfarm.com/farm-animal-care-version-4-0/). Disbudding leads to pain-related distress, which can be measured in behavioral and hormonal changes (Stafford and Mellor, 2005). To control pain, medication should be used and calves should be disbudded prior 8 to wk of age (FARM v. 4.0). Stressful situations activate a hormonal cascade of the hypothalamic–pituitary–adrenocortical axis (Sylvester et al., 1998). As a result, an increased activity of the adrenal glands occurs, which leads to a higher release of glucocorticoids (i.e., cortisol) and

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*Corresponding author: w.heuwieser@fu-berlin.de

catecholamines (Axelrod and Reisine, 1984). Cortisol can be measured in different media such as blood or its metabolites can be measured in feces (Mormède et al., 2007; Palme, 2019). After disbudding, a rapid cortisol release is observed and results in an initial peak of cortisol in plasma within 30 min after disbudding (Stafford and Mellor, 2005). A second plasma cortisol peak occurs between 3 to 8 h after disbudding if local anesthesia is used for prevention of pain (McMeekan et al., 1998; Sutherland et al., 2002; Milligan et al., 2004; Stock et al., 2013). According to Heinrich et al. (2010), pain-associated behavioral changes varied during 44 h with a peak at 6 h after disbudding, implying that calves may be discomforted during this time despite analgesia. Local anesthetic such as lidocaine decreased plasma cortisol levels after disbudding for approximately 2 h and caused a delayed rise of plasma cortisol lasting for 6 h (Sutherland et al., 2002; Stafford and Mellor, 2005). The use of the nonsteroidal anti-inflammatory drug (**NSAID**) meloxicam can cause a significant reduction of plasma cortisol concentration 6 h after disbudding (Heinrich et al., 2009).

In the United States and the EU, only 1.8% and 30 to 40% of farmers reported the use of an analgesic treatment before or after disbudding, respectively (Fulwider et al., 2008; Cozzi et al., 2015). This is due to economic reasons and a lack of training on how to perform disbudding properly including analgesia (Gottardo et al., 2011).

In a randomized controlled trial, a single intravenous treatment using flunixin meglumine (2.2 mg/kg) at the time of disbudding reduced cortisol concentrations in blood plasma (Huber et al., 2013). A second intravenous treatment with flunixin meglumine 3 h after disbudding led to a similar cortisol concentration up to 6 h after disbudding in comparison to the single treatment and the nondisbudded control group. Cortisol concentrations were higher in placebo-treated calves when compared with those in calves receiving 2 administrations of flunixin meglumine 3 h apart and tended to be higher compared with those with a single administration (Huber et al., 2013). Calves treated with flunixin meglumine (once or twice) showed less head shakes and head rubs compared with a placebo-treated control group (Huber et al., 2013). The use of the transdermal flunixin meglumine solution as pain medication at the time of disbudding in dairy calves is currently neither approved in the EU, nor in the United States.

In the EU, flunixin transdermal solution (Finadyne Transdermal, MSD Animal Health; approval number 835578) was licensed in 2014 for treatment of fever, pain, and lameness in cattle associated with respiratory diseases, acute mastitis and interdigital phlegmon. In the United States and Canada, it has been approved for the control of fever associated with bovine respiratory disease and for pain management in lame cattle (US Food and Drug Administration, 2017). This solution is administered across the back line of an animal and absorbed transdermally into the bloodstream, which allows a simple way of administration.

Flunixin meglumine is effective in the treatment of acute inflammation (Thiry et al., 2017) and chronic visceral pain (Cook and Blikslager, 2015). It also serves as a strong antiphlogistic and antipyretic drug. The transdermal solution has a longer half-life (6.42 h) compared with the injectable solution (4.99 h; Kleinhenz et al., 2016). The bioavailability is, however, reduced compared with other administration routes.

The objective of this study was to evaluate the effect of 2 treatments of transdermal flunixin meglumine combined with local anesthesia on blood cortisol concentrations after cautery disbudding in dairy calves. We hypothesize that (1) treatment with transdermal flunixin meglumine at the time of disbudding reduces plasma cortisol concentrations compared with the control group, and (2) a second treatment with transdermal flunixin meglumine 6 h after disbudding extends the effect on plasma cortisol concentrations.

MATERIALS AND METHODS

The reporting guidelines for randomized controlled trials in livestock and food safety by Sargeant et al. (2010) were used as reporting guideline for the manuscript.

Dairy Farm, Animal Enrollment, and Housing

The study was conducted from February to August 2020 on a commercial dairy farm in Northeast Germany. The study protocol was in accordance with the Institutional Animal Care and Use Committee of the Freie Universität Berlin (approval number: 2347–49–2019).

An a priori sample size calculation was performed using MedCalc software (version 15.6.1, MedCalc) considering cortisol concentrations from a previous study. According to Kleinhenz et al. (2017) the sample size calculation was conducted assuming a decrease in cortisol concentration (±SD) to 6.4 ± 7.2 ng/mL between control and treatment group. Presuming 80% power and a confidence level of 95%, total number of 20 dairy calves were required per group. To account for follow-up losses 17 additional calves were enrolled.

Calves were housed in individual calf hutches measuring 2.05 × 1.15 × 1.35 m and bedded with straw. Further, each calf hutch had an individual paddock

with sand bedding (1.50 × 1.10 m). All calves were separated from their dams immediately after birth and placed into an individual calf hutch. Every calf received 3.0 L of colostrum from their dam within 4 h after parturition using a nipple bottle. Colostrum was fed during the first 3 d of life using a teat bucket 3 times/d (4.5 L/d). Feeding took place at 0430, 1230, and 1930 h.

A mixture (70:30) of milk replacer (**MR**; Sprayfo Vitesse 50, Trouw Nutrition Deutschland GmbH; with a concentration of 150 g of MR/L) and colostrum was fed 3 × 2.0 L/d from 4 d until 14 d. From 15 d until 35 d the calves received twice daily 3.0 L of MR (Sprayfo Vitesse 50, Trouw Nutrition Deutschland GmbH; 175 g of MR/L). From 36 d until weaning the feed quantity was reduced to 2 × 2.3 L (4.6 L/d; 175 g of MR/L). Each calf had ad libitum access to water and starter grain from 4 d. The visual and tactile contact with adjoining calves was possible. Feeding took place at 0430 and 1930 h.

Experimental Design and Drug Administration

Holstein Friesian dairy calves (n = 77) had to meet the following inclusion criteria: minimum 265 d of gestation, female, eutocia, and singleton to be enrolled. Calves were randomly assigned to one of 3 groups: (1) control group (**CON**, n = 27), (2) 1-flunixin group (**1-FLU**, n = 26) with a single administration of transdermal flunixin meglumine at disbudding, and (3) 2-flunixin group (**2-FLU**, n = 24) with 2 administrations of transdermal flunixin meglumine, the first treatment at disbudding and the second 6 h afterward. We administered a second dose at 6 h after disbudding to test the hypothesis that the analgesic effect of flunixin meglumine could be extended due to the half-life of 6.42 h to 13.2 h (Wagner et al., 2021). The CON group received a placebo treatment, a mixture of 80% lubricant (Gleitcreme Bengen, WDT e.G.) and 20% water.

The placebo and flunixin meglumine (Finadyne Transdermal, MSD Animal Health) were applied at the top line of the back using the dosage chamber of the bottle. The calculated doses of flunixin transdermal solution were based on actual BW measurements. A range of 3.21 to 3.84 mg/kg (mean 3.45 mg/kg) has been reported when the dosing chamber was used (Martin et al., 2020). To ensure that the solution was not accidentally removed during the disbudding procedures, placebo or transdermal flunixin meglumine was applied directly after disbudding. The treatments were not blinded. A random number list was generated in Excel (Office 2010, Microsoft Deutschland Ltd.) before the initiation of the study to allocate all calves into one of the 3 groups.

Sham Disbudding and Disbudding

For both disbudding procedures the area for the local anesthesia was shaved and cleaned with an alcohol-soaked gauze before administering local anesthesia. The local anesthesia was applied 10 to 15 min before sham disbudding or disbudding took place. The calf was restrained and a cornual nerve block was performed as described by Reedman et al. (2021) at each horn bud. Administered with an 18-gauge, 35-mm disposable needle (B. Braun Melsungen AG) and disposable syringe (5 mL, Henry Schein). The puncture site was located halfway between the lateral corner of the eye and the horn bud of the corresponding site. The puncture direction was perpendicular to the skin, below the palpable external frontal crista. The ramus cornualis of the trigeminal nerve was anesthetized with 5 mL of 2% procaine (Procamidor 20 mg/mL, WDT e.G.) at a depth of approximately 1.5 cm.

A gas dehorner (Buddex, KERBL Corporation) was used for cautery disbudding. Before disbudding, the effect of the local anesthesia was verified by observing the reaction to 5 consecutive needle pricks into the horn bud and the surrounding skin area. Cautery disbudding was performed, if an aversive reaction was absent. Otherwise, a second administration of the local anesthetic was performed. For sham disbudding, the cold gas dehorner was applied to each horn bud for 10 s. For disbudding, the gas dehorner was preheated for 3 min to gain the recommended working temperature of 650°C and the same working procedure was used until a cautery ring around the horn tissue was achieved. After sham disbudding and disbudding, the horn bud area was treated with a topical aluminum spray (Aluminum-Spray, Albrecht GmbH) and monitored for signs of infection the following 8 d. To determine the baseline cortisol concentration and to exclude bias due to manipulation and handling of the animal a sham disbudding procedure was performed before disbudding took place as done in previous studies (Stilwell et al., 2007; Kleinhenz et al., 2017). Disbudding took place one week after sham disbudding. One week was considered as the washout period. Disbudding was performed in the morning hours between 0600 and 0900 h. For a given calf the time of sham disbudding matched with the time of disbudding.

Blood Sample Collection

Blood samples from the jugular vein were withdrawn via venipuncture 30 min before sham disbudding and disbudding (baseline), at sham disbudding and disbudding (0 min) and 30 min, 2 h, 6 h, 12 h, and 24 h after disbudding into BD Vacutainer tubes spray-coated

with lithium heparin (BD Vacutainer). Due to a possible diurnal rhythm of cortisol concentrations (Hays et al., 1975), disbudding was always performed at the same time as sham disbudding. The blood collection times were the same for sham disbudding as for disbudding. The blood samples were carefully inverted 5 times and cooled on ice until centrifugation (approximately 30 to 60 min after sampling). The tubes were centrifuged for 6 min at 4,500 × *g* at room temperature. The obtained plasma was immediately pipetted into cryovials (Cryovial 2 mL, Simport Scientific Inc.) and stored at −18°C. One aliquot was sent on dry ice for the assessment of plasma cortisol concentration to the Unit of Physiology, Pathophysiology and Experimental Endocrinology of the University of Veterinary Medicine in Vienna. Cortisol concentrations in plasma following diethylether extraction (Schöffmann et al., 2009) were analyzed with a cortisol enzyme immunoassay (**EIA**) according to Palme et al. (1997). A detailed description of the EIA, including cross-reactions, is provided in Palme et al. (1997). The standard curve (80 to 20% relative binding) ranged from 1 to 30 pg/well, and the sensitivity of the EIA was 0.3 pg/well. Intra-assay (and interassay) coefficients of variation of high and low concentrated pooled samples were 9.2% (12.7%) and 12.8% (15.7%), respectively.

Daily Weight Gain, Lying, and Standing Behavior

The animals were weighed using an electronic scale (Load bars HD5T with wooden bottom panel, Patura KG) 24 h before and 24 h after both disbudding procedures. To calculate the ADG of each calf, the initial weight was subtracted from the last measured weight and divided by the difference in days.

Lying and standing behavior was recorded using a 3-dimensional (3D) accelerometer (Hobo Pendant G data logger, Onset Computer Corp.) attached to the right hind leg of the calves. A randomized subset of 38 calves (CON, n = 13; 1-FLU, n = 13; 2-FLU, n = 12) was fitted with 3D accelerometers. The logger was mounted and activated 24 h before sham disbudding and left until 7 d after the disbudding procedure. It was fixed at the lateral side of the right hind leg above the metatarsophalangeal joint by using vet wrap bandage (Co-Flex, Andover Healthcare Inc.) and a hook-and-loop tape. To avoid bruises, the logger was wrapped in gauze bandage (Rolta-Soft, Paul Hartmann AG) before fixation. Recording frequency was set to one reading per minute according to Bonk et al. (2013). The data were downloaded from the accelerometers using the manufacturer's proprietary software and exported as one file per calf with comma-separated values. A custom-built script for data processing, written in the Python programming language (van Rossum and Drake, 1995) and utilizing the data analysis and statistics library pandas (McKinney, 2011), was developed. Output was stored as an Excel XLSX file (Office 2010, Microsoft Deutschland Ltd.) containing multiple tables with the aggregated results and processing meta-data. The degree of vertical tilt (y-axis) was used to determine the lying position of the animal, such that readings ≥120° indicated the calf standing, and readings <120° indicated the calf lying down according to Bonk et al. (2013). To verify and ensure accurate data processing of the script, PDF files with y-axis line graphs were generated per calf for visual inspection. Lying bout frequency, average lying bout length, and total lying time per observation period were calculated. Data of 1 h (n = 60) were summarized to a 1-h observation period. The data were further processed in 6-h time periods (0000–0600 h; 0600–1200 h; 1200–1800 h; and 1800–0000 h) until 4 d after sham disbudding and disbudding.

Statistical Analysis

To evaluate the effect of the 2 treatment protocols on plasma cortisol concentrations and on total lying time, number of lying bouts, and average lying bout length a generalized linear mixed model was constructed using the GENLINMIXED procedure of SPSS (version 22.0, SPSS Inc., IBM). The outcome variable was either concentration of plasma cortisol (ng/mL) or total lying time, number of lying bouts, or average lying bout length. Calf was the experimental unit.

The model for plasma cortisol concentration contained the following explanatory variables as fixed effects: treatment procedure (CON, 1-FLU, and 2-FLU); sampling time (hours; continuous); and the interaction of treatment protocol and sampling time. The model for total lying time, number of lying bouts, and average lying bout length contained the following explanatory variables as fixed effects: treatment procedure (CON, 1-FLU, and 2-FLU); time period (6-h section); and the interaction of treatment protocol and time period. A negative binomial model was chosen to analyze number of lying bouts, as it provided the lowest Akaike information criterion value in the GENLINMIXED procedure of SPSS.

Two separate models were used for sham disbudding and disbudding, respectively. To account for multiple comparisons, the *P*-value was adjusted using a Bonferroni correction. A significant difference was specified for variables between the levels of a classification when $P < 0.05$; a tendency was declared as differences between $P \geq 0.05$ and $P \leq 0.10$.

To evaluate significant differences between ADG of the 3 groups, a one-way ANOVA was performed using

Table 1. Descriptive statistics for average plasma cortisol concentration[1] (ng/mL) during sham disbudding of calves (n = 71) at different sampling times

	Time relative to disbudding, h						
Group[2]	−0.5	0	0.5	2	6	12	24
CON	5.42 ± 0.81 (3.83–7.01)	10.65 ± 1.23 (8.23–13.07)	8.92 ± 1.02 (6.91–10.91)	3.8 ± 0.69 (2.47–5.16)	6.08 ± 0.91 (4.30–7.87)	7.89 ± 0.99 (5.94–9.83)	4.23 ± 0.70 (2.99–5.64)
1-FLU	4.01 ± 0.79 (2.45–5.57)	10.29 ± 1.18 (7.97–12.60)	9.22 ± 0.95 (7.31–11.13)	2.76 ± 0.66 (1.47–4.05)	4.19 ± 0.87 (2.48–5.90)	6.51 ± 0.95 (4.65–8.37)	3.30 ± 0.67 (1.98–4.61)
2-FLU	3.91 ± 0.78 (2.39–5.44)	8.94 ± 1.18 (6.63–11.26)	8.19 ± 0.95 (6.28–10.11)	2.97 ± 0.66 (1.68–4.26)	5.45 ± 0.87 (3.74–7.15)	7.50 ± 0.95 (5.64–9.36)	3.57 ± 0.67 (2.25–4.88)

[1]Values are given as mean ± SE (95% CI).

[2]Control group (CON, n = 23), 1-flunixin group (1-FLU, n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at sham disbudding, and 2-flunixin group (2-FLU, n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at disbudding and the second 6 h after sham disbudding.

SPSS. The dependent variable was ADG (kg/d; continuous), the independent variable was treatment procedure (CON, 1-FLU, and 2-FLU). Average, minima and maxima were determined using Excel (Office 2010, Microsoft Deutschland Ltd.).

RESULTS

Overall, 77 calves were enrolled in the study. Six animals were excluded because an adequate local anesthesia was not provided (CON, n = 4; 1-FLU, n = 2). In the final analysis, 71 animals aged 13 ± 2 d with an average weight of 48.9 kg were considered (CON, n = 23; 1-FLU, n = 24; 2-FLU, n = 24).

Plasma Cortisol

The descriptive statistics for average plasma cortisol concentrations (ng/mL) for sham disbudding and disbudding are presented in Tables 1 and 2. Mean plasma cortisol concentrations during sham disbudding were 6.09 ± 2.5 ng/mL with a range of minimum 0.4 ng/mL up to maximum 29.6 ng/mL. Plasma cortisol was affected by time ($P < 0.01$). Treatment tended to have an effect on plasma cortisol concentration during sham disbudding ($P = 0.08$). The single treatment (1-FLU) showed a significant effect compared with CON ($P = 0.05$; −0.97 ng/mL; 95% CI: −1.92 to −0.01), whereas a second treatment (2-FLU) tended to have an effect ($P = 0.06$; −0.93 ng/mL; 95% CI: −1.88 to 0.02) compared with CON, respectively. The 2 treatment groups did not differ ($P = 0.94$; 0.04 ng/mL; 95% CI: −0.89 to 0.97). During disbudding (0 h) the mean plasma cortisol concentration was 5.16 ± 2.8 ng/mL (range: 0.2–26.4 ng/mL). Plasma cortisol concentration was affected by treatment ($P < 0.01$) and sampling time ($P < 0.01$). There was no interaction between treatment and sampling time ($P = 0.95$). The single treatment (1-FLU) showed a significant effect in comparison to CON ($P < 0.01$; −1.29 ng/mL; 95% CI −2.15 to −0.44), as well as a second treatment (2-FLU; $P < 0.01$; −1.29 ng/mL; 95% CI: −2.14 to −0.43) compared with CON, respectively. The 2 treatment groups did not differ significantly ($P = 0.99$; 0.01 ng/mL; 95% CI: −0.84 to 0.85).

Two hours after disbudding, plasma cortisol was reduced by a single treatment (1-FLU, $P = 0.01$) compared with CON as well as 6 h after disbudding (P

Table 2. Descriptive statistics for average plasma cortisol concentration[1] (ng/mL) during disbudding of calves (n = 71) at different sampling time points

	Time relative to disbudding, h						
Group[2]	−0.5	0	0.5	2	6	12	24
CON	3.86 ± 0.65 (2.59–5.13)	8.82 ± 1.20 (6.47–11.17)	8.98 ± 1.01 (6.99–10.97)	4.30 ± 0.50 (3.32–5.83)	6.02 ± 0.67 (4.71–7.32)	5.58 ± 0.74 (4.11–7.04)	4.59 ± 0.80 (3.01–6.15)
1-FLU	3.41 ± 0.63 (2.17–4.65)	8.61 ± 1.17 (6.31–10.91)	7.07 ± 0.99 (5.12–9.02)	2.48 ± 0.49 (1.52–3.44)	3.69 ± 0.65 (2.42–4.97)	4.67 ± 0.71 (3.27–6.07)	3.17 ± 0.76 (1.67–4.68)
2-FLU	2.75 ± 0.63 (1.50–4.00)	8.29 ± 1.17 (5.99–10.59)	6.44 ± 0.99 (4.49–8.39)	2.89 ± 0.49 (1.93–3.90)	4.18 ± 0.65 (2.90–5.45)	5.38 ± 0.71 (3.98–6.78)	3.21 ± 0.76 (1.70–4.70)

[1]Values are given as mean ± SE (95% CI).

[2]Control group (CON, n = 23), 1-flunixin group (1-FLU, n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at disbudding, and 2-flunixin group (2-FLU, n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at disbudding and the second 6 h after disbudding.

= 0.01). The plasma cortisol concentration was also affected and reduced by a double treatment 2 h after disbudding (2-FLU, $P = 0.05$) compared with CON as well as 6 h after disbudding (2-FLU, $P = 0.05$). During disbudding, the mean plasma cortisol concentration maxima from different sampling times were 8.98 ng/mL in CON (0.5 h), 8.61 ng/mL in 1-FLU (0 h), and 8.29 ng/mL in 2-FLU (0 h), respectively (Table 2).

After the initial peak during disbudding procedure (0 h) the plasma cortisol concentrations reached baseline levels 2 h after sham disbudding and disbudding in all groups except for control calves after disbudding (Figures 1 and 2). After another increase of plasma cortisol concentrations 2 h after disbudding a second peak occurred 12 h after disbudding except for CON in disbudding. For control calves during disbudding the second peak was observed 6 h after disbudding (Figures 1 and 2).

Daily Weight Gain, Lying, and Standing Behavior

As one calf of the control group had to be excluded from the data set of daily weight gain due to implausible data, 70 calves were included in the statistics for ADG (CON, n = 22; 1-FLU, n = 24; 2-FLU, n = 24).

Mean (±SD) BW was 52.7 ± 4.10 kg for CON calves, 51.1 ± 4.50 kg for 1-FLU calves, and 52.1 ± 3.81 kg for 2-FLU calves. There was no statistical difference between ADG and treatment ($P = 0.74$; Table 3).

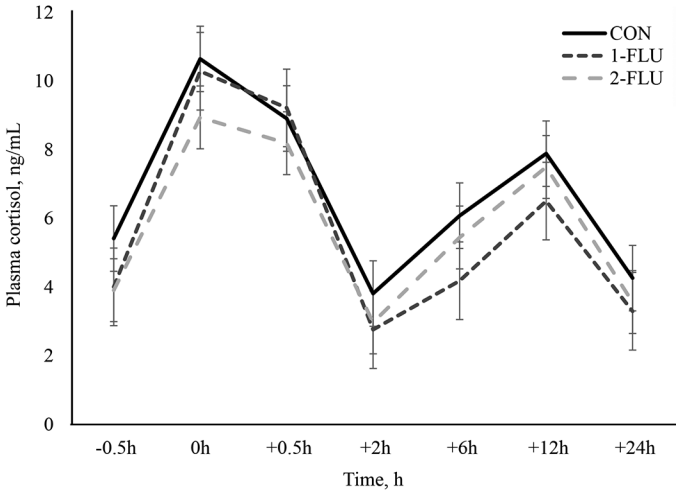

Figure 1. Plasma cortisol concentrations (mean ± SE) after sham disbudding for control group (CON, n = 23), 1-flunixin group (1-FLU, n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at sham disbudding, and 2-flunixin group (2-FLU, n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at sham disbudding and the second 6 h after sham disbudding. The treatment procedures tended to have an effect on plasma cortisol concentrations ($P = 0.08$). Plasma cortisol concentrations were affected by sampling time ($P < 0.01$). There was no interaction between treatment and sampling time ($P = 0.99$).

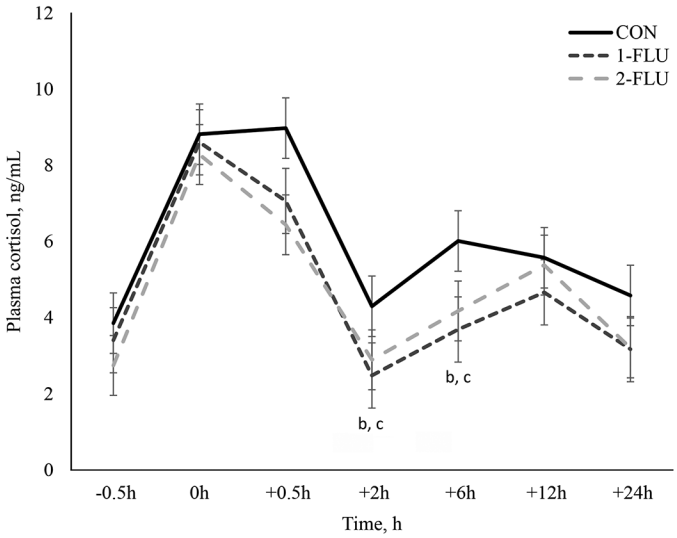

Figure 2. Plasma cortisol concentrations (mean ± SE) after disbudding for control group (a, CON, n = 23), 1-flunixin group (b, 1-FLU, n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at disbudding, and 2-flunixin group (c, 2-FLU, n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at disbudding and the second 6 h after disbudding. Plasma cortisol concentrations were affected by treatment procedures ($P < 0.01$) (letters b, c) and sampling time ($P < 0.01$) (letters b, c). There was no interaction between both ($P = 0.95$).

For the lying and standing behavior, a total of 38 animals were analyzed (CON, n = 13; 1-FLU, n = 13; 2-FLU, n = 12). In total, 608 time periods (CON, 208 time periods; 1-FLU, 208 time periods; and 2-FLU, 192 time periods) of measurement were collected. Due to the high number of collected data on lying and standing behavior per calf, there was enough power to perform the statistical analysis without the need of a Hobo Logger for each calf. Therefore, 38 calves were randomly selected by using Excel and the loggers were distributed among the 3 experimental groups. Treatment after sham disbudding had an effect on total lying time ($P = 0.01$). Calves that received a double treatment with flunixin meglumine (281.8 ± 2.2 min/6 h) had increased lying time compared with CON (273.7 ± 2.1 min/6 h; $P = 0.01$) and 1-FLU (273.4 ± 2.1 min/6 h; $P = 0.01$). Treatment also had an effect on average lying bout length in calves ($P = 0.01$) after sham disbudding. The average lying bout length of calves that received a double treatment with flunixin meglumine (57.1 ± 2.3 min/6 h) increased compared with CON (46.6 ± 2.2 min/6 h; $P = 0.01$) and 1-FLU (52.2 ± 1.8 min/6 h; $P = 0.02$). Treatment had an effect on number of lying bouts ($P = 0.02$). Calves that received a double treatment of flunixin meglumine (6.3 ± 0.2 observa-

Table 3. Descriptive statistics for average BW (kg) and ADG (kg) of 70 female Holstein dairy calves during sham disbudding and disbudding distributed in 3 groups

Group [1]	Parameter[2]	n	Mean	SD	Minimum	Maximum
CON	Weight 1 (before sham disbudding)	22	49.5	4.97	39.0	57.0
	Weight 2 (after disbudding)	22	55.3	3.90	48.0	64.0
	ADG	22	0.57	0.30	−0.05	1.11
1-FLU	Weight 1 (before sham disbudding)	24	48.0	3.80	39.6	56.5
	Weight 2 (after disbudding)	24	53.8	4.89	46.5	68.0
	ADG	24	0.64	0.31	0.36	1.28
2-FLU	Weight 1 (before sham disbudding)	24	49.1	3.80	41.5	54.5
	Weight 2 (after disbudding)	24	54.7	3.67	47.5	61.0
	ADG	24	0.62	0.27	0.61	1.06

[1]Control group (CON; n = 22), 1 treatment with transdermal flunixin meglumine at disbudding (1-FLU; n = 24), and 2 treatments with transdermal flunixin meglumine at disbudding and 6 h after disbudding (2-FLU; n = 24).

[2]Weight was recorded 24 h before sham disbudding and 24 h after disbudding.

tions/6 h) showed a decreased number of lying bouts compared with CON (6.8 ± 0.2 observations/6 h; $P = 0.02$). There was a difference between CON and 1-FLU ($P = 0.02$) but not for 1-FLU and 2-FLU ($P = 0.85$).

After disbudding, treatment had no effect on total lying time ($P = 0.31$). The average total lying time was 273.7 ± 2.5 min/6 h for CON, 274.1 ± 2.7 min/6 h for 1-FLU, and 278.8 ± 2.7 min/6 h for 2-FLU. Treatment also had no effect on average lying bout length in calves ($P = 0.41$) after disbudding. The average lying bout length was 51.7 ± 2.6 min/6 h for CON, 54.3 ± 2.3 min/6 h for 1-FLU, and 56.8 ± 2.9 min/6 h for 2-FLU. Moreover, treatment also had no effect on number of lying bouts ($P = 0.28$). Lying behavior was affected by time during sham disbudding ($P < 0.01$) and disbudding ($P < 0.01$) for all groups.

DISCUSSION

The increase in plasma cortisol concentrations after sham disbudding might be related to a novel experience, unknown personnel, the restraining for drug administration, or the sham disbudding procedure (Vickers et al., 2005; Heinrich et al., 2009; Huber et al., 2013). A cortisol response in blood could be seen after several minutes after ACTH administration (Negrão et al., 2004) and after restraining and handling (Huber et al., 2013). In our study the plasma cortisol samples (0 h) were collected immediately after sham disbudding or disbudding and sampling took less than 3 min. Therefore, we conclude that the time-zero samples mainly reflected the stress associated with restraining and drug administration, rather than with the disbudding process itself. Nevertheless, the results showed that even during sham disbudding, the administration of transdermal flunixin meglumine tended to have an effect on plasma cortisol ($P = 0.08$). Heinrich et al. (2009) recorded similar results with the use of meloxicam, which decreased heart rates in combination with local anesthesia during sham disbudding.

After disbudding, peak plasma cortisol concentrations were measured in 1-FLU and 2-FLU at 0 h, whereas in control calves, the peak plasma cortisol concentration was measured at 0.5 h after cautery disbudding. This is consistent with a previous study (Huber et al., 2013), in which cortisol concentrations (7.6 ng/mL) in a placebo-treated group peaked 0.5 h after the disbudding procedure. Thirty to 60 min after procaine injection, the anesthetic effect wears off (Skade et al., 2021) and sensitivity returns (Stafford and Mellor, 2005; Stock et al., 2013), which can explain the increase in plasma cortisol concentrations 0.5 h after disbudding in CON.

Two and 6 h after disbudding, the plasma cortisol concentrations were affected by treatment for 1-FLU and 2-FLU calves. And a return to baseline plasma cortisol concentrations occurred in 1-FLU and 2-FLU after 2 h. According to Wagner et al. (2021) the maximum concentration of transdermal flunixin meglumine occurred 1.66 to 2.14 h (Tmax-concentration) after administration. This timing might have influenced the return of plasma cortisol concentrations to baseline levels in 1-FLU and 2-FLU 2 h after disbudding in our study. Due to a delay between the drug administration and the full effect of transdermal flunixin meglumine, Kleinhenz et al. (2017) suggested an administration 2 h before disbudding.

Because of the half-life of 6.42 to 13.2 h (Wagner et al., 2021), we administered a second dose at 6 h postdisbudding to test the hypothesis that the analgesic effect of flunixin meglumine could be extended. Inflammation induced prostaglandin E_2 production peaks 24 h as well as 48 h postdisbudding (Allen et al., 2013; Stock et al., 2015). According to Thiry et al. (2017) the inflammation-inhibiting effect of transdermal flunixin meglumine persisted for up to 48 h. However, the second treatment 6 h postdisbudding did not have an ad-

ditional effect on the cortisol concentrations compared with a single treatment.

The treatment with transdermal flunixin meglumine in combination with local anesthesia reduced plasma cortisol after cautery disbudding in 1-FLU and 2-FLU group compared with the placebo-treated CON group. Our results showed that a local anesthetic in combination with a NSAID was more effective in reducing the stress response after cautery disbudding in calves compared with a local anesthetic alone.

Standing and lying behavior in the present study was monitored using a 3D accelerometer according to Bonk et al. (2013). Due to individual variation in lying and standing time, Coetzee et al. (2012) compared the behavior of each calf before and after the disbudding procedure. Discomfort after disbudding or castration was associated with decreased lying time (Heinrich et al., 2010; Theurer et al., 2012). After sham disbudding treatment increased total lying time by 10 min in 6 h. This effect was rather small and can be considered as irrelevant. After disbudding, treatment had no effect on total lying time ($P = 0.31$), average lying bout length in calves ($P = 0.60$) and number of lying bouts ($P = 0.49$). Total lying time was similar for all presented groups and was not affected by treatment. This is not in accordance to a previous study (Sutherland et al., 2018) in which meloxicam affected lying behavior 2 h after disbudding. Furthermore, calves treated with meloxicam had up to 10% greater lying time on the first 4 d postdisbudding compared with a nontreated control group (Theurer et al., 2012). Therefore, we presume that flunixin meglumine is less effective in reducing discomfort after disbudding than meloxicam as measured by lying behavior. Further research is warranted.

Study Limitations

In the present study, there was no difference in ADG among the treatment groups. This result was likely confounded by the restrictive feeding program (maximum of 6.0 L/d) of the dairy farm and the short interval (2 wk) between the 2 weight assessments. The milk amount of the feeding protocol and ADG of our study calves was lower than recommended (Khan et al., 2011).

CONCLUSIONS

In this study, we demonstrated that transdermal flunixin meglumine decreased plasma cortisol concentrations following cautery disbudding of calves. Further research is warranted to evaluate analgesic effects compared with other NSAID medications. To ensure adequate pain relief more research is warranted to explore the effect of a second dose of flunixin meglumine administered at different times after disbudding. However, a second dose of transdermal flunixin meglumine 6 h after disbudding had no additional effect on plasma cortisol concentrations. Average daily gain and total lying time were not affected.

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