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Research Article

Plasma Cortisol Levels as a Measure of Stress in Rumen Impaction in Sheep

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Abstract

Scavenging ruminants are susceptible to rumen impaction from ingestion of indigestible plastic bags which affects feed intake and digestion, induces stress that compromise their survival. Yet, physiological evidence of stress consequent to impaction is not known, hence the aim of this study. Fifteen sheep divided into 3 groups of 5 each were coded as RIP, RC and C. Each individual in RIP had 166g of plastic bags implanted into its rumen through rumenotomy to simulate impaction while the positive control group (RC) only underwent rumenotomy without plastic bags implanted. The negative control group (C) underwent neither rumenotomy nor plastic bag-implantation. Plasma samples from each individual during the first 72 hours then weekly for 8-week post-impaction period were analyzed for cortisol as a measure of stress using an ELISA kit. Results showed elevated plasma cortisol in RIP by 4-fold (316.0 ± 4.0nmol/L, $p < 0.0001$) to 2-fold (162.6 ± 17.9nmol/L, $p = 0.0010$) between 6 and 72-hour post-impaction respectively compared to their baseline value (62.3 ± 8.5nmol/L). Group C at 72 hours recorded 85.4 ± 10.7nmol/L. Elevated plasma cortisol in RIP persisted the subsequent 3 weeks (153.6 ± 35.1nmol/L, $p < 0.0001$) compared to C, declining to normal levels by the 8th week. In RC increased levels were noted 6 hours post-rumenotomy (174.4 ± 48.6nmol/L, $p = 0.0125$) compared to C (85.0 ± 3.1nmol/L), but after week 1 declined to normal levels. In conclusion, sheep with impacted-rumen have acutely elevated plasma cortisol, indicative of stress but declines to normal if survived.

INTRODUCTION

Improper disposal of non-biodegradable materials particularly waste plastic bags continues to be an environmental and health concern. This concern is heightened in most urban and peri-urban areas especially in Africa, where sheep and goats scavenge on waste dumping sites, mainly as a result of diminished grazing grounds. Ruminants grazing in such environments are likely to ingest these plastic bags that will gradually accumulate to cause rumen impaction which interferes with digestion and general health of the animal [1,2]. Presence of these indigestible plastic bags in the rumen interferes both with the digestive processes and the overall energy balance in the body [1,3]. They inevitably cause physiological disturbances that could culminate into stress for the animal and may even lead to death [4,5]. The physiological alterations caused by stress affect the well-being of the animal [6].

Stress is defined as a disease of adaptation [7], which ensues when the mechanisms to cope with a stressor become overextended resulting in an eventual break down of the stress response system. The stressor may be an internal stimulus within the animal's body or external stimulus within the environment of the animal [8]. The Hypothalamic-Pituitary-Adrenal (HPA) axis together with the autonomic nervous system referred to as the neuroendocrine system mediates stress response [9,10]. Cortisol is the primary active stress hormone produced by the HPA axis in most mammals while corticosterone is produced in birds and laboratory rodents [10,11]. Both of these stress hormones are glucocorticoids which are cholesterol-derived steroids synthesized in the zona fasciculata of the adrenal cortex.

Any stressor that continually stimulates the HPA axis for several days would subsequently lead to chronic elevation in cortisol secretion. Increased cortisol level for prolonged period is detrimental to animal health, inhibiting inflammatory processes and suppressing immune response, which increases susceptibility of the animal to pathogens [12]. Accumulation of indigestible plastic bags in the rumen of sheep as a result of

scavenging on dumping sites may cause stress that would lead to a sustained rise in cortisol levels in the body, culminating in physiological disturbances. The purpose of this research was to carry out cortisol assays in plasma to indicate if rumen impaction causes stress in the animal or not. There is no available literature that reports on relationship between rumen impaction and stress animals.

MATERIALS AND METHODS

Experimental design and animals

Fifteen castrated male sheep aged between 12-15 months were purchased from Gicheha farm Nairobi-Kenya, which had pastures free of plastic bag wastes. All sheep were born and raised within the farm. The animals were confirmed by a veterinarian to be clinically healthy. They were housed in stalls within the Animal Unit of the Department of Clinical Studies, University of Nairobi and allowed a 5-week acclimatization period during which period they were de-wormed, vital parameters monitored daily and restrained regularly to accustom them to handling.

They were randomly divided into 3 experimental groups of 5 sheep in each group. Each sheep was ear-tagged with individual and group identification. Group 1 coded RIP (rumen impacted with plastic bags through rumenotomy) was the experimental group and the 5 sheep were numbered RIP-1, RIP-2, RIP-3, RIP-4 and RIP-5; Group 2 was coded RC (rumen underwent rumenotomy without impaction with plastic bag) as the positive control group and sheep numbered RC-1, RC-2, RC-3, RC-4 and RC-5; Group 3 was coded C (no rumenotomy no impaction with plastic bags) as the negative control group and the sheep numbered C-1, C-2, C-3, C-4 and C-5. The sheep in RIP were each implanted with 166 g of plastic bags into their rumen through rumenotomy. The Sheep in RC were subjected to rumenotomy but no plastic bags were implanted in the rumen. The sheep in C were not subjected to any procedure but were left whole. The sheep in each group were housed together throughout the experiment and fed with Rhodes grass, commercially produced concentrates (UNGA AFYA Meal®, UNGA Farm Care Ltd, Nairobi, Kenya), supplemented with mineral lick and provided with water *ad libitum*.

Implantation of plastic bags into the rumen

Rumenotomy was performed in groups RIP and RC using the standard procedure described by Baird [13]. The plastic bags that were implanted into the rumen of Group 1 (RIP) were the type used for packaging foods. They were soft, thin, transparent non-perforated type (Malaika Poly Bags®, KEBS producers, Nairobi, Kenya) and each measured 215 mm × 360 mm. For each sheep, a total of 166 g consisting of individual plastic bags temporarily rolled together was implanted into the rumen. The plastic bags were rolled in a way that allowed them to separate to individual pieces and mix freely with rumen contents. Post-operatively, penicillin-streptomycin (PenStrep® Norbrook, Ireland) was administered intramuscularly in each sheep for 5 consecutive days to prevent possible infection and 20% phenylbutazone (Phenylbutazone® Agrar, Holland BV) was administered intramuscularly every alternate day to manage pain until there was evidence of wound healing. Aerosol Oxytetracycline HCL (Alamycin®, Norbrook, Kenya) was applied on the skin wound daily until skin sutures were removed 14 days post-surgery.

Collection of blood samples

Four milliliters of blood was collected from the jugular vein of each sheep under sterile conditions using 18 gauge needles. The blood was immediately transferred into vacutainer tubes containing anticoagulant EDTA and properly labelled. The vacutainer tubes were kept in ice cold temperature until centrifuged at 2500 g for 15 minutes to obtain plasma within 1-2 hours after collection. The plasma was aliquoted out into Eppendorf tubes and stored at -20°C.

Baseline blood samples: Blood samples collected from all groups before rumenotomy served to provide baseline values.

Short-term blood sampling after implantation: Once rumenotomy was completed, 4 ml of blood was collected from each sheep in all groups over a period of 72 hours post-implantation. These blood samples were collected at 6 hours, 24 hours, 48 hours and 72 hours post-implantation and the results considered as short-term values.

Long-term blood sampling after implantation: Weekly blood samples were collected from each individual sheep in the experimental groups (08:00 hours). They were collected weekly beginning at week-1 to week-8 post-implantation for the three groups.

Plasma cortisol assay: Total plasma cortisol concentration was measured using commercial cortisol ELISA kit (Creative Diagnostics, NY, USA; Cat. No: DEIA0708H). Intra-assay and inter-assay coefficients of variation ranged from 5.6 to 14.7 % and 6.3 % to 10.9 % respectively. The limit of detection was determined as 45.4 pg/mL. Sensitivity of the assay was determined as 17.3 pg/mL at a wavelength of 450 nm. The concentrations of the standards were log-transformed and a standard curve was plotted using GraphPad Prism software version 6.0 (GraphPad, San Diego, USA). The total cortisol concentration for each sample was interpolated from the standard curve as log-transformed and converted back to normal values by antilog function in Microsoft excel.

Data management and statistical analysis

The raw data collected for all parameters were verified, validated and entered into Microsoft Office 2010 excel spread sheet. The data sets were coded with letters and numbers representing each sample in an experimental group and calculated values expressed as mean ± S.E.M. The data obtained were imported into GraphPad Prism software version 6.0 (GraphPad Prism Statistical Software, Inc. California, USA) for graphical presentation and statistical analysis. Two-way Analysis of variance (ANOVA) with Tukey's Multiple Comparison (Post hoc) Test were performed using GraphPad Prism software (version 6.0) to compute simple associations and comparisons between the different parameters and the 3 experimental groups. Unpaired t-test of GraphPad Prism was used to compute differences in mean values within the same experimental group. The differences of means were considered significant at the level of $P < 0.05$.

Ethical approval

The treatment of animals and study protocol was approved

by the Biosafety, Animal Use and Ethics Committee of the Faculty of Veterinary Medicine, University of Nairobi, Kenya.

RESULTS

Short-term effects of rumen impaction on plasma cortisol levels

The mean values of plasma cortisol concentrations measured over 72 hours in sheep with impacted rumen (RIP) compared to those in the positive control group (RC) and negative control group (C) are presented in Table 1. RIP showed persistently elevated concentration of cortisol in their plasma when compared with their baseline value of 62.3 ± 8.5nmol/L, as well as compared to RC and C within 72 hours post-impaction. In comparison with its baseline values, the increased concentration of plasma cortisol was 4-fold higher [316.0 ± 4.0 nmol/L, t(8) = 27.01, p <0. 001] at 6 hours and 2-fold higher [162.6 ± 17.9 nmol/L, t(8) = 5.062, p <0.001] at the end of 72-hours. Similarly, RIP showed elevated levels of plasma cortisol at 6 hours (p <0.0001) and 72 hours (p = 0.0355) post-impaction compared to the respective mean values of C (85.0 ± 3.1 nmol/L, 6 hours; 85.4 nmol/L ± 10.7, 72 hours). In comparison with RC plasma cortisol concentration in RIP was still higher at 6 hours (p <0.0001) and 24 hours (p = 0.0003) post-impaction (Table 1).

RC had significantly elevated levels (p = 0.0125) of mean plasma cortisol 6 hours after rumenotomy compared to C. This increase was also about 100 % (148.5 nmol/L, t(8) = 3.829, p = 0.0050) from their mean baseline value of 71.1 nmol/L. However, the concentration of cortisol in the plasma of RC gradually declined over the remaining period to the extent that at 72 hour end-point, it was close to normal reference values.

Long-term effects of rumen impaction on plasma cortisol concentration

The mean plasma cortisol concentrations during the 8-week experimental period in group1 (RIP) with rumen impaction, group 2 (RC) positive control with only rumenotomy and group 3 (C) negative control are presented in Figure (1).

The mean values of plasma cortisol concentration in RIP significantly increased (p <0.05) over the first 3 weeks then progressively declined to values considered normal (Table 2). One week after rumen impaction, the concentration of plasma cortisol in RIP increased 2-fold (189.0 ± 33.0 nmol/L, t(8) = 3.78, p = 0.0059) compared to the baseline value of 62.3 ± 8.5nmol/L. The elevated levels in RIP persisted over the first 3 weeks which was significantly higher (p <0.0001) compared to the levels in C (44.9± 7.7 nmol/L, week 3). Similarly, mean plasma cortisol concentration in RIP were significantly elevated at week-2 (181.7± 28.6 nmol/L, p = 0.0026) and week-3 (153.6 ± 35.1 nmol/L, p = 0.0003) compared to the respective values of 108.1 ± 28.0 nmol/L and 65.7 ± 13.7 nmol/L in RC. However, by the 4th week post-impaction the levels of plasma cortisol in RIP had dropped to normal (66.1 ± 6.4 nmol/L), which was fairly maintained thereafter to the end of the 8-week experimentation.

One week after rumenotomy, mean plasma cortisol concentrations in RC rose about 100 % (p = 0.0074) from their baseline value, but thereafter declined to normal values and remained static to the end of the 8th experimental week.

In the first week of the experiment, C also showed 63 % increase in plasma cortisol concentration from baseline value but the levels decreased to normal in the weeks that followed.

DISCUSSION

The present study has shown for the first time that severe rumen impaction in sheep would cause acute stress in the first 72 hours, which was demonstrated by significant spontaneous 4-fold increase in levels of plasma cortisol concentration. The key role of the rumen in microbial fermentation of forages for the production and absorption of volatile fatty acids which contributes about 80% of the total energy requirement in sheep **[**15**]** may be compromised. The acute elevation in plasma cortisol levels in this study are partially comparable to those reported previously in sheep stressed by confinement on the first day but markedly reduced to normal values by the third day **[**16**]**. However, in the present study, plasma cortisol concentration significantly remained high beyond the third day owing to the

Table 1: Mean plasma cortisol concentration (nmol/L) in 3 experimental groups of sheep during the first 72-hour post-impaction period.

Time post-implantation (hours)	Mean (± SEM) plasma cortisol concentration (nmol/L)			*P* value	Normal values in sheep** (nmol/L)
	RIP (n = 5)	RC (n = 5)	C (n = 5)		
Baseline Measurements	62.3 ± 8.5	71.6 ± 10.2	63.6 ± 6.2	0.9624	42 – 82
6	316.0[a] ± 4.0*	174.4[b] ± 48.6	85.0[c] ± 3.1	< 0.0001 < 0.0001 0.0125	
24	274.3[a] ± 49.9*	148.5[b] ± 17.3*	100.9 ± 9.6	< 0.0001 0.0003	
48	172.0[a] ± 20.5*	122.9 ± 14.8	99.1 ± 6.4	0.0499	
72	162.6[a] ± 17.9*	113.8 ± 17.3	85.4 ± 10.7	0.0355	

** Source: [14]
*Significance at p < 0.05; compared to baseline measurement
[a]*P*-value = test group RIP compared with negative control group C
[b]*P*-value = test group RIP compared with positive control group RC
[c]*P*-value = positive control group RC compared with negative control group C

Table 2: Mean plasma cortisol concentration (nmol/L) in 3 experimental groups of sheep over an 8-week post-impaction period.

Period post-implantation (Week)	Weekly mean (± SEM) plasma cortisol concentration (nmol/L)			*P* –value	Normal values in sheep** (nmol/L)
	RIP (n = 5)	RC (n = 5)	C (n = 5)		
Baseline Measurements	62.3 ± 8.5	71.6 ± 10.2	63.6 ± 6.2	0.9267	42 – 82
1	189.0[a] ± 33.0*	169.8± 23.5	103.5[c] ± 3.6	0.0004 0.0074	
2	181.7[a] ± 28.6*	108.1[b] ± 28.0	92.8 ± 6.5	0.0002 0.0026	
3	153.6[a] ± 35.1*	65.7[b] ± 13.7	44.9± 7.7	< 0.0001 0.0003	
4	66.1 ±6.4	48.8 ± 4.3	55.4 ± 11.7	0.8728	
5	43.6 ± 5.1	40.8 ± 6.1	42.7 ± 4.7	0.9991	
6	69.1 ± 25.0	42.3 ± 8.0	29.0 ± 4.8	0.1895	
7	75.9 ± 5.4	33.7 ± 3.5	50.4 ± 6.0	0.5621	
8	82.3 ± 12.7	54.0 ± 6.8	48.6 ± 5.3	0.3678	

** Source: [14]
*Significance at $p < 0.05$; compared to baseline measurement
[a]*P*-value = test group RIP compared with negative control group C
[b]*P*-value = test group RIP compared with positive control group RC
[c]*P*-value = positive control group RC compared with negative control group C

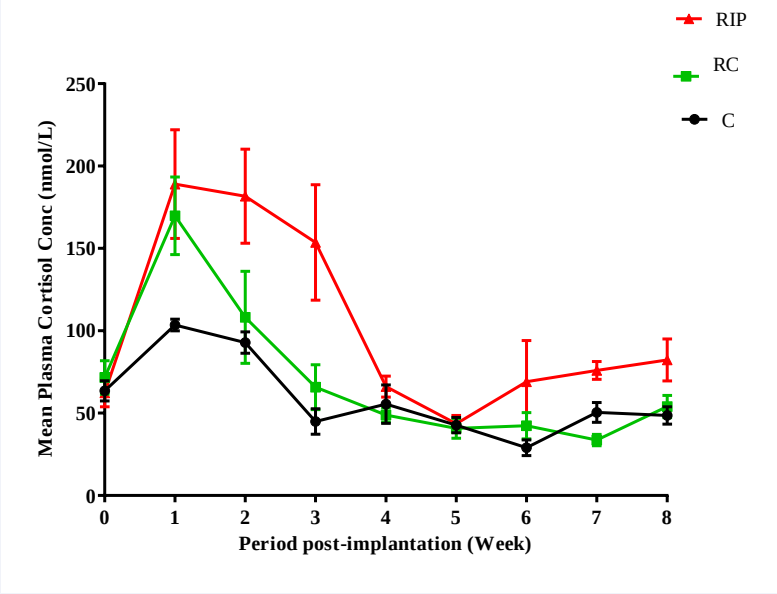

Figure 1 Comparative mean ± SEM plasma cortisol concentration (nmol/L) in sheep with plastic-bag impacted rumen (RIP) and in the positive control group which had rumenotomy (RC) as well as negative control group which had no procedure (C) over an 8-week experimental period. Note the acute elevation of levels of plasma cortisol in RIP.

excessive stress from rumen impaction. The differences in observations from the previous reports could be attributed to variations in the type and intensity of the stress stimulus [17].

The pressure exerted by the plastic bags served as internal stressor in the rumen, which caused stimulation of the HPA axis to initiate a stress response, consequently causing the adrenal gland to secrete excess cortisol. The ability of an animal to produce enough cortisol on stimulation of the HPA axis by a stressor is key for the animal to be able to quickly adapt to the stressor [10]. Thus increased secretion of cortisol in response to an acute stressor may not necessarily be pathological, except when the intensity and persistent period of the stressor causes prolonged presence of excessive cortisol, resulting in detrimental changes to the animal's physiological functions [18]. It is therefore implied that the increased levels of plasma cortisol concentration in the sheep was a reflection of their HPA axis' responsiveness to those foreign plastic bags in the rumen. The results support numerous earlier reports that plasma cortisol may be a good estimator of acute stress [16,19-24].

The increased plasma cortisol concentration found in the

positive control group can be attributed to stress from the surgical procedure of rumenotomy even without presence of plastic bags in the rumen. Surgical stress is likely to be immediate but later as the animal adjusts and recovers, the stress diminishes. This is probably the reason plasma cortisol concentration increased spontaneously 6 hours after surgery but declined thereafter to normal values within the 2-week recovery period in this group. This observation concurs with previous findings of increased plasma cortisol concentration in Holstein-Friesian dairy cows that were subjected to surgery on the abdomen [25]. Since the highest concentrations of plasma cortisol occurred immediately after surgery, it is likely that severe pain just after surgery, may have served as the potent stressor [26].

The marginal increase in levels of plasma cortisol in the negative control group during the first 72 hour period of the experiments could be a result of incidental circadian fluctuating patterns of cortisol secretion as time progresses [27]. Other contributing factors to the marginal rise of plasma cortisol levels could also be time of the day when blood samples were collected, duration taken to sample each animal or probably stress of repeated blood sampling despite the fact that all sheep were acclimatized before commencement of the study.

Induction of long-term stress by rumen impaction manifested through increase in plasma cortisol concentration during the first 3 weeks followed by steady decline towards normal levels in the next five weeks, may imply that the animals became adapted to the situation, hence the adrenal gland failing to respond further to the stressor. This can be compared to a similar report which indicated low levels of plasma cortisol concentration in sheep that had fully adapted to their new environment as compared to high cortisol levels in non-adapted sheep [28]. The latter authors also reported that sheep may require a period of 7 - 28 days to completely adapt to a new stressor, an observation that agrees with the current study. Similar findings in baboons revealed that decreased production of cortisol is present in chronic stress [29]. Conversely, continuous elevation of plasma cortisol concentration over a period of 35 days was reported in sheep exposed to stress of loud noise [20].

It has been suggested that animals tend to adapt to prolonged stress, which results in the reduction of plasma cortisol levels [30]. This is because increased concentration of plasma cortisol inhibits further release of ACTH through negative feedback mechanism [31-33], subsequently inhibiting further secretion of cortisol by the adrenal glands in order to maintain homeostasis. It has also been suggested that low plasma cortisol in prolonged stress does not mean absence or reduced stress, but could imply exhausted adrenal glands [34]. Others suggest that the sensitivity of adrenal glands present in acute stress declines as time progresses when the stressor persists [35,36]. These reports indeed corroborate the findings of the current study which also agree with earlier opinions that plasma cortisol concentration may not be a good estimator for assessment of long-term or prolonged stress [37-40]. Hence there is the need to investigate other biomarkers of chronic stress.

CONCLUSIONS

To our knowledge, the present study is the first to determine the effect of rumen impaction on plasma cortisol concentration in sheep. We concluded that:

a) Rumen impaction by any sudden cause is likely to induce acute stress in sheep, manifested by increased plasma cortisol levels.

b) Sheep with persistent or slow occurring rumen impaction are likely to adapt well to prolonged stress shown by return to normal levels of the concentration of plasma cortisol.

c) Plasma cortisol concentration is a good estimator for assessment of acute stress rather than chronic stress.

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