Acute behavioral and biochemical responses of sheep to S/C ivermectin injection

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This study was designed to compare between the effects of subcutaneous injection of ivermectin at the left neck region versus behind the left elbow on the acute behavioural and biochemical responses of sheep, with the aim of selecting the most suitable injection strategy causing the least adverse effects on the animal health and welfare. Twenty-five sheep were assigned to 5 groups: one control group (C, without injection), and two groups injected with 0.9% NaCl either at neck (SN) or elbow (SE), and two groups injected with ivermectin (IVM) at a dose of 0.2 mg kg^{-1} BW either at neck (IN) or elbow (IE). Results reflected that head shaking and neck scratching showed significant increases in the IN group, while standing was significantly lower in the IE group compared to the C group. Pawing was significantly higher in both SE and IE groups compared to the C group. Plasma levels of cortisol, glucose and lactate were significantly increased in both IN and IE groups. There were no obvious changes in the levels of other stress markers among the different treated groups. In conclusions, the magnitude of acute stress reactivity was not significantly different between IVM injections behind the elbow and at the neck region.

1   Introduction

Ivermectin is a member of the macrocyclic lactones widely used as a broad-spectrum drug against a wide range of endo- and ectoparasites in ruminant animals (Gokbulut et al., 2008; Jameel et al., 2014). It is commercially available in different formulations including injections, oral, and rumino-reticular and cutaneous delivery systems (Mestorino et al., 2003). Although previous studies showed that subcutaneous (S/C) injection is a more efficient route for this drug in terms of drug bioavailability, efficacy, and persistency compared to other routes of administration (Lespine et al., 2005; Gokbulut et al., 2007), its irritability at the site of injection represents a major concern (Bokisch and Walker, 1986).

Given that much attention has been paid to animal welfare, selection of injection site depending on the satisfaction of this demand is of outmost significance. However, it is well established that the preferred site for S/C injections in sheep is the skin just behind the elbow; In Egypt, the preference of S/C injection at the neck region over that behind the elbow is a common trend among veterinarians without a well-established scientific basis. Therefore, this investigation sheds light on the potential difference between S/C injections at the neck region versus behind the elbow manifested by modulations in acute behavioural patterns and stress endpoints measured as a reflection of animal welfare.

2   Material and methods

2.1 Experimental groups

This experiment was conducted in the Hospital of Veterinary Medicine, Assiut University, Assiut, Egypt. Twenty multiparous, non-pregnant and non-lactating adult ewes and five native Egyptian breed rams (*Ovis aries*) weighing (35–45 kg) and aged 3–5 years, were used in this experiment. The sheep were randomly assigned to five groups [each had 4 ewes and one ram]; namely, a control group (C, without injection), two saline groups (SN and SE) (injected with 0.9% NaCl) and two IVM groups (IN and IE) [IVM at a dose of 0.2 mg kg^{-1} BW, 1% solution (Ivomec® injection Merk & Co. Inc., Rahway, NJ, USA)]. The injection was at the left neck region or behind the left elbow. The animals were penned individually for all ethological observations and collection of all data and samples.

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Table 1   The animal numbers, average body weight, and age of different experimental groups

Group Data	C	SN	SE	IN	IE
Ewe numbers	4	4	4	4	4
Rams number	1	1	1	1	1
Number of pens	5	5	5	5	5
Average body weight/kg	40.20 ±1.16	39.00 ±1.30	40.60 ±1.63	40.00 ±1.48	40.80 ±1.24
Age/years	3.80 ±0.37	3.80 ±0.49	3.80 ±0.20	3.60 ±0.40	3.60 ±0.24

C – control group; SN – group injected saline at the neck region; SE – group injected saline behind the elbow; IN – group injected ivermectin at the neck region; IE – group injected ivermectin behind the elbow

2.2 Behavioural pattern recording

Before the time of injection, the animals were housed in a single large room. However, directly after the injection, each individual animal was hold inside a small separate pen with visual and auditory contact with other sheep. Each group had five animals which were housed in five different pens and each animals pen was used as statistical unite. The behavioural activities of individual sheep were observed continuously throughout the first thirty minutes post-injection. The frequency of sheep behavioural patterns including standing, walking, pawing, biting fleece, head shaking and neck shaking were recorded and quantified as the number of occurrences of these behaviours. The frequency of a particular behaviour in a 30-minute session was calculated. The sheep were considered to be in a normal standing position if animal in a straight-back posture with the head raised higher than the level of the back line. A hunched back posture with the head lower than the highest level of the back was considered abnormal. Locomotion was recorded as normal, stiff walking and running. Pawing was registered when the sheep pawed at the ground. Biting fleece was presented at biting the sides of the body, back line and sometimes the upper part of the legs. Head shaking was noted as the period of violent shaking and nodding of the head. Neck scratching was defined as sudden scratching to neck skin with the hind leg (occasionally including head), or rubbing the neck against solid surrounding objects.

2.3 Biochemical indicators of stress

Blood samples (20 mL) were collected from each sheep via the jugular vein immediately after 30 minutes behavioural observation. The blood samples were transferred to sterile test tubes with an anticoagulant and allowed to set for about 30 minutes in the refrigerator (4 °C). Then, all blood samples were centrifuged at 3000 × g for 25 minutes. The plasma samples were transferred to plastic Eppendorf tubes and stored frozen at -83 °C until the analysis.

Cortisol measurements were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Immunospec Corporation, USA) according to the manufacturer's instructions and protocol previously published (Watts and Tindall, 1988). Total cholesterol, triglycerides and lactate were estimated based on the published enzymatic colorimetric methods (McGowan et al., 1983; Young et al., 1975; Field et al., 1966) using various commercially available reagent kits (Egyptian Company for Biotechnology, Cairo, Egypt). Total protein was assessed based on the reaction of copper with the peptide bonds of proteins in alkaline medium to form a pink to purple biuret complex in which colour intensity is directly proportional to the protein concentration in the sample (Gornall et al., 1949). Urea was measured according to modified urease berthlot method (Shephard and Mazzachi, 1983) and creatinine and was analyzed by the colorimetric method with deproteinization (Bowers and Wong, 1980).

### 2.4 Statistical analysis

The data were represented as means ± standard error of the mean. The differences between groups were statistically analyzed by one-way analysis (ANOVA) of variance followed by Duncan posthoc after testing the data for normality using Anderson Darling test and for variance homogeneity to be sure that the data are normally distributed and variances would be homogenous using SPSS program version 16 (SPSS, Richmond, VA, USA). Behavioural data was log transformed before analysis because it was not normally distributed. Differences were considered statistically significant at $P < 0.05$.

3   Results and discussion

3.1 Behavioural changes

3.1.1 Mobility behaviours

The overall relationships between the sites of S/C injections and mobility behaviors, including standing and walking were presented in Figure 1. The results show that S/C injection of saline or IVM at the neck region or
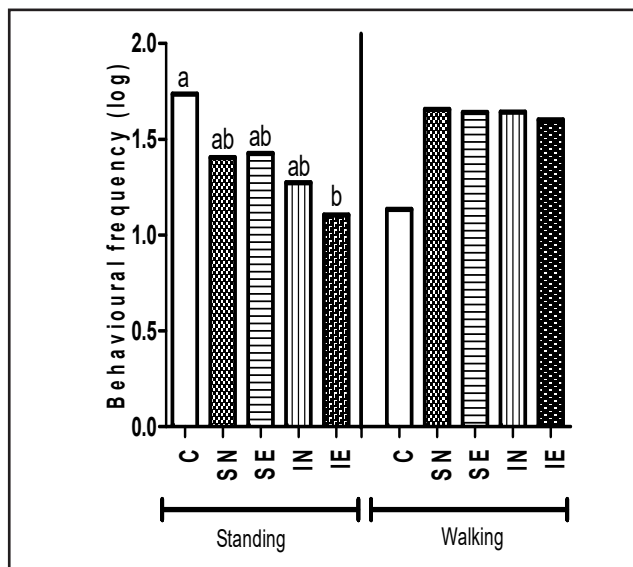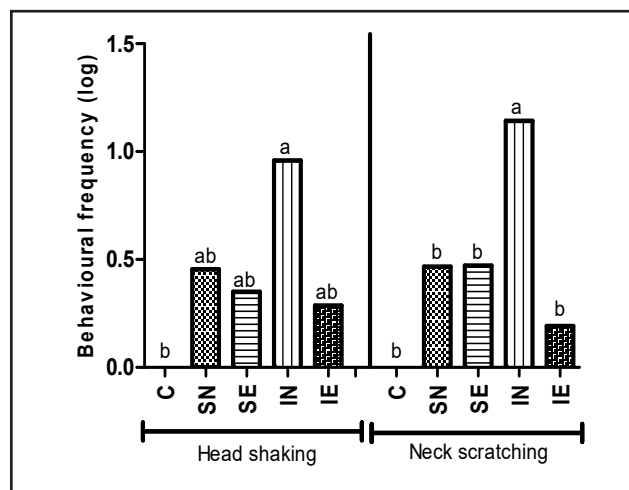
Figure 1 The frequency of standing and walking activities in the different experimental groups

Results are represented in logs of sheep activities frequency/30 minutes [of five sheep per group ($n = 5$)]

Different letters indicate a significant difference at $P <0.05$ (one-way ANOVA by Duncan posthoc)

C – control group; SN – group injected saline at the neck region; SE – group injected saline behind the elbow; IN – group injected ivermectin at the neck region; IE – group injected ivermectin behind the elbow

Figure 2 The frequency of head shaking and neck scratching in the different experimental groups

Results are represented in logs of sheep activities frequency/30 minutes [of five sheep per group ($n = 5$)]

Different letters indicate a significant difference at $P <0.05$ (one-way ANOVA by Duncan posthoc)

C – control group; SN – group injected saline at the neck region; SE – group injected saline behind the elbow; IN – group injected ivermectin at the neck region; IE – group injected ivermectin behind the elbow

behind elbow joint did not significantly ($P >0.05$) affect the walking activities. However, the results of standing activities show that S/C injection of IVM behind the elbow joint significantly ($P ≤0.05$) reduced the frequency of standing activities in sheep compared to the C group (Figure 1 and Table 2).

3.1.2 Head shaking and scratching the neck with hind legs

The results of the present study indicated that the neck scratching in the IN group was significantly ($P = 0.015$) higher than the rest of other groups, whereas head

shaking in this group was significantly ($P = 0.002$) higher than that of the C group only (Figure 2 and Table 2).

3.1.3 Pawing and biting fleece

Results show that S/C injection of IVM or saline behind the elbow joint significantly ($P = 0.025$) increased the pawing activities in comparison with the C group. However, the biting fleece activities have not been affected by the different treatments (Figure 3 and Table 2).

3.2 Biochemical changes

As shown in Table 3, plasma cortisol and glucose levels were significantly increased in the IN and IE groups when compared to the SN, SE and C groups. The SN and SE

Table 2 The frequency (Log) of different behavioural patterns in different experimental groups

Group / Pattern	C	SN	SE	IN	IE
Walking	1.134	1.655	1.639	1.642	1.601
Standing	1.736[a]	1.404[ab]	1.425[ab]	1.274[ab]	1.105[b]
Scratching the neck	0[b]	0.467[b]	0.472[b]	1.143[a]	0.192[b]
Pawing	0.320[b]	1.028[ab]	1.088[a]	0.585[ab]	1.325[a]
Biting fleece	0.320	0.492	0.219	0.184	0.524
Head shaking	0[b]	0.455[ab]	0.351[ab]	0.959[a]	0.287[ab]

Results are represented as means (logs) of sheep activities frequency/30 minutes [of five sheep per group ($n = 5$)]. Different letters indicate a significant difference at $P <0.05$ (one-way ANOVA by Duncan posthoc)

C – control group; SN – group injected saline at the neck region; SE – group injected saline behind the elbow; IN – group injected ivermectin at the neck region; IE – group injected ivermectin behind the elbow
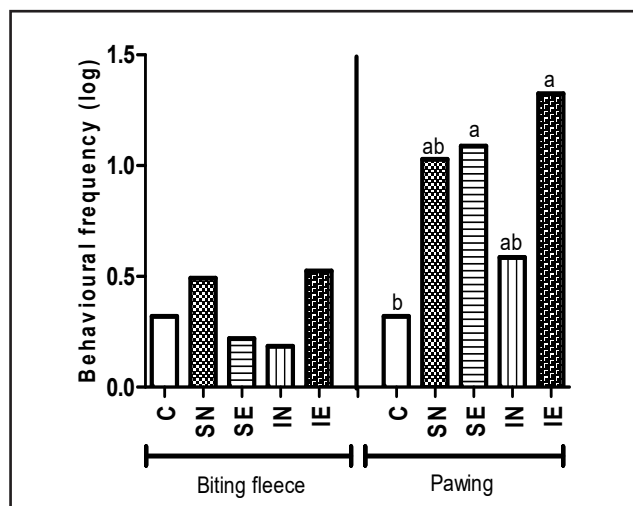
Figure 3 The frequency of biting fleece and pawing in the different experimental groups

Results are represented in logs of sheep activities frequency/30 minutes [of five sheep per group ($n = 5$)]
Different letters indicate a significant difference at P <0.05 (one-way ANOVA by Duncan posthoc)
C – control group; SN – group injected saline at the neck region; SE – group injected saline behind the elbow; IN – group injected ivermectin at the neck region; IE – group injected ivermectin behind the elbow

groups had a significant hypoproteinemia in comparison with C, IN and IE groups. A significant hypolactemia was observed in the SN, SE, IN and IE groups compared to the C group. The levels of other stress markers such as total cholesterol, triglyceride, urea and creatinine have not been changed significantly between all the experimental groups.

Up to our knowledge the acute effects of S/C injection on the behavioural activities and biochemical changes in sheep have not been investigated before. In the current study the behavioural and biochemical responses were assessed in sheep undergoing S/C saline or IVM injection either at the neck region or behind elbow. Our findings showed IVM injection behind the elbow significantly reduced standing activities and increased pawing activities compared to the C group. In contrast, IVM injection at the neck region significantly increased head shaking and neck scratching. On contrary, the differences between the C, SN and SE groups were not significant except for pawing activities which were significantly increased in the SE group. These findings may indicate that IVM injection is more stressful than saline injection and at the same time, the altered type of behavioural activities and severity of alteration depend on the site of S/C injection. The behavioural changes observed in the current study may be attributed to the fact that injection of S/C irritant substance like IVM may stimulate inflammation, and cause swelling, itching and pain (Pulliam and Preston, 1989). Moreover, the increased pawing and reduced standing activities in both SE and IE groups may be explained based on the knowledge that the injection site behind the elbow in sheep is richly supplied with superficial nervous plexus (Ghoshal and Getty, 1967) along with suggested noceiceptive abundance given a solid-based rationality for more susceptibility for pain expression, irritation and neurological distress following injection. During the observation session the ingestive (feeding, drinking and rumination) and resting activities were not considered. The sheep had not been observed long enough to see the ingestive behavioral changes associated with injections, since they were observed for only 30 minutes immediately post-treatment.

Higher cortisol and glucose levels following IVM injection at the neck or behind the elbow than the saline or C groups reveal stress-induced difference between the treatments with a higher degree of discomfort and pain in case of the former. The ability of IVM injection to

Table 3 The levels of biochemical stress indicators in the plasma of different experimental groups

Parameter / Group	C	SN	SE	IN	IE
Cortisol (nmol/l)	97.32±4.17[b]	64.14±2.02[b]	65.66±2.34[b]	180.83 ±9.24[a]	227.30±54.64[a]
Glucose (mmol/l)	3.55 ±0.11[b]	3.41 ±0.32[b]	3.86 ±0.24[b]	5.84 ±0.35[a]	6.14 ±0.51[a]
Lactate (mmol/l)	5.40 ±0.27[a]	1.67 ±0.10c	1.49 ±0.25c	2.70 ±0.20[b]	2.83 ±0.42[b]
Total protein (g/dl)	3.50 ±0.12[a]	2.08 ±0.07[b]	2.15 ±0.06[b]	3.66 ±0.10[a]	3.49 ±0.06[a]
Total cholesterol (mmol/l)	3.02 ±0.17	4.03 ±0.73	2.78 ±0.72	3.70 ±0.27	3.42 ±0.52
Triglyceride (mmol/l)	2.99 ±0.71	3.38 ±0.52	2.48 ±0.48	2.58 ±0.34	3.16 ±0.85
Urea (mmol/l)	5.41 ±0.15	3.93 ±0.09	4.17 ±0.32	4.40 ±0.72	5.26 ±0.81
Creatinine (mmol/l)	0.13 ±0.01	0.98 ±0.01	0.09 ±0.01	0.09 ±0.01	0.13 ±0.03

Results are represented as means ±SEM of five sheep per group ($n = 5$). Different letters indicate a significant difference at P <0.05 (one-way ANOVA by Duncan posthoc)
C – control group; SN – group injected saline at the neck region; SE – group injected saline behind the elbow; IN – group injected ivermectin at the neck region; IE – group injected ivermectin behind the elbow

augment cortisol secretion is compatible with a previous finding recorded in cow (Sadek and Shaheen, 2015) and could be attributed to pain-induced stimulation of the hypothalamic-pituitary-adrenal axis (Embi and Scherlag, 2014).

The hyperglycemia-associated IVM injection may be secondary to increased cortisol secretion during stress (Lakshmi and Sudhakar, 2010; Al-Qarawi, 2004). It is well known that cortisol inhibits insulin secretion through a genomic action in β cells, activates the key enzymes involved in hepatic gluconeogenesis, inhibits glucose uptake in peripheral tissues, and increases the glucogenic precursors as amino acids through muscle proteolysis (Lambilotte et al., 1997; Shpilbert et al., 2012; Dungan et al., 2009; Yoshioka et al., 2005; Larsen and Kristensen, 2013). In addition, epinephrine and norepinephrine may also contribute to the hyperglycemic status by enhancing glycolysis and gluconeogenesis. Moreover, stimulation of lipolysis by norepinephrine supplies glycerol which channels towards the glucogenic pathway (Marik et al., 2015). Contrary to these findings, Jin et al. (2013) reported that IVM displays antidiabetic activities in mice by reducing blood glucose and cholesterol levels, and also by improving insulin sensitivity in FXR dependent manner.

In contrast to previous data in the literature (Min et al., 2016; Tian et al., 2015), the marked hypolactemia following S/C injection of either saline or IVM represents a major surprise in this study and explanation of this outcome needs more investigation.

Hypoproteinemia in the saline-injected groups reflected the obvious debilitating action of stress (Khazadihe et al., 2014). The increase in the stress response-related hormones has adverse impacts on protein kinetics by inducing proteolysis (Paddon-Jones et al., 2006; Bessey and Lowe et al., 1993; Brillon et al., 1995). Owing to the increased protein turnover rate, greater inclusion of glucongenic amino acids could provide another metabolic building block for the gluconeogenic process in the liver (Chevalier et al., 2006).

4   Conclusions

The findings of the current study suggested that site of S/C injection of IVM significantly affected the behavioral activities of the sheep, while there were no differences in the biochemical indicators of stress between S/C injections of IVM at the neck region or behind the elbow joint. However, further investigation is still required to give information about the chronic effect of S/C injection at the neck region and behind the elbow comparing between the behavioural and biochemical indicators of stress before and after injection in the same animal.

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