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Transient apnoea in sheep: an alternative method for serial urine sample collection

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ABSTRACT

Urine samples are necessary in order to carry out many veterinary diagnostic and research protocols. In ovine, these samples are usually obtained through catheterization, but a transitory apnoea could be an easy way to obtain urine samples. This is achieved by preventing air from entering through the nostrils and mouth in sheep, for a short period of time, always less than 1 minute. The results of this transitory apnoea demonstrated that urine characteristics (pH, density and proteinuria) were not different from samples obtained by bladder catheterization. There were no differences in the blood parameters studied (cortisol, glycaemia, total proteins, haematocrit and total and differential leucocyte counts) either, when compared with the control group of catheterized animals, or with the measured values before or after sampling. In conclusion this system could be considered an easy and effective method to obtain urine samples, without causing stress to the sheep and avoiding the alterations to the blood and urinary parameters measured.

Key words: transient apnoea, urethral catheterization, urine, blood, urinalysis, sheep

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Introduction

Urine is the final product of blood renal filtration, and its analysis is one of the more useful procedures as an health or disease indicator at any animal life stage (LASO, 2002). A complete urinalysis consists of at least three phases: 1) physical evaluation (colour,

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turbidity, pH and density), 2) chemical analysis and 3) sediment microscopic examination, either directly or after sample centrifugation (MADEJÓN et al., 2006).

From the clinical point of view, pets (cats and dogs) are the species that most demand urinalysis, followed by sport horses, while farm species are those that cause the least interest (KASARI, 2002). However, some research protocols carried out on farm animals require the collection of data on the metabolic state of the animals by analysing blood and urine (DEL VALLE et al., 1983; RADOSTITS et al., 2007).

When a routine urine analysis is performed, a single sample is used, but in some cases a marked circadian rhythm in excretion and the need to quantify certain substances eliminated by the kidneys make it necessary to determine the amount of substance excreted over 24 hours (KASARI, 2002). In veterinary medicine, unlike in human medicine, obtaining urine samples serially or continuously is not an easy task, resulting, therefore, in it being very cumbersome to collect them throughout the day.

There are different urine collecting techniques and the choice of one or another procedure depends on the animal species, their state or health, and the type of analysis performed (KASARI, 2002). The three main urine collecting techniques are the use of a clean and dry sample container, urethra catheterization and the cystocentesis, depending on the necessity for a sterile or non-sterile sample (WILLARD and TVEDTEN, 2004; RAMOS and FERRER, 2007). An i.v. injection of furosemide (0.5-0.8 mg/kg BW) produces urination in most animals in about 20 minutes, but its composition is drastically altered by the diuretic (RADOSTITS et al., 2007).

In sheep, the aim of the research and experimental design was to determine a method used for urine sampling (GONZALEZ-MONTAÑA et al., 1998; PRIETO-MONTAÑA and GARCÍA-PARTIDA, 1999). In females of this species urethral catheterization is usually recommended when a single sample is needed, with catheter removal once the sampling is performed (KASARI, 2002). However, in some research protocols it is necessary to obtain serial urine samples, within certain time intervals.

Also it has to be taken into account whether it is necessary to measure the volume of urine passed in a period of time, when other techniques have to be used. Thus metabolic cages have been employed, which allow urine collection in suitable containers (DELIBERTO and URNESS, 1995; LOUVANDINI and VITTI, 1996), a bladder retention catheter with a collection bag (McKINLEY et al., 2000) or a peristaltic pump, which requires the placement of a urinary catheter and limitation of the animal´s movements (TEBOT et al., 2002). Furthermore, rams usually cannot be catheterized because of the inaccessibility of the penis and the small diameter of the urethra (RADOSTITS et al., 2007).

Under field conditions, a technique used by veterinary practitioners for the collection of sheep urine samples is transient apnoea, which is based on temporary occlusion of the

mouth and nostrils of the sheep by the hands of an operator, while another collects the excreted urine in a suitable container. There are experimental studies that have used this technique as a method to induce urination in sheep and rams (KASARI, 2002; BENECH, 2007; RAMOS and FERRER, 2007).

In humans, different mechanisms have been postulated to explain the urine output that occurs with transient apnoea, among which are a decrease in renin-angiotensin-aldosterone, an increase in plasma levels of atrial natriuretic peptide (ANP) secondary to hypoxemia (UMLAUF and CHASENS, 2003; OZTURA et al., 2006) and changes in intrathoracic pressure (OZTURA et al., 2006; KANG et al., 2012).

None of the veterinary authors reviewed provided details regarding the mechanism by which sheep (both male and female) urinate after a few seconds of apnoea, nor do they refer to whether this method produces alterations in the characteristics of the sample obtained, nor its repercussions on sheep haematology and blood chemistry.

Materials and methods

The experimental procedures were undertaken under field conditions at the Veterinary Faculty, University of La República, Libertad, Department of San José, Uruguay (34º 38´S; 56º39´W), upon approval of the local animal welfare committee (Ethics Committee on the Use of Animals, CEUA).

Fourteen Corriedale adult ewes, 4-6 years of age, were selected at random from a flock maintained under the usual production conditions, with body condition scores between 2.5 and 3.5 on a 1-5 scale (MARTIN and AITKEN 2002), and average body mass 52.6 ± 4.4 kg. The ewes were randomly divided into two groups: Apnoea group (Ap Group, n = 7) and Urethral Catheterization group (UC group, n = 7). The experimental protocol was carried out over 24 hours, from 6 pm of the first day to 6 pm of the second day.

In the Apnoea group (Ap) urine samples were obtained by the method of transient apnoea (forced apnoea), by occluding the nostrils with both thumbs, and the mouth with the palms of the operator's hands, until the sheep urinated in a clean, dry, graduated glass container. This technique was repeated every 6 hours for 24 hours (six total samples, where sample 1 was the first). In each animal, the apnoea time lapsed until urination started and the urine volume passed was recorded. Whenever urine samples were collected, two blood samples from the jugular vein were taken, one before starting the apnoea (sample Ap-Pre) and another immediately after urination (sample Ap-Post).

In sheep in the Urethral catheterization Group (Group UC) a Foley bladder catheter (Rusch, nº 14) was inserted via the urethra of the ewe and attached to a sterile polyvinyl bag for cumulative urine collection (WONG et al., 2001). As with the Ap group, urine samples were taken every 6 hours over 24 h. Prior to the urine sample collection a blood

sample from the jugular vein was taken. Thirty minutes before taking each urine sample, the content of the collection bags was emptied in order to obtain fresh urine samples.

In all animals, before the collection of samples, rectal temperature was recorded using a digital thermometer.

Urine samples collected were analysed immediately by strips (Uriscan ® Pro using Gen 10 SGL strips), evaluating the pH, urine density and total proteins.

Blood samples were collected with a 10 mL syringe with 18G needles and divided into 5 mL fluorinated EDTA tubes, for determination of glucose, haematocrit value and total and differential leukocyte count (WBC), and dry tubes (5 mL) to obtain plasma. This sample, centrifuged at 2,200×g at 25 °C for 10 minutes, was used for determination of total protein and cortisol.

The total protein value was determined using a Total Protein liquicolor, photometric colorimetric test, Biuret method (Human®), using a digital photocolorimeter (Humalyser Junior) at λ = 520 nm.

Cortisol was quantified by radioimmunoassay (RIA, DPC, Coat-A-Count, Los Angeles, USA), with a coefficient of variation of 5 % intra-assay and a detectability level of 3 ng/mL, defined as 90 % of buffer control). Blood glucose value was determined using an enzymatic colorimetric method (Glucose Liquicolor; Enzymatic colorimetric test, GOD-PAP method (Human®) with the previously defined photocolorimeter a λ = 500 nm.

The haematocrit value was determined by the microhaematocrit method, centrifuging in a microcentrifugue (Centralit, JP Selecta, Barcelona, Spain) at 14.000×g for 10 min, while the leukocyte values were determined using a Celltac counter (Nihon Kohden Corporation, model MEK S103K, Tokyo, Japan. A blood smear was made and was used to calculate the leucocyte differential count by means of manual reading, May Grunwald-Giemsa's stain, ×1000, counting 200 leucocytes differentially, and expressing the result as a percentage (ALONSO et al., 1997).

Statistical analyses were performed using Statistica 6.0 software (Stat Soft Inc, 1995). All values are presented as mean ± standard deviation (χ ± SD). After checking the normal distribution of the data found, we used the *t*-Student test for independent groups to compare Apnoea group results *vs* Catheterized group results. The *t*-test for dependent groups was used to compare the blood samples results of the Apnoea group, before and after urine sampling. Differences were considered significant when $P<0.05$.

Results

The apnoea time required for obtaining the urine samples in the Apnoea group animals was less than one minute in all cases. Table 1 shows the time in seconds (mean ± SD) that it took for the animals to urinate and volume in mL (mean ± SD) collected in each sample.

Table 1. Time necessary to obtain urine sample using apnoea.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	Day 1, 18:00 h	Day 2, 24:00 h	Day 2, 6:00 h	Day 2, 12:00 h	Day 2, 18:00 h
Apnoea time (sec)	30.4 ± 18.8	29.0 ± 12.4	44.9 ± 15.1	34.2 ± 10.2	35.4 ± 10.5
Volume (mL)	95.6 ± 45.3	86.2 ± 34.3	97.1 ± 42.1	110.3 ± 47.1	90.7 ± 30.4

Mean apnoea time necessary, in each sampling, to achieve urine emission and volume in the Ap group animals (mean ± SD).

Fig. 1. Urination in sheep after forced apnoea.

Rectal temperature at the start of the test was 39.4 ± 0.4 ºC in the Catheterized group and 39.9 ± 1.0 ºC in the Apnoea group, while at 24 hours it was 40.3 ± 0.8 and 39.4 ± 0.2 ºC, respectively. Body temperature did not change significantly during the protocol either within the same group or between the two experimental groups.

At the start of the experimental protocol none of the parameters analysed in the urine showed any significant differences between the groups (Apnoea *vs* Catheterized). As shown in Table 2, urine pH and density did not show differences either intragroup or between groups, within the 24 hour period of this protocol. However, in the Catheterized group significantly ($P<0.01$) higher proteinuria was recorded 6 hours after the catheter had been placed, and this difference was maintained until the end of the protocol. In one

of the sheep of the catheterized group the presence of blood in the collection bag was observed macroscopically from sample 4 (at 18 hours from the beginning of the protocol) and continued until the end. Therefore urine samples numbers 4 and 5 from this animal were not considered in the evaluation of the urinary parameters.

Fig. 2. Sheep with bladder catheter and polyvinyl bag

The evolution of the blood variables studied is presented in Fig. 3. The haematocrit values (Fig. 3a) did not show any differences between groups in the initial sample, with 30.3 ± 6.0 % in the UC group, 34.5 ± 4.6 % in the Ap-Pre and 35.4 ± 1.7 % in the sample after the sheep apnoea period (Ap-Post). There were no significant differences in this blood parameter throughout the experimental protocol. No differences were observed in total leukocyte count throughout the test, either between experimental groups or in the samples before and after, in the Apnoea group (Fig. 3b). The results of the differential count are presented in Table 3 and they did not show any significant differences between groups or between the initial sample (sample 1) and the sample measured at the end of the experiment (sample 5).

Table 2. Urinary parameters in samples obtained by catheterization or apnoea

Parameter	Group	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
		Day 1, 18:00 h	Day 2, 24:00 h	Day 2, 6:00 h	Day 2, 12:00 h	Day 2, 18:00 h
pH	UC	6.6 ± 1.3	6.0 ± 0.9	6.9 ± 0.6	6.4 ± 0.7	6,0 ± 0,5
	Apnoea	6.5 ± 0.7	5.4 ± 0.5	6.1 ± 0.9	6.6 ± 1.0	7,4 ± 0,7
Urine density	UC	1.019 ± 0.004	1.027 ± 0.002	1.026 ± 0.002	1.025 ± 0.003	1.026 ± 0.004
	Apnoea	1.021 ± 0.002	1.019 ± 0.007	1.024 ± 0.002	1.018 ± 0.007	1.019 ± 0.004
Total proteins (g/L)	UC	0.286 ± 0.049	1.471 ± 1.075*	1.186 ± 0.841*	1.443 ± 1.113*	2.443 ± 0.335*
	Apnoea	0.143 ± 0.035	0.140 ± 0.038	0.121 ± 0.135	0.100 ± 0.100	0,257 ± 0,035

Evolution of pH, density (mean ± SD, g/L) and total proteins (mean ±SD, g/L) in urine samples obtained by catheterization or apnoea. Different letters indicate significant differences (*P<0.01). UC - urethral catheterization

Table 3. Leukocyte counts of animals with urine collection by means of apnoea or catheterization during the experimental protocol

Group	Neutrophils		Lymphocytes		Eosinophils		Monocytes		Basophils	
	At first, sample 1	At end, sample 5	At first, sample 1	At end, sample 5	At first, sample 1	At end, sample 5	At first, sample 1	At end, sample 5	At first, sample 1	At end, sample 5
UC	34.3 ± 3.2	29.3 ± 2.5	57.0 ± 5.0	63.0 ± 4.3	4.7 ± 1.5	3.9 ± 0.4	2.3 ± 1.2	1.3 ± 0.8	0.5 ± 0.3	1.0 ± 0.4
Ap-Pre	35.5 ± 1.3	40.8 ± 2.8	52.0 ± 0.5	51.8 ± 3.6	5.8 ± 1.8	4.2 ± 0.4	0.5 ± 0.5	0.2 ± 0.0	1.0 ± 0.5	0.7 ± 0.5
Ap-Post	37.8 ± 4.3	40.2 ± 2.5	54.8 ± 1.3	51.5 ± 4.8	3.5 ± 0.5	4.2 ± 1.3	1.2 ± 0.3	2.0 ± 1.0	1.2 ± 0.6	0.5 ± .03

Leukocyte counts in UC and Apnoea group before (Ap-Pre) and after (Ap-Post) urine sampling, baseline (Sample 1) and end of the experiment (Sample 5). The result is expressed as a percentage (%). Between Sample 1 and 5, 24 h are passed. UC - urethral catheterization

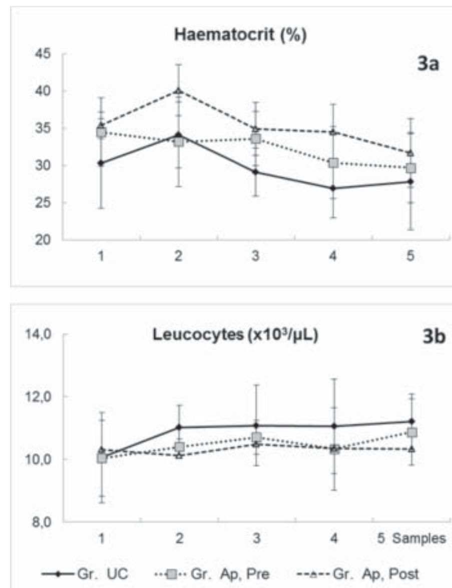

Fig. 3. Sheep haematological parameters in Ap and UC groups.

We did not find any significant variations in the blood biochemical parameters studied. The initial blood glucose value did not differ between groups, with concentrations of 3.30 ± 0.65 mmol/L in UC group and 3.29 ± 0.37 mmol/L in the Ap-Pre and 3.57 ± 0.71 mmol/L in the sample Ap-Post. Although glucose value increased in both groups at the end of the protocol (sample 5), this increase was not significant with respect to its initial value (Fig. 4a). In the same way, no differences in total protein were recorded between groups in the initial sampling (76.7 ± 7.9 g/L) in UC group, 78.5 ± 9.1 g/L in the Ap-Pre, and 88.1 ± 6.6 g/L in the Ap-Post. This parameter did not change significantly during the experimental protocol (Fig. 4b). There were no significant differences in blood cortisol at the start of the experimental protocol between the UC group and the Ap group, or in the latter group before and after urination (66.22 ± 39.73 nmol/L) in UCG, 43.32 ± 33.38 nmol/L in Ap-Pre and 54.63 ± 13.24 nmol/L in Ap-Post). However, after 24 hours the UC group recorded blood cortisol levels significantly ($P < 0.05$) higher (78.63 24.28 nmol/L) than those of the Ap-Pre values (44.70 ± 29.25 nmol/L), although this difference was not recorded when compared with Ap-Post values (61.53 ± 29.52 nmol/L), see Fig. 4c.

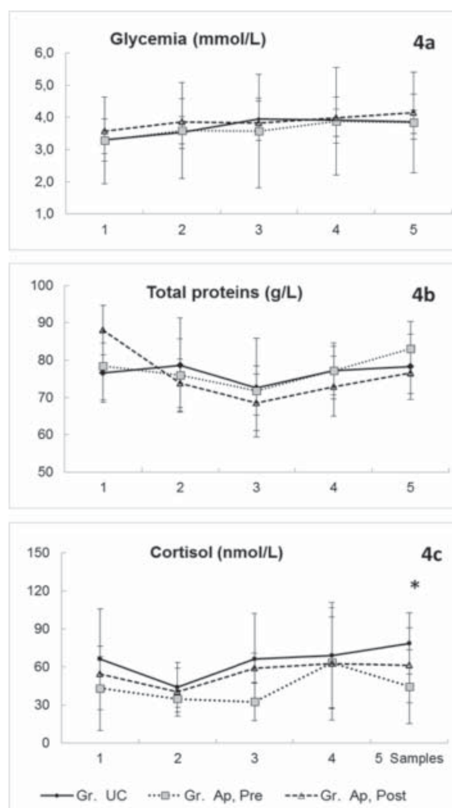

Fig. 4. Sheep biochemical parameters in Ap and UC groups. (* indicate significant differences, P<0.05).

Discussion

The blood parameters evaluated in the initial samples (haematocrit, glycaemia, total protein and cortisol) were within the normal range for sheep raised in similar conditions (ALONSO et al., 1997; RADOSTITS et al., 2007; MARTIN and AITKEN et al., 2002; RAMOS and FERRER, 2007). Neither glucose nor total blood proteins showed significant differences between experimental groups or variations throughout the protocol.

The blood cortisol showed a significant increase in the UC group compared with the Apnoea group, especially with Ap-Pre in the last sample (sample 5). VAN LIER (2003) recorded an increase of cortisol in animals in which jugular venipuncture was performed, when this handling procedure took longer than a minute, due to the stress of the necessary

restraining. Furthermore SANGER et al. (2011) found a significant increase in sheep cortisol after 24 h of being subjected to acute stress (shearing). The duration of apnoea handling in all animals always lasted less than a minute, which could explain that cortisol values in this group did not show any significant increase. Moreover the increase recorded in the UC group in blood cortisol may be due to urethral mucosal irritation caused by the catheter, acting as a stressor. This is also evidenced by the marked increase in the urinary protein values recorded in this group.

While it is suggested that there is a circadian variation in cortisol secretion, with its highest peak discharge at midnight and lowest in the morning (FULKERSON and TANG, 1979; PARRAGUEZ et al., 1989), in our protocol we did not observe such as hormonal pattern, possibly because the sampling frequency was not sufficient to detect such a phenomenon. On the other hand, ALILA-JOHANSSON et al. (2003) found that, in the goat, this variation has a marked effect due to external factors, so that light conditions have no immediate effects.

Although TORNQUIST and RIGAS (2010) after catheterization and forced apnoea, and SANGER et al. (2011) at 24 h of shearing sheep claim to have found stress leukogram (mild mature neutrophilia, lymphopenia, eosinopenia and variable monocytosis), in this protocol there were no such changes. Stress leukogram could be observed in these ruminants after handling, due to the excitation and the fear that the sheep suffers (TORNQUIST and RIGAS, 2010). Total and differential leukocyte counts did not show any variations during the test, and the values were also consistent with the literature reviewed (ALONSO et al., 1997; RADOSTITS et al., 2007; MARTIN and AITKEN, 2002; RAMOS and FERRER, 2007). The differences between our results and the leukogram variations found by the authors mentioned above could be due to the fact that transient apnoea *per se* is a factor that produces a small stress reaction compared with the magnitude of shearing or catheterization.

The pH and urine density values were considered normal for the species in the initial sample (RADOSTITS et al., 2007). However, according to WALDROP (2008), the definition of “normal” is difficult to determine in urine as there are different factors intra and extra-individual which can modify the values of solutes and water in a healthy animal sample. On the other hand, according to RAMOS and FERRER (2007) renal disorders in sheep produce minor variations in pH (7.5-8.4), always higher than the values found in this study.

The urine density values recorded were in accordance with the values of specific density (1.028 to 1.032) reported for most species (RADOSTITS et al., 2007) and that, in the opinion of RAMOS and FERRER (2007), indicates normal urine, which means that the renal tubules are fully functional and can remove solutes (KASARI, 2002). The presence of pus, blood or other solids in the urine increases the specific density (KASARI, 2002),

a fact that did not occur in our sheep, except in one animal in which blood was detected macroscopically.

We think that the presence of blood in one of the urine collection bags was due to the rupture of the submucosa bladder or lower urinary tract vessels caused by the catheter, which increases the stress hypothesis due to mucosa tract irritation. Haematuria, if it is due to post-renal causes, can originate from vascular damage, and so special care must be taken not to traumatize the urethral mucosa and not to cause urinary tract infections with the catheter used (RADOSTITS et al., 2007).

Normal urine contains small amounts of protein, which are insufficient to be detected using standard testing, but proteins may be present in hemoglobinuria and haematuria, and when urinary tract infections are present or when plasma proteins reach the urine due to increased glomerular permeability (RADOSTITS et al., 2007). The urinary protein values were normal in the initial sample of both groups, increasing rapidly after the second sample in the UC group. It is possible that this increase was due to urethral mucosa irritation (ASCHBACHER, 1970; KURIEN et al., 2004).

Metabolic male restraining stalls for cattle, sheep, goats and swine, and collecting urine and faeces separately are available. In these stalls, cross-contamination of excreta occurs in varying degrees. Other systems, such as indwelling catheters and apparatus attached to animals using a harness arrangement, have been developed to minimize or prevent cross contamination of excreta. However, these methods sometimes cause discomfort for animals or bladder infection, or the apparatus does not remain in place for extended urine collection (ASCHBACHER, 1970; KURIEN et al., 2004). In some experimental protocols sheep have to be maintained under grazing conditions and require urine samples serially collection. Under these conditions the use of these devices is very difficult, so many researchers use transient apnoea, which has proven to be efficient, rapid and repeatable.

The mechanism by which sheep urinate when apnoea is produced has not yet been elucidated. However, sheep transient apnoea is a similar situation to that occurring in humans with obstructive sleep apnoea, which can produce nocturia. In these patients the urge to urinate is attributed to apnoea or hypopnea (MARGEL et al., 2006). In this way, 76 % of night time urination episodes were immediately preceded by an episode of obstructive apnoea (OZTURA et al., 2006), and also the greater the number of obstructive apnoea events, the greater the urine volume (UMLAUF and CHASENS, 2003). ARAI et al. (1999) observed the existence of urodynamic changes corresponding to acute respiratory efforts during obstructive apnoea.

Obstructive sleep apnoea is characterized by repeated episodes of complete or partial upper airway occlusion, causing pauses in breathing, or apnoea, in which the patient attempts to inhale air, but, as the upper airway remains closed, a negative intrathoracic pressure is generated, causing the heart to receive a false signal of volume overload

(KANG et al., 2012). In response to this signal, hormonal secretion of ANP (atrial natriuretic peptide) is increased, which therefore increases urinary output (CHASENS and UMLAUF, 2003; MARGEL et al., 2006; FITZGERALD et al., 2006). De BOLD et al. (1981) indicates that this protein, which normally circulates through the bloodstream, could exert a prompt and strong natriuretic effect. The ANP is a short-acting substance with 2 ± 3 min circulating half-life (UMLAUF and CHASENS, 2003). This cardiac hormone increases sodium and water excretion and also inhibits other hormonal systems that regulate liquid volume, as vasopressin and renin-angiotensin-aldosterone complex (UMLAUF and CHASENS, 2003).

Regardless of the theories described in human medicine, to our knowledge, when submitted to transient apnoea, sheep eliminated the urine already contained in the bladder and emptying occurs as a result of intraabdominal pressure changes produced by respiratory effort. However, ARAI et al. (1999) postulated that the increased intraabdominal pressure caused by respiratory effort against a closed airway is insufficient to explain the mechanism of enuresis (uncontrolled urination) in adult human patients with obstructive sleep apnoea, suggesting that these patients present a significant increase in nocturnal urine production and anatomical altered urethra.

Based on our results, we conclude that the use of transient apnoea for obtaining sheep urine samples seems to be an easier handling procedure for the investigator, harmless to the animal and does not alter the urine or blood parameters. Taking all into account, this proves to be a simple and effective method to use in research protocols where it is necessary to obtain serial urine samples, when quantification of total urine volume produced is not required.

In conclusion we can say that the transient forced apnoea technique, covering the sheep's mouth and nostrils for a short period of time (always less than a minute), causes animal micturition without changes in the urine composition or blood conditions of these animals. Therefore this technique could be valid for both clinical and research, on account of its characteristics of being easy, fast and safe, both for the practitioner and the animal.

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BENECH, A., L. CAL-PEREYRA, S. DA SILVA, J. ACOSTA-DIBARRAT, J. R. GONZÁLEZ-MONTAÑA: Prolazna apneja u ovaca: alternativna metoda za uzastopno uzimanje uzoraka mokraće. Vet. arhiv 85, 293-307, 2015.

SAŽETAK

Prikupljanje uzoraka mokraće nužno je radi provođenja mnogih veterinarskih dijagnostičkih postupaka i znanstvenih istraživanja. Ti se uzorci u ovaca obično uzimaju kateterizacijom. No, prolazna apneja može biti jednostavan način uzimanja uzoraka mokraće u ovaca. To se može postići zatvaranjem nosnica i gubice u kratkom vremenu, uvijek manje od jedne minute. Rezultati pretrage mokraće prikupljene nakon prolazne apneje pokazali su da značajke mokraće (pH, gustoća i proteinurija) nisu bile različite od onih dobivenih pretragom uzoraka uzetih kateterizacijom mokraćnog mjehura. Nije bilo razlike u pretraženim krvnim pokazateljima (kortizolu, glikemiji, ukupnim proteinima, hematokritu, ukupnim leukocitima i diferencijalnoj krvnoj slici) u usporedbi s kontrolnom skupinom kateteriziranih životinja, a ni u usporedbi s izmjerenim vrijednostima prije i nakon uzimanja uzoraka. Može se zaključiti da je opisani način uzimanja uzoraka jednostavan i učinkovit bez izazivanja stresa u ovaca i ne dovodi do promjena u pokazateljima krvi i mokraće.

Ključne riječi: prolazna apneja, kateterizacija uretre, mokraća, krv, ovce

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