Plasma concentrations of steroid precursors, steroids, neuroactive steroids, and neurosteroids in healthy neonatal foals from birth to 7 days of age

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Abstract

Background: Transient hypothalamic-pituitary-adrenal axis dysfunction occurs in critically ill foals with sepsis and neonatal maladjustment syndrome (NMS). Cortisol is the most commonly measured steroid. However, a complex interaction of various steroid compounds might play a role in pathophysiology of this disorder.

Objective: To identify steroid compounds present at high concentrations at birth that rapidly and steadily decrease within the first 7 days of life in healthy foals and that might be supportive diagnosis of NMS and other neonatal disorders.

Animals: Ten healthy neonatal Quarter Horse foals (5 females and 5 males).

Methods: Prospective study. Blood was collected in heparinized tubes within 30 minutes after birth, and at 12, 24, 48, 72, 96, 120, 144, and 168 hours of age. Plasma was separated and a panel of steroid compounds was analyzed using liquid chromatography-mass spectrometry. A nonlinear regression model was used to determine decay concentrations over time. Confidence intervals (CIs) were calculated and significance was set at a \( P \leq 0.05 \).

Results: Five compounds were identified: pregnenolone, progesterone, deoxycorticosterone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate. Pregnenolone and progesterone concentrations rapidly decreased by 24 hours of age and remained low throughout the first 7 days of life. Their half-life (95% CI) was short at 3.7 (3.4, 4.0) and 4.5 (2.8, 6.1) hours, respectively. No statistical differences in the concentrations of these compounds were found between males and females.

Conclusions and Clinical Relevance: Progesterone might be a useful marker for identifying continuous endogenous production of neuroactive steroids in foals with suspected NMS and other neonatal disorders.

KEYWORDS
brain, equine, maladjustment, progesterone, progestins

Abbreviations: 95% CI, 95% confidence interval; ACTH, adrenocorticotropic hormone; CNS, central nervous system; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DOC, deoxycorticosterone; GABA, gamma aminobutyric acid; HPAA, hypothalamic-pituitary-adrenal axis; ICU, intensive care unit; KMC, kangaroo mother care; NMS, neonatal maladjustment syndrome; NAS, neuroactive steroids; NS, neurosteroids.
1 | INTRODUCTION

Two of the most common reasons for admission of neonatal foals into intensive care units (ICU) are sepsis and neonatal maladjustment syndrome (NMS). Two of the most common reasons for admission of neonatal foals into intensive care units (ICU) are sepsis and neonatal maladjustment syndrome (NMS). This syndrome encompasses foals suffering from perinatal hypoxia, as well as those suspected of experiencing impaired transition from intra- to extra-uterine life, resulting from persistent increases in neurosteroid (NS) concentrations. Alterations of NS concentrations over the first 48 hours after birth. Significant differences in NS concentrations are observed in infants born by vaginal delivery compared to those born by Cesarean section, and in those receiving skin to skin contact as compared to those not receiving contact, often termed kangaroo mother care (KMC). The reported benefits of KMC include mother and infant bonding, improved thermoregulation, enhanced survival of preterm infants, and improved neurodevelopmental outcome, all of which might be related to neurosteroid concentrations. Increased steroid concentrations in plasma have been detected in neonatal foals with NMS and sepsis. The lack of transition from high concentrations of NS in the fetus during uterine life to the rapid decrease of NS after birth might affect the neurologic behavior of neonatal animals, resulting in maladaptation to extrauterine life. This alteration results in clinical signs ranging from lack of affinity and bonding of the foal to the mare, not nursing, unawareness of the environment, obtundation, recumbency, altered sleep, and, in some cases, seizures. Experimental IV administration of the neurosteroid allopregnanolone in healthy neonatal foals induced similar changes in neurologic behavior and state of consciousness as those observed in foals with NMS, supporting the important role that NS play in neuronal function.

Various disorders including NMS in neonatal foals can present with similar signs, and the role of NS warrants further investigation. Therefore, our objective was to identify steroid compounds including NS and NAS in healthy foals that are found in high concentrations at birth, and that undergo a rapid steady decrease within the first days of life, and subsequently remain at low concentrations and that might be used as reference compounds to support a diagnosis of NMS and other neonatal disorders.

2 | MATERIALS AND METHODS

2.1 | Animals

After approval by the Colorado State University (CSU) Institutional Animal Care and Use Committee, foals born at CSU during the 2016 breeding season were included in the study. Inclusion criteria consisted of foals born from healthy mares with no placentitis, normal gestational period, uneventful birth, and having normal physical and neurological examination findings. The foals had to successfully stand and nurse within 2 hours of birth and remain clinically healthy during the study period (7 days).

2.2 | Plasma collection and handling

Jugular venous whole blood was collected in heparinized tubes within 30 minutes after birth, and sequentially at 12, 24, 48, 72, 96, 120, 144, and 168 hours of age. At each collection time, a jugular venous blood sample (3 mL) was collected using a 20 gauge needle in a 3 mL syringe and transferred into a glass vacuum heparinized blood tube. Blood was centrifuged for 10 minutes at 1000g. Plasma was aliquoted into 2 mL cryotubes and stored at −80°C until further analysis.

2.3 | Sample analysis

Plasma samples were analyzed for concentrations of steroid precursors, steroids, NAS, and NS and included cholesterol, cholesterol-3-SO₄, pregnenolone, pregnenolone-SO₄, pregnanediol, 17-hydroxypregnenolone, 21-hydroxypregnanolone, 20-hydroxypregnenolone, allopregnanolone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-SO₄ (DHEA-S), progesterone, allopregnanolone, dehydroepiandrosterone-SO₄, dihydroprogesterone, 20α-dihydroprogesterone, 17α-dihydroprogesterone, 5α-dihydroprogesterone, 5β-dihydroprogesterone, 17α,20α-dihydroprogesterone, 5α-dihydroprogesterone, 20α-dihydroprogesterone, 17α,20α-dihydroprogesterone, 5β-dihydroprogesterone, 17α,20α-dihydroprogesterone, 5β-dihydroprogesterone, 17α,20α-dihydroprogesterone, 5β-dihydroprogesterone, 17α,20α-dihydroprogesterone, 5β-dihydroprogesterone, 17α,20α-dihydroprogesterone.
dihydroprogesterone, cortexolone, corticosterone, deoxycorticosterone (DOC), aldosterone, androstenedione, testosterone, allopregnanolone, epitestosterone, estrone, 17ß-estradiol, 17α-estradiol 3-SO₄, estrone-3-SO₄, estradiol, estradiol-3-SO₄, and etiocholanolone-3-glucuronide using liquid chromatography-mass spectrometry with online sample extraction by turbulent flow chromatography and detection by standard reference material on a triple quadrupole mass spectrometer. This method has been described in detail elsewhere.³³

2.4 | Statistical analysis

Normality of the data was evaluated using the Shapiro-Wilk normality test. Scatter plots were constructed to determine distribution of concentrations of each compound over the 7 days. Initially, exponential decay, and second or third polynomial models were considered for all compounds. Choice of which model to use for final representation for each compound was based on the coefficient of determination ($R^2$). Half-life of the compounds was determined using nonlinear regression with random effects for initial concentration of each compound at the first blood collection (within 30 minutes after birth)³⁴ Compounds after a 1-phase exponential decay model were considered preferable targets for use as references supportive for the diagnosis of NMS and other neonatal disorders. The general predicted nonlinear regression model was:

$$Y = (Y_0 - \text{Plateau}) \times e^{-K \times X} + \text{Plateau}$$

where $Y$ is the concentration of the compound at different time points, $Y_0$ is the value of $Y$ when $X$ is equal to zero; Plateau is the $Y$ value at infinite time, $K$ is the rate constant, $X$ is the time variable, and $e$ is the exponential function. Half-life (hours) for each compound was calculated as ln(2)/$K$, where ln is the natural logarithm function. Wherever possible, differences between males and females were evaluated by comparing the rate constants ($K$) using an $F$ test. In contrast, compounds after a decay that fitted best with a second or third polynomial model were considered poor targets for use as references supportive for the diagnosis of NMS and other neonatal disorders. All statistical analysis was performed using commercial statistical software (GraphPad Prism, Version 7, LaJolla, CA). Significance was set at $P \leq .05$.

3 | RESULTS

A convenience sample of 10 healthy Quarter Horse foals, 5 females and 5 males, was included in the study. These foals were born from February to April as follows: February N = 2 (1 filly, 1 colt), March N = 4 (2 fillies, 2 colts), April N = 4 (2 fillies and 2 colts). Seven foals were born between 1:00 and 4:30 am, and 3 between 6:00 and 8:00 pm Daily evaluations showed that these foals remained clinically healthy and continued to grow eventfully. Of the panel of steroid compounds measured, 5 were identified as potential markers of continuous endogenous production of steroids based on the narrow concentration range among foals with a rapid progressive decrease in concentrations as the foals aged. In some foals, these NS decreased to concentrations below the level of detection by 96 hours of age (Table 1). Potential markers included pregnenolone (Figure 1A), progesterone (Figure 1B), DOC (Figure 1C), DHEA (Figure 1D), and DHEA-S (Figure 1E). Of these 5 compounds, pregnenolone and progesterone rapidly decreased to low concentrations by 24 hours of age and remained low through the first 7 days of life (Figure 1A,B). Half-lives (95% confidence interval [CI]) for pregnenolone, progesterone, DOC, DHEA, and DHEA-S were 3.7 (3.4, 4.0), 4.5 (2.8, 6.1), 18.2 (10.0, 34.9), 27.1 (11.5, 144), and 41.5 (25.1, 87.4) hours, respectively. Cortisol also was found in high concentrations at birth with a rapid decrease within the first 24 hours after birth. However, this decrease was highly variable among foals (data not shown). Decay of all other compounds fitted a second or third polynomial model with variable decay over time, and therefore are not presented here. Plasma all-opregnanolone concentrations in these foals were below the level of detection (<0.24 ng/mL). Differences in the concentrations of the compounds between males and females were not significant for pregnenolone ($P = .81$), progesterone, ($P = .64$), DOC ($P = .49$), DHEA ($P = .81$), and DHEA-S ($P = .85$).

4 | DISCUSSION

Based on our results, healthy neonatal foals are born with high concentrations of steroid compounds, which decrease within a few days of life, with progesterone, DOC, DHEA-S, DHEA, and pregnenolone rapidly decreasing within the first 48 hours of life and thereafter remaining at low concentrations. Of these compounds, progesterone and pregnenolone had the most precipitous decrease by 24 hours after birth. A rapid decrease in concentrations of certain steroids might be necessary in the postnatal period to allow development of normal state of consciousness and behavior in newborn foals.⁷,¹⁰,₂⁰,³⁵-⁴² The short half-life of the NS in neonatal foals supports the rapid decrease observed within the first days of life, and likely reflects a placental origin or termination of endogenous production within the central nervous system (CNS).⁴³,⁴⁴ The contribution of the foal’s fetal gonads and adrenal cortex is unknown. Suppression of fetal level of consciousness by progesterone through the modulation of the CNS in the prenatal period has been investigated in lambs, and presumed to be similar in fetal foals.⁸,⁴⁵ This suppression of fetal consciousness is necessary to avoid injury to the uterus, cervix, and vaginal tissues during gestation and the peripartum period.⁴⁵ However, a rapid transition from the neurologic dampening effects of NS and NAS resulting in a decreased state of consciousness to an alert and active state must occur rapidly in the postnatal period to increase the chance of survival of prey animals in the wild.⁸,⁴⁵ Recently, high concentrations of NS have been documented in diseased neonatal foals.⁷,²⁰,³⁶ This finding raises awareness of the importance and role of NS and NAS in states of disease. A persistent increase in NS and NAS concentrations in foals with NMS suggests likely endogenous production of these compounds. A cause for this persistent increase remains unknown but is suspected to be associated...
| Neurosteroid (ng/mL) | Age (HR) | T0  | T12 | T24 | T48 | T72 | T96 | T120 | T144 | T168 |
|---------------------|----------|-----|-----|-----|-----|-----|-----|------|------|------|
| Pregnenolone        | N foals  | 10  | 10  | 10  | 10  | 10  | 10  | 10   | 10   | 10   |
| Mean                | 9498.4   | 2122.1 | 1372.2 | 1297 | 1153.1 | 1268.8 | 1363.5 | 1245.9 | 1291.2 |
| SD                  | 10       | 116.4 | 1188 | 1354.5 | 1063.2 | 1283.4 | 1321.7 | 996   | 1424.9 |
| Median              | 3554.1   | 1485.8 | 872.3 | 738.1 | 697.4 | 704.6 | 357.6 | 441.3 | 408.5 |
| Range               | 2840.5-14507 | 550.7-55285 | 360.3-2969.2 | 346.8-2671.2 | 207.7-2277.2 | 115.5-2125.7 | 869.1-1986.7 | 834.8-1952 | 618.5-1734.1 |
| Progesterone        | N foals  | 10  | 10  | 10  | 10  | 9   | 10  | 9    | 10   | 10   |
| Mean                | 76.3     | 14.1 | 9.5 | 7.2 | 6.4 | 2.4 | 2.3 | 2.3  | 2.3  | 2    |
| SD                  | 30.3     | 6.2  | 4   | 4   | 4.2 | 0.9 | 0.7 | 0.7  | 0.7  | 1    |
| Median              | 72.9     | 11.8 | 7.8 | 5.7 | 5.3 | 2.4 | 1.9 | 1.9  | 1.9  | 1.7  |
| Range               | 28.3-127.6 | 7.1-24 | 5.1-15 | 3-16.1 | 3.2-16.6 | 1-4.2 | 1.5-3.2 | 1.3-5.9 | 0.7-3 |
| Deoxycorticosterone | N foals  | 10  | 10  | 10  | 10  | 9   | 7   | 5    | 5    | 8    |
| Mean                | 25.7     | 13.6 | 10.2 | 8.3 | 4.2 | 1.9 | 1.1 | 1.1  | 1.1  | 1.1  |
| SD                  | 12       | 6.8  | 4.6  | 4.8 | 2.7 | 0.7 | 0.3 | 0.3  | 0.3  | 0.4  |
| Median              | 25.7     | 12.1 | 9.5  | 7.7 | 4.2 | 1.8 | 1   | 1    | 1    | 1    |
| Range               | 13.2-49.4 | 5.6-26.3 | 37.18.5 | 4.4-19.7 | 1.5-10.1 | 0.7-3.2 | 0.9-1.7 | 0.6-3.3 | 0.5-1.8 |
| DHEA                | N foals  | 10  | 9   | 9   | 8   | 4   | 3   | 1    | 5    | 5    |
| Mean                | 502.1    | 655.9 | 322.6 | 75.1 | 31  | 30  | 12.9 | 25   | 29.1 |
| SD                  | 550.2    | 676.2 | 315.7 | 49.7 | 9.8 | 17.4 | 12.9 | 13   | 12.5 |
| Median              | 401.4    | 474.6 | 239.3 | 59.6 | 32.6 | 38.7 | 12.9 | 26.5 | 29.1 |
| Range               | 10.1-1681.1 | 63.2-2205 | 19.8-908.8 | 33.2-1774 | 19.5-39 | 10-41.3 | 12.9 | 10.8-42.1 | 10.2-44 |
| DHEA-S              | N foals  | 10  | 9   | 10  | 10  | 8   | 8   | 6    | 2    | 3    |
| Mean                | 365.3    | 330.2 | 287.8 | 183.6 | 167.5 | 109.3 | 90.6 | 123.2 | 67.1 |
| SD                  | 207.1    | 151.1 | 155  | 81.6 | 73.2 | 54  | 46.1 | 50.7 | 21.7 |
| Median              | 318.9    | 309.8 | 266.2 | 157  | 164.7 | 98.5 | 83.9 | 123.2 | 57   |
| Range               | 67.1-695.5 | 120.8-559.6 | 56.6-626 | 68.8-318.9 | 533.262.2 | 70.5-238.9 | 51.176.9 | 87.4-159.1 | 52.4-92 |

Abbreviations: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate.
with inadequate time elapsed and compression of the neonate’s body during passage through the birth canal. The end result is hypothesized to be insufficient or altered triggering of the signal mechanisms involved in the lowering of NS and NAS. This presumed association might be supported by the observation that some neonatal foals with NMS have experienced rapid births. A similar hypothesis has been proposed for human infants born by Cesarean section. Transition of consciousness from fetal to neonatal life also has been studied in humans and requires the establishment of important thalamocortical connections.

Concentrations of steroid compounds are different in plasma, cerebrospinal fluid, and brain tissue. Concentrations of these compounds also vary within the different areas of the brain (e.g., hippocampus and thalamus). Pregnenolone, the first hormone produced in the steroidogenesis cascade, is known to occur at high concentrations in the brain. Pregnenolone subsequently is converted to progesterone. Progesterone can be produced by the ovaries, placenta, and adrenal glands; DHEA and DHEA-S are produced in the adrenal glands, gonads, and brain; and, DOC is produced in the adrenal glands and brain. Furthermore, pregnenolone and DHEA have been found to be the most abundant steroids in the fetal blood of horses. Several acronyms have been given to these compounds. For instance, DHEA-S is also known as androstenedione sulfate, DHEA as androstenedione, and DOC as 21-hydroxyprogesterone, deoxycortone, or cortexone. In addition to these compounds, androstenedione and epitestosterone also have been reported to be increased in plasma from foals with NMS < 48 hours of age. These NS also were increased at birth in our current study but their decline was not consistent and concentrations fluctuated after 48 hours of age (data not shown), making these NS less useful as a reference for comparison with diseased foals.

Allopregnanolone, a neuroactive metabolite of progesterone, is known to increase during pregnancy and peaks in late gestation in rats and mares. Allopregnanolone is responsible for signaling pregnancy to the brain, stimulates opioid production by the brainstem, and enhances gamma aminobutyrate (GABA) action in the paraventricular nucleus to attenuate the HPAA response to stress in late pregnancy. This pregnancy adaptation is believed to protect the fetus from adverse early life programming by maternal glucocorticoids. However, allopregnanolone must decrease rapidly after birth. Allopregnanolone concentrations in neonatal foals in our study were below the level of detection. Signs comparable to NMS were induced in 2 healthy neonatal foals after the IV administration of allopregnanolone. The signs displayed by foals included abnormal behavior such as lack of nursing and bonding with the mare, obtundation (1 foal becoming recumbent), somnolence, and altered sleep (1 foal sleeping while standing). Furthermore, an electroencephalogram showed induced drowsiness and slow wave sleep during allopregnanolone infusion. The observed signs were transient and
fully reversible with no complications within a few minutes after the conclusion of the infusion. A similar outcome (relatively rapid full recovery with no short or long term sequelae) has been observed in foals with NMS if supportive care is provided.2

The mode of action of steroid compounds in neuronal activity involves 1 of 2 pathways: genomic and nongenomic.57 Genomic mechanisms consist of steroid binding to intracellular receptors located in the nucleus or cytoplasm that regulate gene transcription. This effect occurs within minutes to hours.57 Nongenomic mechanisms consist of binding to plasma membrane receptors resulting in rapid modulation within milliseconds to seconds.57 Neurosteroids and NAS are highly lipophilic and modulate neuronal excitability of the brain by direct interaction with GABA-A receptors and their mode of action depends on concentration, type of compound (agonist versus antagonist, sulfated versus nonsulfated forms), and type of receptor (synaptic versus extrasynaptic).25,26 The GABA-A receptors are pentameric ligand-gated chloride channels responsible for the majority of inhibitory currents in the brain.26 Despite distinct binding sites to GABA-A receptors for GABA, benzodiazepines, and barbiturates, NS have similar anesthetic and anticonvulsant effects.26 Two of the compounds identified here, progesterone and DOC, are known to have sedative, anesthetic, and anticonvulsant actions.58,59 Persistent high concentrations of these NS could explain clinical effects observed such as lack of bonding with the mare, failure to nurse, and obtundation in foals with NMS.

Opposite to sedative and anesthetic modes of action, DHEA-S inhibits the GABA-A receptor and the clinical effects could be different.22 For instance, seizures might be observed in some foals with NMS.9 It is possible that a more complex interaction of various NS and NAS might play a role in the development of seizures in a subset of foals, and warrants further investigation. Steroid compounds with known anticonvulsant effects include progesterone, allopregnanolone, pregnanolone, dihydropregesterone, androstanediol, etiocholanolone, dihydrotestosterone, DOC, dihydrodeoxycorticosterone, and allo- trihydrodeoxycorticosterone.60,61 Proconvulsant steroids include estradiol, pregnenolone sulfate, DHEA-S, cortisol, and 11-deoxycortisol.60 Pregnenolone and its sulfate derivative (pregnenolone sulfate) have a multitude of actions involving inhibition of voltage-gated calcium channels in the brain.60-62 The influence of NS on function of the nervous system goes beyond the CNS. Neurosteroids also contribute to the modulation of the intrinsic enteric nervous system through inhibition by GABAergic synaptic input from the nucleus tractus solitarius.63 Some foals with NMS display gastrointestinal motility disturbances that resolve as neurological status improves.

Sensitivity to NS and NAS between females and males in mice models and humans has been reported to be different and dependent on concentrations and receptor targets.26,64,65 A sex predisposition in foals with NMS has not been reported, and differences in NS and NAS concentrations between fillies and colts have not been investigated.2,7 In our study, no differences were found between females and males in the concentrations and decay patterns of the 5 compounds identified as potential markers. Concentrations of the compounds in our study however were below detectable levels in some foals particularly after 48 hours of age, which might have affected the decay models and predicted half-lives.

Based on our findings in healthy foals, we propose that progesterone be considered as a useful clinical reference compound for the investigation of NMS and other neurological disorders of neonatal foals. Progesterone assays have been validated in equine blood for use in monitoring the estrous cycle in mares, with relatively sensitive immunoassay methods.66,67 This is not the case for a number of other steroid compounds for which assays might not be available or sensitivity might be low if compound concentrations are low. One limitation of progesterone commercial kits is the use of semiquantitative methods. Furthermore, concentrations of progesterone above the detection levels cannot be accurately determined without proper dilutions or linearity studies. Routine progesterone measurements rely on immunoassays (eg, ELISA, radioimmunoassay [RIA]) and it is unknown if such results would be comparable with those reported in our study using mass spectrometry.57 A recent study showed that progestagen and cortisol concentrations decreased in healthy foals over a 7-day period compared to sick foals (sepsis) born to mares with experimental bacterial placentitis.36 However, that study measured steroids using ELISA and RIA. Those results suggest that other mechanisms such as shifts in adrenocortical steroidogenesis and HPAA activity might be involved. In our study, progesterone concentrations rapidly and consistently decreased within 24 hours of birth, and remained low throughout the 7-day observation period. During this time, the behavior and state of consciousness of the foals was normal. Similarly, another study found a rapid decrease in progesterone concentrations in healthy foals, using an RIA assay.10 This decrease was not observed in foals with NMS in which progesterone remained persistently increased and foals displayed abnormal neurologic status.10 In a different study, plasma concentrations of progestagens were measured in pregnant mares, fetuses, and newborn foals, and progestagen concentrations were undetectable within 1-2 days of life.43

In conclusion, pregnenolone and progesterone were found to decrease rapidly within the first 24 hours of life and remained at low concentrations for the first 7 days of life. Deoxycorticosterone, DHEA, and DHEA-S concentrations decreased within the first 48 hours of life and continued to decrease to low concentrations throughout the first 7 days of life. Other NS such as androstenedione and epitestosterone also decreased within the first 48 hours of life, but the decrease was more variable and concentrations did not remain consistently low throughout the first few days of life. Progesterone rapidly decreased to a mean concentration of <10 ng/mL within 24 hours of life and remained at concentrations of approximately 2 ng/mL after 4 days of age. The short half-life (4.5 hours) of progesterone in circulating blood of healthy neonatal foals after birth makes it an ideal marker for detection of continued endogenous production in foals suspected of NMS and other neonatal disorders. Furthermore, determination of progesterone concentrations is readily available in most laboratories. An advantage of our study as compared to others consists of using mass spectrometry to more precisely identify the various steroid compounds in comparison to other methodologies (eg, ELISA and RIA) in which progestogen compounds might cross-react with other compounds. Also,
progesterone concentrations were evaluated at several time points within the first 7 days of life compared to single time points in other studies. Limitations of our study consist of the inclusion of a small convenience sample and using a single breed of foals. Studies using a larger population and representing multiple breeds of foals are needed.

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CONFLICT OF INTEREST DECLARATION
Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION
Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION
Colorado State University approved Animal Care and Use Protocol #16-6505A.

HUMAN ETHICS APPROVAL DECLARATION
Authors declare human ethics approval was not needed for this study.

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