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Effects of Supplementation with Sodium Sulfate, Trace Elements, and Their Combinations on Daily Excretion of Three Estrogen Sulfates in Kazakh Pregnant Mares: Postprint

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Abstract

This study aimed to investigate the effects of dietary supplementation with sodium sulfate, trace elements, and their combination on the concentrations of estrone sulfate, equilin sulfate, and 17 -dihydroequilin sulfate in plasma and urine, as well as the daily excretion of these three estrogen sulfates, in Kazakh mares during mid-to-late gestation. Thirty-four Kazakh mares in mid-gestation, aged 5–7 years with 2–4 parities, were selected and randomly assigned to four groups: control group (9 mares), Test Group I (9 mares), Test Group II (8 mares), and Test Group III (8 mares). Under identical feeding conditions, a 90-day supplementary feeding trial was conducted. The control group received 50 g cornmeal per mare daily during days 1–30, and 100 g cornmeal during days 31–90. Test Group I received 50 g cornmeal + 6 g sodium sulfate per mare daily during days 1–30, and 100 g cornmeal + 12 g sodium sulfate during days 31–90. Test Group II received 50 g cornmeal + 5 g trace elements per mare daily during days 1–30, and 100 g cornmeal + 5 g trace elements during days 31–90. Test Group III received 50 g cornmeal + 6 g sodium sulfate + 5 g trace elements per mare daily during days 1–30, and 100 g cornmeal + 12 g/d sodium sulfate + 5 g trace elements during days 31–90. The results showed: 1) Compared with the control group, dietary supplementation with sodium sulfate, trace elements, and their combination increased the plasma concentrations of estrone sulfate, equilin sulfate, and 17 -dihydroequilin sulfate in pregnant mares, but no significant differences were observed among groups ($P > 0.05$). 2) At each sampling time point during the trial period, no significant differences in urinary estrone sulfate, equilin sulfate, and 17 -dihydroequilin sulfate concentrations were detected between the test groups and the control group ($P > 0.05$); however, the urinary concentrations of these three estrogen sulfates in each test

group were numerically higher than those in the control group. Test Group III exhibited the highest urinary concentrations of these three estrogen sulfates on day 30. 3) On days 30, 60, and 90 of the trial, the daily excretion of the three estrogen sulfates in Test Group III was significantly or extremely significantly higher than that in the control group ($P < 0.05$ or $P < 0.01$). These results indicate that dietary supplementation with the combination of sodium sulfate and trace elements to Kazakh pregnant mares can increase the urinary concentrations of estrone sulfate, equilin sulfate, and 17 -dihydroequilin sulfate, thereby significantly or extremely significantly increasing the daily excretion of estrone sulfate, equilin sulfate, and 17 -dihydroequilin sulfate.

Full Text

Effects of Supplemental Feeding Sodium Sulfate, Trace Elements and Their Combination on Three Estrogen Sulfate Daily Outputs of Kazak Pregnancy Horses

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Abstract

This study investigated the effects of supplemental feeding sodium sulfate, trace elements, and their combination on plasma and urinary concentrations of sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate, as well as the daily excretion of these three estrogen sulfates in mid-to-late gestation Kazak mares. Thirty-four pregnant Kazak mares aged 5–7 years and carrying their second to fourth foals were selected and randomly divided into four groups: control (n=9), Trial Group I (n=9), Trial Group II (n=8), and Trial Group III (n=8). Under identical feeding conditions, a 90-day supplementation trial was conducted. From days 1–30, the control group received 50 g corn flour per horse daily, while Trial Groups I, II, and III received 50 g corn flour supplemented with 6 g sodium sulfate, 5 g trace elements, and 6 g sodium sulfate + 5 g trace elements, respectively. From days 31–90, the control group received 100 g corn flour daily, while Trial Groups I, II, and III received 100 g corn flour supplemented with 12 g sodium sulfate, 5 g trace elements, and 12 g sodium sulfate + 5 g trace elements, respectively. The results showed: (1) Compared with the control group, supplemental feeding of sodium sulfate, trace elements, and their combination increased plasma concentrations of sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate, but differences among groups were not significant ($P > 0.05$). (2) At each sampling time point during the trial, urinary concentrations of the three estrogen sulfates in the treatment groups

showed no significant differences from the control group ($P > 0.05$), though numerical values were higher. On day 30, Trial Group III exhibited the highest urinary concentrations of all three estrogen sulfates. (3) On days 30, 60, and 90, the daily excretion of the three estrogen sulfates in Trial Group III was significantly or extremely significantly higher than in the control group ($P < 0.05$ or $P < 0.01$). These findings indicate that combined supplementation of sodium sulfate and trace elements can increase urinary concentrations of sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate in pregnant Kazak mares, thereby significantly or extremely significantly enhancing their daily excretion rates.

Keywords: Kazak pregnancy horse; sodium sulfate; trace elements; estrogen sulfate

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Pregnant mare urine contains multiple bioactive components including hormones, organic acids, inorganic acids, and inorganic salts. Hormonal substances exist in both conjugated and unconjugated forms, including estrogens, progestogens, and androgens [1-3]. Studies have shown that estrogen concentrations in pregnant mare urine range from 70–130 mg/L, with total estrogen production reaching 50–100 g per mare per pregnancy [4], making it a primary source for natural estrogen extraction. Conjugated estrogens represent one of the most biologically effective components in pregnant mare urine, formed through glucuronidation and sulfation of estrogens. The predominant forms are sodium estrone sulfate (Es-S), sodium equilin sulfate (Eq-S), and sodium 17 -dihydroequilin sulfate (2H-Eq-S), which together account for over 90% of total hormonal content and serve as marker compounds for conjugated estrogens in pregnant mare urine. Clinically, conjugated estrogens are primarily used in hormone replacement therapy (HRT) to alleviate symptoms caused by estrogen deficiency, including treatment and prevention of menopausal syndrome, osteoporosis [6], Alzheimer' s disease, and coronary heart disease [7] in postmenopausal women.

Current research on conjugated estrogens in pregnant mare urine has focused primarily on extraction methods, analytical techniques, and variations across gestational stages [8-10], with limited studies on enhancing their content. The Kazak horse is a major breed in Xinjiang, valued for its milk, meat, and draft capabilities, characterized by roughage tolerance, cold resistance, and strong

adaptability. As the primary source of pregnant mare urine in Xinjiang, increasing conjugated estrogen content in Kazak mares could significantly enhance herders' economic returns. Therefore, this study examined mid-to-late gestation Kazak mares to investigate how supplemental feeding of sodium sulfate, trace elements, and their combination affects plasma and urinary concentrations of three estrogen sulfates (sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate), providing a reference for maintaining normal hormone secretion during pregnancy and increasing conjugated estrogen content in pregnant mare urine.

1.1 Experimental Period and Location

The trial was conducted from November 2012 to March 2013 in Alemale Township, Xinyuan County, Ili Kazak Autonomous Prefecture, Xinjiang.

1.2 Experimental Animals

Thirty-four pregnant Kazak mares aged 5–7 years, carrying their second to fourth foals and in mid-gestation, were selected based on similar breeding dates.

1.3 Experimental Design

The 34 pregnant mares were randomly divided into four groups: control (n=9), Trial Group I (n=9), Trial Group II (n=8), and Trial Group III (n=8). All mares were maintained under identical feeding conditions. During days 1–30, the control group received 50 g corn flour per horse daily, while Trial Groups I, II, and III received 50 g corn flour supplemented with 6 g sodium sulfate (analytical grade, from Tiankang Biological Co., Ltd.), 5 g trace elements (from Tiankang Biological Co., Ltd., containing 1.34% Cu, 5.38% Zn, 6.72% Fe, 5.38% Mn, and 0.014% Se), and 6 g sodium sulfate + 5 g trace elements, respectively. During days 31–90, the control group received 100 g corn flour daily, while Trial Groups I, II, and III received 100 g corn flour supplemented with 12 g sodium sulfate, 5 g trace elements, and 12 g sodium sulfate + 5 g trace elements, respectively. The total experimental period was 90 days. Corn flour was used as a carrier to facilitate consumption of sodium sulfate and trace elements.

1.4 Feeding Management

All experimental horses were housed in stalls with daily access to exercise areas for 2 hours during watering (12:00–13:00 and 18:00–19:00). Daily supplements of corn flour, sodium sulfate, and trace elements were divided into two equal portions and fed at 09:00 and 20:00 using feed buckets to ensure accurate intake and complete consumption. Roughage consisted of hay and alfalfa at a 4:1 ratio, offered ad libitum five times daily at 09:00, 12:00, 16:00, 20:00, and 00:00. Nutrient levels of the roughage are shown in Table 1 .

1.5 Sample Collection and Processing

Blood samples were collected via jugular venipuncture at 08:30 on day 30 of the trial after overnight fasting. Heparinized vacuum tubes were used, gently agitated, then centrifuged at 2,190×g for 15 minutes to obtain plasma, which was stored at -20°C.

Urine samples were collected on days 0 (the day before trial initiation), 30, 60, and 90. Total daily urine output from each mare was collected in buckets, thoroughly mixed, and a 250 mL aliquot was transferred to plastic bottles and stored at -20°C.

1.6 Sample Analysis

Plasma and urinary concentrations of conjugated estrogens—sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate—were determined by high-performance liquid chromatography (HPLC).

1.6.1 Chromatographic Conditions Column: Ultimate® XB-C18 (4.6 mm × 100 mm, 5 µm) (Welch Materials); detection wavelength: 215 nm; column temperature: 20°C; flow rate: 1 mL/min; injection volume: 10 µL.

1.6.2 Mobile Phase Buffer preparation: 2.00 g potassium hydrogen phosphate trihydrate (K HPO · 3H O) and 1.00 g potassium dihydrogen phosphate (KH PO) were dissolved in a 1 L volumetric flask with double-distilled water, adjusted to pH 7.00 ± 0.01 with HCl or KOH if necessary, and mixed thoroughly.

Mobile phase: buffer:acetonitrile (HPLC grade):methanol (HPLC grade) = 680:240:80 (V/V/V), mixed thoroughly, filtered through a 0.45 µm membrane, and degassed by ultrasonication for 1 hour.

1.6.3 Plasma Processing and Injection Volume Frozen plasma samples were thawed and mixed thoroughly. A 0.5 mL aliquot was placed in a 5 mL capped plastic tube, mixed with 2 mL pre-chilled chloroform (4°C), vortexed thoroughly, and centrifuged at 3,500 rpm for 15 minutes. The upper layer (chloroform has higher density and forms the lower layer) was carefully aspirated (0.3 mL) and transferred to a 1.5 mL Eppendorf tube, mixed with an equal volume (0.3 mL) of HPLC-grade methanol, and stored at 4°C until analysis. Before injection, samples were centrifuged at 12,000 rpm for 5 minutes, and 10 µL of the supernatant was injected.

1.6.4 Urine Processing and Injection Volume Frozen urine samples were thawed and mixed thoroughly. A 0.2 mL aliquot was placed in a 1.5 mL Eppendorf tube, diluted with 0.8 mL distilled water, mixed, and centrifuged at 12,000 rpm for 10 minutes at 4°C. Then 0.4 mL of the supernatant was transferred to a

new 1.5 mL Eppendorf tube, diluted with 0.4 mL distilled water, and mixed. Finally, 0.2 mL of the diluted supernatant was transferred to another tube, mixed with 0.2 mL HPLC-grade methanol, and 10 µL was injected for analysis.

1.7 Data Processing

All data are expressed as mean ± standard deviation (mean ± SD). One-way ANOVA was performed using SPSS 19.0 software, followed by Duncan's multiple comparison test.

2.1 Effects of Supplemental Feeding Sodium Sulfate, Trace Elements and Their Combination on Plasma Estrogen Sulfate Concentrations in Pregnant Kazak Mares

As shown in Table 2 , no significant differences were observed among groups in plasma concentrations of sodium estrone sulfate, sodium equilin sulfate, or sodium 17 -dihydroequilin sulfate ($P > 0.05$). However, Trial Group I (sodium sulfate supplementation) and Trial Group III (combined sodium sulfate and trace elements) showed higher concentrations than the control group, with increases of 8.33%, 10.00%, and 8.11% in Trial Group I, and 12.50%, 17.50%, and 8.11% in Trial Group III, respectively.

2.2 Effects of Supplemental Feeding Sodium Sulfate, Trace Elements and Their Combination on Urinary Estrogen Sulfate Concentrations in Pregnant Kazak Mares

Table 3 shows that urinary concentrations of the three estrogen sulfates increased initially then decreased as pregnancy progressed. At each sampling time point, no significant differences were found between treatment groups and the control group ($P > 0.05$), though numerical values were higher in all treatment groups. The highest concentrations occurred on day 30, when Trial Group III reached 155.35 µg/mL, 75.48 µg/mL, and 40.92 µg/mL for sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate, respectively.

2.3 Effects of Supplemental Feulfate Daily Excretion in Pregnant Kazak Mares

Table 4 shows that daily urine volume ranged from 3.54–5.34 L (mean 4.33 L), with no significant differences among groups at any sampling time point ($P > 0.05$). Regarding daily estrogen sulfate excretion, on day 0, Trial Groups II and III showed extremely significantly lower sodium 17 -dihydroequilin sulfate excretion compared to the control and Trial Group I ($P < 0.01$). All groups exhibited peak excretion on day 30, when Trial Groups I and III showed significantly or extremely significantly higher excretion of sodium estrone sulfate and sodium equilin sulfate than the control ($P < 0.05$ or $P < 0.01$), and Trial Group III also showed significantly higher sodium 17 -dihydroequilin sulfate excretion

($P < 0.05$). On day 60, Trial Groups I and III exhibited extremely significantly higher excretion of all three estrogen sulfates compared to the control ($P < 0.01$). On day 90, Trial Group III showed significantly higher excretion of sodium estrone sulfate and sodium 17 -dihydroequilin sulfate ($P < 0.05$) and extremely significantly higher excretion of sodium equilin sulfate ($P < 0.01$). These results demonstrate that combined supplementation of sodium sulfate and trace elements can significantly or extremely significantly increase daily urinary excretion of estrogen sulfates in pregnant mares.

Discussion

The body synthesizes two main types of conjugated estrogens: metabolites containing glucuronic acid groups formed via glucuronidation at the estrogen C-3 position, and those containing sulfate groups formed via sulfation at the same position. Conjugated estrogens lose their ability to bind estrogen receptors and are excreted in urine as sulfates and glucuronides. Studies indicate that estrogen sulfate concentrations in normal human plasma exceed those of estrogen glucuronides [11], establishing estrogen sulfates as the dominant form of conjugated estrogens in the body.

Estrogen sulfation represents the primary pathway for estrogen sulfate synthesis [12-13] and a major mechanism for regulating endogenous steroid hormone homeostasis [14-15]. Estrogen sulfates are metabolites formed when sulfotransferases transfer sulfate groups to the C-3 position of estrogens [16]. The sulfate donor for conjugated estrogen synthesis is 3' -phosphoadenosine-5' -phosphosulfate (PAPS), which is synthesized in two steps: (1) ATP and sulfate (SO^{2}) form adenosine 5' -phosphosulfate (APS) via ATP sulfurylase, and (2) APS reacts with ATP via APS kinase to produce PAPS and ADP. PAPS serves as the sulfate group donor in sulfation reactions occurring in the Golgi apparatus, where sulfate is transferred to hydroxyl groups of tyrosine residues under enzymatic catalysis. Berglund [17] reported that renal reabsorption is the most critical factor for sulfate homeostasis, while estrogen sulfate formation occurs in both kidneys and liver. Sulfate cannot directly bind to estrogens; it must first form PAPS through ATP-dependent enzymatic reactions before PAPS donates the sulfate group to estrogens via estrogen sulfotransferase.

Pregnant mare urine is rich in estrogenic substances [4], with concentrations of 70–130 mg/L and total production of 50–100 g per pregnancy. These estrogens exist primarily as conjugated sodium salts—sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate—which together comprise over 90% of total estrogen content. Factors influencing conjugated estrogen content include age, parity, gestational stage, and feeding management [18]; under similar physiological conditions, feeding management may be the primary factor affecting synthesis. Research has shown that sodium sulfate supplementation increases blood sulfate and hepatic PAPS concentrations in rats administered acetaminophen [19-20]. Klaassen et al. [21] reported that PAPS availability under normal physiological conditions determines estrogen sulfate concentrations,

with tissue sulfate content being the most important factor influencing PAPS synthesis. Therefore, increasing free sulfate content in body tissues may represent a viable approach to enhance conjugated estrogen content in pregnant mare urine.

In this study, supplemental feeding of sodium sulfate, trace elements, and their combination to mid-to-late gestation Kazak mares resulted in numerically higher plasma concentrations of all three estrogen sulfates compared to the control group, though differences were not statistically significant. Urinary estrogen sulfate patterns showed peak concentrations on day 30 (sixth month of gestation), consistent with findings by Yao et al. [22] and Muratali · Kelimu et al. [23], confirming that mid-gestation represents the period of highest urinary estrogen sulfate excretion, followed by a decline. The concentrations observed in the sodium sulfate and combined supplementation groups exceeded those reported by Yao et al. [22] and Muratali · Kelimu et al. [23], demonstrating that supplementation can increase urinary estrogen sulfate content. Control group values were similar to those of Muratali · Kelimu et al. [23] but slightly higher than Yao et al. [22], possibly due to differences in mare age and parity.

According to Guo Haijuan [24], average daily urine volume in various breeds during weeks 27–29 of gestation ranged from 3.42–4.08 L, with sodium estrone sulfate excretion of 76.37–213.81 mg/d, sodium equilin sulfate of 27.54–171.31 mg/d, and sodium 17 -dihydroequilin sulfate of 33.22–136.03 mg/d. Based on the 34 mares in this study, Kazak mares averaged 4.84 L daily urine volume in mid-gestation. Using Muratali · Kelimu et al.' s [23] data as reference, mid-gestation Kazak mares excreted approximately 604.76 mg/d sodium estrone sulfate, 260.20 mg/d sodium equilin sulfate, and 117.42 mg/d sodium 17 -dihydroequilin sulfate, indicating that similar breeds under comparable gestational stages produce consistent estrogen sulfate levels, while supplementation can effectively increase these values.

The sodium sulfate and combined supplementation groups exhibited higher urinary estrogen sulfate concentrations than both the control group and the trace element-only group, indicating that sodium sulfate supplementation enhances urinary estrogen sulfate content while trace element supplementation alone has no positive effect. However, the combination of sodium sulfate and trace elements provides additional enhancement beyond sodium sulfate alone. Pregnancy is a complex physiological process requiring coordinated nutrient interactions to maintain gestation. Studies show that trace elements not only support reproductive needs but also regulate reproductive hormones and maintain pregnancy [25-26]. Trace element supplementation may increase secretion of estrone and equilin, while sodium sulfate supplementation increases PAPS availability, with estrogen sulfates formed via sulfotransferase action. This may explain why the sodium sulfate and combined supplementation groups outperformed the control and trace element-only groups.

Conclusions

1. In mid-gestation Kazak mares, urinary concentrations of sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate increase initially then decrease as pregnancy progresses.
2. Under the conditions of this study, combined supplementation of sodium sulfate and trace elements to pregnant Kazak mares increased urinary concentrations of sodium estrone sulfate, sodium equilin sulfate, and sodium 17 -dihydroequilin sulfate, thereby significantly or extremely significantly enhancing their daily excretion rates.

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