

## RESEARCH ARTICLE

# The Effect of Midazolam and Its Reversal Flumazenil on Sedative and Cardiopulmonary Variables in Sheep

Ünal YAVUZ <sup>1,a</sup>(\*) Kerem YENER <sup>1,b</sup> Adem ŞAHAN <sup>2,c</sup><sup>1</sup> Department of Surgery, Faculty of Veterinary Medicine, Harran University, TR-63200 Şanlıurfa - TURKEY<sup>2</sup> Department of Internal Medicine, Faculty of Veterinary Medicine, Harran University, TR-63200 Şanlıurfa - TURKEY  
ORCID: <sup>a</sup> 0000-0002-4981-2355; <sup>b</sup> 0000-0002-6947-0356; <sup>c</sup> 0000-0002-4779-0893

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## Abstract

The aim of this study was to investigate the effects of flumazenil antagonism after midazolam administration on sedative and cardiopulmonary variables in ewes. Six Awassi ewes were studied at least 14 days apart. The ewes were randomly divided into two groups as midazolam/saline (MDS) and midazolam/flumazenil (MDF). Hemodynamic values, blood gas, metabolic and electrolyte variables and reflex values were determined in both groups before midazolam (0.6 mg/kg, IV) administration (baseline) and after the 5<sup>th</sup> (T5) and 25<sup>th</sup> min (T25) of administration. At T25, saline was injected into the MDS and flumazenil (0.02 mg/kg, IV) into the MDF. The same measurements were repeated at the 5<sup>th</sup> min (T30), 35<sup>th</sup> min (T60), and 65<sup>th</sup> min (T90) of saline and flumazenil applications. Midazolam produced deep sedation and a significant increase in reflex scores in both groups at the indicated times (P<0.05). It caused an increase in heart rate (HR), a decrease in systemic and diastolic arterial pressure (SAP, DAP), hypoventilation, mild respiratory acidosis, and adverse effects on metabolic variables at a transient and clinically acceptable level. It was observed that these effects returned to baseline within 5 min with the use of flumazenil in MDF. Flumazenil (0.02 mg/kg, IV) was sufficient to reverse cardiopulmonary adverse effects within 5 min during deep sedation by high-dose midazolam (0.6 mg/kg, IV) in ewes.

**Keywords:** *Cardiopulmonary, Flumazenil, Midazolam, Sedation, Sheep*

## Koyunda Midazolam ile Reversali Flumazenilin Sedatif ve Kardiyopulmoner Değişkenlere Etkisi

### Öz

Bu çalışmada koyunlarda midazolam uygulamasının ardından flumazenil antagonizmasıyla sedatif ve kardiyopulmoner değişkenlerdeki etkilerin değerlendirilmesi amaçlanmıştır. Çalışmada en az 14 gün aralıkla İvesi ırkı 6 erkek koyun kullanıldı. Koyunlar midazolam/izotonik serum (MDS) ve midazolam/flumazenil (MDF) olarak rastgele 2 gruba ayrıldı. Her iki grupta midazolam (0.6 mg/kg, IV) uygulamasından önce (baseline) ve uygulamanın 5. (T5) ve 25. dk'sında (T25) hemodinamik değerler ile kan gazı, metabolik ve elektrolit değişken değerleri belirlendi ve refleks değerleri ölçüldü. T25'de MDS'ye izotonik serum solüsyonu, MDF'ye flumazenil (0.02 mg/kg, IV) enjekte edildi. İzotonik serum ve flumazenil uygulamalarının 5. dk (T30), 35. dk (T60) ve 65. dk (T90)'larında aynı ölçümler tekrarlandı. Belirlenen zamanlarda midazolam her iki grupta derin sedasyon ve refleks değerlerde önemli yükselme sağladı (P<0.05). Kalp vuruş sayısında (HR) artış, sistemik ve diastolik arteriyel basınç (SAP, DAP)'da azalma, hypoventilasyon, hafif şekilde respiratorik asidoz ve metabolik değişkenler üzerinde geçici ve klinik olarak kabul edilebilir düzeyde olumsuz etkilere neden oldu. MDF'de flumazenil uygulanmasıyla bu etkilerin 5 dk içinde başlangıç seviyesine döndüğü görüldü. Flumazenil (0.02 mg/kg, IV) koyunlarda yüksek doz midazolamın (0.6 mg/kg, IV) oluşturduğu derin sedasyon ile kardiyopulmoner olumsuz etkilerin 5 dk içinde tersine çevrilmesine yeterli oldu.

**Anahtar sözcükler:** *Flumazenil, Kardiyopulmoner, Koyun, Midazolam, Sedasyon*

## INTRODUCTION

Thanks to their skeletal growth in a short time, their similarity to humans in weight, size, and anatomical and physiological aspects, ewes are a frequently used large

animal model for *in vivo* biomedical studies, especially in surgical sciences <sup>[1-3]</sup>. Between 2015 and 2017, 60.158 ewes were used for experimental and scientific purposes in the EU Member States <sup>[4]</sup>. The complexity of surgical research, preoperative preparation, anesthetic protocols,

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### (\*) Corresponding Author

Tel: +90 414 318 3921 Cellular phone: +90 505 311 4310

E-mail: unalyavuz@harran.edu.tr (Ü. Yavuz)



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and monitoring, and postural changes (lateral position) can lead to significant morbidity and mortality in ewes [2,5]. These procedures require close monitoring of anesthesia and hemodynamic parameters [3].

Sedatives are commonly used in small ruminants. The most commonly used sedatives for this purpose are  $\alpha$ -adrenoceptor agonists. However,  $\alpha$ -adrenoceptor agonists have many side effects such as hypoventilation and hypoxemia as well as cardiopulmonary depression [5-8]. Small ruminants are more vulnerable to the pulmonary side effects of  $\alpha$ -adrenoceptor agonists. Since they are thought to have minimal adverse effects on the cardiovascular and respiratory systems, many anesthesiologists recommend benzodiazepines (BDZs) as an alternative sedative agent in ewes [8-11].

Midazolam, a benzodiazepine with a high margin of safety, produces anxiolytic and muscle relaxant effects by interacting with anticonvulsant and sedative effects, as well as glycine-mediated inhibitory routes in the brain and spinal cord. It acts indirectly by optimizing an existing physiological inhibitory route rather than directly suppressing neuronal activity, and rapidly crosses the blood-brain barrier [12-15]. While its low doses (0.05-0.1 mg/kg IV) can be used to induce mild sedation during painless procedures (e.g., X-ray, ultrasound) in ewes, major surgery can be performed by adding local analgesia to the high-dose (0.4 mg/kg and above, IV) midazolam injection or midazolam-opioid combination [8,16-18]. One of its main advantages is that its effects can be reversed by using flumazenil, a benzodiazepine antagonist [8,14]. Flumazenil has a high affinity for the benzodiazepine receptor site of the GABA<sub>A</sub> receptor and reverses the effects of benzodiazepines throughout the central nervous system (CNS) [19,20]. This increases the importance of flumazenil as a clinical antagonist in ewes.

The aim of this study was to investigate the effects of reversal administration of flumazenil (0.02 mg/kg, IV) in ewes after high-dose midazolam (0.6 mg/kg, IV) administration on sedative effects and cardiopulmonary, arterial blood gas, metabolic, and electrolyte variables. We hypothesized that in healthy ewes, IV administration of midazolam would produce a rapid and deep induction of sedation with minimal changes in cardiopulmonary variables, followed by the administration of flumazenil, these changes would soon return to baseline values.

## MATERIAL AND METHODS

### Ethical Statement

The study was conducted at Experimental Animal Implementation and Research Center (HDAM) Farm Animals Unit with the permission of Harran University Animal Experiments Local Ethics Committee (HRU-HADYEK) with the number 2020/003/11.

### Animals

Six male Awassi ewes aged 6-8 months and weighing 34-41 kg (38.6±2.6 kg) were studied. The health status of the ewes to be included in the study was checked by clinical examination and complete blood count. One month prior to the study, ewes were divided into pairs or groups of three and housed in wide boxing. During this time, they were fed *ad libitum* with hay and concentration. Because the ewes were extremely anxious and restless when separated from flock, they were kept in pairs in the experimental room for 1 h at least 2 times per week for 2 weeks before the start of the study to minimize stress, and they were allowed to acclimate to environmental conditions. Meanwhile, physiological changes in the ewes were measured and electrocardiogram (ECG) leads were introduced in the baseline apex configuration for habituation. The hair at the withers was shaved and ewes were adapted to the application of a clamp modified to the withers and metacarpus. For 12 h prior to each experiment, ewes were not allowed to eat food, but water was available *ad libitum*.

### Instrumentation

The experiment was conducted at a constant ambient temperature of 22°C, in a quiet, air-cooled closed cabin with fluorescent lighting, without attempting to maintain the body temperature of the sheep. To ensure standardization, injections were performed during the light phase of the cycle between 09:00 and 11:00 am. In the experimental room, two ewes were placed together in adjacent cabins of sufficient width to perform the procedure. Catheterization (Bıçakçılar®, Istanbul, Turkey) was performed with a 20 G branule in the left jugular vein for drug injections, with a 22 G branule in the left auricular artery for blood sampling, and with a 22 G branule in the right auricular artery for IBP measurement. 5% lidocaine (Sandoz® ilaç, Istanbul, Turkey) was pomaded to the auricular artery 45 min before catheter insertion to facilitate catheter placement. Just before the injection of midazolam (baseline, T0), a multi-parameter monitor (Mindray® uMEC12VET, Shenzhen Mindray Bio-Medical Electronics Co, Shenzhen, China) was used to monitor values for hemodynamic parameters such as heart rate (HR, beats per minute), systemic arterial pressure (SAP, mmHg), diastolic arterial pressure (DAP, mmHg), respiratory rate (RR, breaths per minute), Oxy-hemoglobin saturation (SpO<sub>2</sub>, %), end-tidal CO<sub>2</sub> (EtCO<sub>2</sub>, %), and body temperature (BT, °C) values with ECG lead II. SpO<sub>2</sub> was monitored using a pulse oximeter with a clip placed in the ear, and BT was monitored via an electronic rectal probe from the rectum. Epoc® Blood Analysis device (Epocal Inc, Ottawa, Canada) and the BGEM® Test Card kit (Epocal Inc, Ottawa, Canada) were used to measure the values of arterial blood gas and metabolic and electrolyte variables such as arterial pH, partial pressure of carbon dioxide in arterial blood (pCO<sub>2</sub>), partial pressure of oxygen in arterial blood (pO<sub>2</sub>), bicarbonate (HCO<sub>3</sub><sup>-</sup>), base excess

(BE), hematocrit (Hct), hemoglobin (Hb), lactate, glucose, sodium (Na<sup>+</sup>), potassium (K<sup>+</sup>), chloride (Cl<sup>-</sup>).

### Experimental Design

Ewes were randomly divided into 2 groups as MDS and MDF. The study was conducted in two phases with a minimum interval of 14 days. The MDS group was studied first followed by the MDF group. In both groups, the hemodynamic changes were recorded at the 5<sup>th</sup> (T5) and 25<sup>th</sup> min (T25) after slow IV injection of midazolam (Zolamid® 5 mg/5 mL IV ampul, Defarma İlaç, Ankara, Turkey) at a dose of 0.6 mg/kg within 15 sec, and for the analysis of arterial blood gases and metabolic and electrolyte variables, 1 mL blood samples were taken from the auricular artery.

At the 25<sup>th</sup> min of midazolam administration (immediately after blood collection, T25), saline (Kanfleks® 0.9% isotonic sodium chloride solution, Kansuk Laboratory, Istanbul, Turkey) was slowly injected within 15 sec into the sheep of the MDS group (n:6) via the IV route in an amount equal to the volume of flumazenil.

At the 25<sup>th</sup> min of midazolam administration (immediately after blood collection, T25), benzodiazepine antagonist flumazenil (Mazenil® 0.5 mg/5 mL IV ampul, VEM İlaç, Ankara, Turkey) was slowly injected within 15 sec into the sheep of the MDF group (n:6) via the IV route at the dose of 0.02 mg/kg.

The hemodynamic parameters were re-recorded at the 5<sup>th</sup> (T30), 35<sup>th</sup> (T60), and 65<sup>th</sup> (T90) min after the injection of saline to the ewes in MDS and flumazenil to the ewes in MDF, later, 1 mL blood samples were collected for analysis of arterial blood gasses and metabolic and electrolyte variables. The collected blood samples were processed without delay via the Epoc® Blood Analysis instrument and the results were recorded.

### Degree of Sedation

The scoring system proposed by Carroll et al.<sup>[21]</sup> was used to determine sedation and posture degree. Accordingly, the degree of sedation was scored as 0 = No sedation (A normal warning attitude; can be frightened, moves the ears in response to toy gun firing); 1 = Mild sedation (A depressive attitude; may be frightened, movements may be observed in the ears or face in response to the sound of the toy gun); 2 = Moderate sedation (A depressive attitude and sternal recumbency. May be aware of the sound of the toy gun, but has little reaction); 3 = Profound sedation (Lateral recumbency), and the degree of posture was scored as 0 = Standing; 1 = Recumbency (without movement). Scorings were recorded by the same observer, who had no knowledge of the study materials or methods. Sedation and postural scoring and behavioral scores were determined immediately after baseline (T0), T5, T25, T30, T60, and T90 hemodynamic values and blood sample collection.

Sedation scores were determined by firing a toy gun from a distance of 1 m from the head of the ewe. A modified tester with an adapted electronic force transducer was used to measure the applied isometric force when determining changes in mechanical threshold, similar to the modified hoof tester proposed by Carroll et al.<sup>[21]</sup>. The modified tester, powered by two batteries, was about 30 cm long and had a tester gap diameter of 7 cm. The accuracy of the tester was 1 N and it was capable of measuring up to a maximum of 1 kN. In baseline, T5, T25, T30, T60 and T90, pressure was applied using the modified tester to the withers and metacarpus of the ewe in less than 5 sec and the force applied could be visualized by a signal processor. If the sheep made a purposeful movement or emitted a sound during the application of force, the force was terminated and the reading was recorded as a force tolerance test value.

The ewes were also monitored for signs of agitation (vocalization, piloerection, rolling), salivation, ruminal tympany, and urination.

### Statistical Analysis

Because the data did not meet the parametric test assumptions, the difference between groups (MDS and MDF) for all quantitative traits measured was compared using the Mann-Whitney U test. The difference between repeated measurements made on the same ewe as a function of time was compared separately within each group using the Freidman test. Pairwise comparisons of variables with significant differences as a result of the Freidman test were made using the Wilcoxon test, only the differences between the baseline measurement and the later measurements. Changes were considered significant when  $P < 0.05$ . All values were given as median.

## RESULTS

Time-dependent median results of the MDS and MDF groups in sedation and postural scoring and changes in mechanical threshold of reflexes were summarized in *Table 1*.

The difference between the MDS and MDF groups in sedation and postural variables proved significant ( $P < 0.05$ ) in scoring (T30, T60 and T90) after the administration of flumazenil. In the within-group evaluation, lateral recumbency and deep sedation (220 sec in MDS, up to 260 sec in MDF) occurred with the absence of the palpebral reflex from the administration of midazolam to T5 in both groups and the return of the Bulbus oculi to the full ventromedial position ( $P < 0.05$ ).

At the onset of sedation, stargazing and mydriasis were observed in all sheep in both group, but no signs of apnea, agitation (vocalization, piloerection, rolling), or convulsions were observed.

**Table 1.** Time-dependent median results of sedation and postural and analgesia levels determined by force application at the withers and metacarpus in 6 ewes receiving MDS and MDF by the IV route

Time Points	Sedation		Posture		Withers		Metacarpus	
	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF
BL (T0)	0.0	0.0	0.0	0.0	9.5	10.5	3.5	4.0
T5	3.0*	3.0*	1.0*	1.0*	41.0*	43.5*	11.0*	11.5*
T25	3.0*	3.0*	1.0*	1.0*	44.5*	47.5*	14.0*	13.0*
T30	3.0*	0.0#	1.0*	0.0#	44.5*	11.0#	14.0*	4.0#
T60	3.0*	0.0#	1.0*	0.0#	35.5*	10.5#	10.5*	4.0#
T90	2.0*	0.0#	1.0*	0.0#	22.5*	10.0#	6.5*	4.0#

Sedation and postural scoring and changes in mechanical reflex threshold were evaluated after blood collection with determination of cardiopulmonary variables; **MDS**: injection of saline solution equal to the volume of flumazenil 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **MDF**: injection of flumazenil (0.02 mg/kg, IV) 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **BL (T0)**: Baseline measurement value immediately before midazolam injection; **T5**: Measured value 5 min after midazolam injection; **T25**: Measured value 25 min after midazolam injection, immediately before flumazenil injection; **T30**: 30 min after midazolam injection and 5 min after flumazenil injection; **T60**: Measured value 60 min after midazolam injection and 35 min after flumazenil injection; **T90**: Measured value 90 min after midazolam injection and 65 min after flumazenil injection; #: Significantly different from MDS at indicated times, ( $P < 0.05$ ); \* Significantly different from baseline (T0), ( $P < 0.05$ )

**Table 2.** Time-dependent median results for cardiopulmonary variables in 6 ewes receiving MDS and MDF by the IV route

Time Points	HR (beats per min)		SAP (mmHg)		DAP (mmHg)		RR (breaths per min)		SpO <sub>2</sub> (%)		EtCO <sub>2</sub> (%)		BT (°C)	
	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF
BL (T0)	97.5	95.5	141.0	129.0	93.0	92.5	34.5	31.5	94.0	94.5	40.5	38.0	39.4	39.1
T5	106.0*	104.5	127.0	124.0	57.0	74.5*	27.0*	25.0*	91.5*	90.5*	43.5*	40.5*	39.2*	38.9
T25	110.0*	109.0	112.5	129.0	64.0	69.5*	29.0*	26.0*	88.5*	86.0*	40.5	37.5*	39.2*	38.6*
T30	112.0*	88.5#	110.0	144.0	68.5	113.0	29.0*	34.0*	86.5*	95.0#	45.0*	33.5#	39.0*	39.0
T60	93.0	95.0	106.5	119.0	68.5	93.5#	31.5*	35.0	89.5*	96.0#	42.5	37.5#	38.8*	39.1
T90	97.5	95.0	114.0	122.0	58.5	86.5#	33.5*	33.0	93.5	95.0	44.0	39.5	38.9*	39.0

**MDS**: injection of saline solution equal to the volume of flumazenil 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **MDF**: injection of flumazenil (0.02 mg/kg, IV) 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **BL (T0)**: Baseline measurement value immediately before midazolam injection; **T5**: Measured value 5 min after midazolam injection; **T25**: Measured value 25 min after midazolam injection, immediately before flumazenil injection; **T30**: 30 min after midazolam injection and 5 min after flumazenil injection; **T60**: Measured value 60 min after midazolam injection and 35 min after flumazenil injection; **T90**: Measured value 90 min after midazolam injection and 65 min after flumazenil injection; **HR**: heart rate; **SAP**: systolic arterial pressure; **DAP**: diastolic arterial pressure; **RR**: respiratory rate; **SpO<sub>2</sub>**: oxy-haemoglobin saturation; **EtCO<sub>2</sub>**: end-tidal CO<sub>2</sub>; **BT**: body temperature; # Significantly different from MDS at indicated times ( $P < 0.05$ ); \* Significantly different from baseline (T0), ( $P < 0.05$ )

The difference between the MDS and MDF groups in analgesia scores measured at the withers and metacarpus was significant ( $P < 0.05$ ) for measurements after the administration of flumazenil (T30, T60, and T90), as well as for sedation and posture scores. In the within-group evaluation related to analgesia scores (both in withers and metacarpus), the measured increase from baseline to T90 in MDS was significant ( $P < 0.05$ ), while the increase in MDF from baseline to flumazenil administration (T5 and T25) was significant ( $P < 0.05$ ). In MDS, two ewes were standing (ewes 2 and 4) and did not lie down again in T90, whereas in MDF, one ewe for 36 sec (ewe 11), two ewes for 44 sec (ewes 9 and 12), and three ewes for 53 sec after flumazenil (ewes 7, 8 and 10) were standing and not lying down again (at T30, T60 and T90) ( $P < 0.05$ ). During the recovery period, mild agitation was observed in two ewes (ewes 5 and 6) in MDS, but no signs of agitation were observed in MDF.

Hypersalivation was noted in all ewes in both groups. Hypersalivation, which started at the 6<sup>th</sup> min in ewes in MDS, continued until the 34<sup>th</sup> min. In MDF, on the other hand, hypersalivation, which started at the 8<sup>th</sup> min, ended after flumazenil application (T30). It was observed that hypersalivation was higher in the first 10 min and gradually decreased. Mild ruminal tympany was observed between 16 and 60 min in the MDS in 2 ewes (ewes 2 and 4) and between 12 and 30 min in the MDF in 3 ewes (ewes 9, 11 and 12). Urination was observed between 22 and 70 min in MDS in 3 ewes (ewes 2, 3 and 6) and between 27 and 64 min in MDF in 2 ewes (ewes 7 and 9).

The time-dependent median results of the MDS and MDF groups in relation to cardiopulmonary variables and BT were summarized in Table 2.

With regard to cardiovascular variables, the difference

**Table 3.** Time-dependent median results for blood gases and metabolic variables in 6 ewes receiving MDS and MDF by the IV route

Time Points	pHa		pCO <sub>2</sub> (mmHg)		pO <sub>2</sub> (mmHg)		HCO <sub>3</sub> <sup>-</sup> (mmol/L)		BE (mmol/L)		Hct (%)		Hb (g/dL)		Lactate (mmol/L)		Glucose (mg/dL)	
	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF	MDS	MDF
BL (T0)	7.481	7.513	30.50	31.15	81.75	80.25	24.65	25.40	3.65	4.65	33.50	32.50	8.00	7.60	1.50	2.33	95.00	88.50
T5	7.458	7.462	31.30*	31.80*	78.90*	78.20*	24.15	24.40	1.90*	2.30*	32.50	31.50	7.65	7.20	1.32*	1.86*	97.00	89.00
T25	7.426*	7.454	31.75*	33.10*	76.15*	77.20*	24.10	24.75	1.30*	1.25*	33.50	32.50	8.05	7.60	1.25*	1.34*	103.50*	94.00
T30	7.446*	7.462 <sup>#</sup>	32.05*	30.30*	76.25*	81.60	25.60	26.10	2.70	2.20	34.00	32.00	8.30	7.45	1.18*	1.69*	102.50*	97.00
T60	7.453	7.534**	32.40*	29.35**	77.75*	83.45*	25.50	27.85*	2.40	3.30	34.50	32.50	8.45	7.60	1.20*	2.10 <sup>#</sup>	100.50*	91.00 <sup>#</sup>
T90	7.461	7.521**	31.90*	30.20*	81.50	82.60	25.75	29.35*	2.10	4.05 <sup>#</sup>	35.50	32.00	8.70	7.15	1.29*	2.06 <sup>#</sup>	108.50*	91.50 <sup>#</sup>

**MDS:** injection of saline solution equal to the volume of flumazenil 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **MDF:** injection of flumazenil (0.02 mg/kg, IV) 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **BL (T0):** Baseline measurement value immediately before midazolam injection; **T5:** Measured value 5 min after midazolam injection; **T25:** Measured value 25 min after midazolam injection, immediately before flumazenil injection; **T30:** 30 min after midazolam injection and 5 min after flumazenil injection; **T60:** Measured value 60 min after midazolam injection and 35 min after flumazenil injection; **T90:** Measured value 90 min after midazolam injection and 65 min after flumazenil injection; **pHa:** arterial pH; **pCO<sub>2</sub>:** partial pressure of carbon dioxide in arterial blood; **pO<sub>2</sub>:** partial pressure of oxygen in arterial blood; **HCO<sub>3</sub><sup>-</sup>:** bicarbonate; **BE:** base excess; **Hct:** hematocrit; **Hb:** hemoglobin; # Significantly different from MDS at indicated times ( $P < 0.05$ ); \* Significantly different from baseline (T0), ( $P < 0.05$ )

**Table 4.** Time dependent median results of electrolyte variables in 6 ewes receiving MDS and MDF by the IV route

Time Points	Na <sup>+</sup> (mmol/L)		K <sup>+</sup> (mmol/L)		Cl <sup>-</sup> (mmol/L)	
	MDS	MDF	MDS	MDF	MDS	MDF
BL (T0)	144.50	144.00	4.10	4.20	108.00	110.00
T5	145.50	144.00	3.75*	3.70*	108.00	108.50
T25	145.00	144.50	3.75*	3.90*	108.50*	109.00
T30	144.00	144.00	3.90	3.90	107.50	109.00
T60	145.00	144.00	4.20	4.05	108.50	108.50
T90	145.00	144.50	4.40	4.05	110.00	108.00

**MDS:** injection of saline solution equal to the volume of flumazenil 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **MDF:** injection of flumazenil (0.02 mg/kg, IV) 25 min (T25) after midazolam (0.6 mg/kg, IV) injection; **BL (T0):** Baseline measurement value immediately before midazolam injection; **T5:** Measured value 5 min after midazolam injection; **T25:** Measured value 25 min after midazolam injection, immediately before flumazenil injection; **T30:** 30 min after midazolam injection and 5 min after flumazenil injection; **T60:** Measured value 60 min after midazolam injection and 35 min after flumazenil injection; **T90:** Measured value 90 min after midazolam injection and 65 min after flumazenil injection; **Na<sup>+</sup> (mmol/L):** sodium, **K<sup>+</sup> (mmol/L):** potassium, **Cl<sup>-</sup> (mmol/L):** chloride; # Significantly different from MDS at indicated times ( $P < 0.05$ ); \* Significantly different from baseline (T0), ( $P < 0.05$ )

between the MDS and MDF groups in HR was significant ( $P < 0.05$ ) only immediately after flumazenil administration (T30). The increase in HR, which began immediately after midazolam administration in both groups, continued in MDS until T30 ( $P < 0.05$ ) and remained at a level close to baseline from T60. A sudden drop was observed in MDF after flumazenil administration, and all changes within the group were found to be insignificant compared with baseline ( $P > 0.05$ ). No arrhythmia was monitored in ECG in either group. The difference between and within groups in SAP was insignificant ( $P > 0.05$ ). While a decrease was observed in MDS up to T60, a decrease was seen in MDF at T5, and after flumazenil administration (T30), it increased with a sudden rise above baseline. The difference between the groups in DAP at T60 and T90 was found to be significant ( $P < 0.05$ ). It was observed to be lower than the baseline value from T5 to T90 in MDS ( $P > 0.05$ ). While a decrease ( $P < 0.05$ ) was observed in MDF until the application of flumazenil (T25), the sudden increase that started at T30 continued until T90 at a level close to baseline. Midazolam was observed to cause mild, transient, but statistically

significant cardiovascular depression and insignificant hypotension in MDS, and these effects were normalized by flumazenil administration in MDF.

With respect to respiratory variables, midazolam resulted in transient and nondramatic changes. The difference between the MDS and MDF groups in RR was insignificant ( $P > 0.05$ ). It was observed that RR in T5 showed a sudden decrease in both groups ( $P < 0.05$ ). This decrease in MDS remained below baseline until the end of anesthesia ( $P < 0.05$ ). In contrast, in MDF, a decrease ( $P < 0.05$ ) was observed until the administration of flumazenil (T25), whereas the sudden increase ( $P < 0.05$ ) that began at T30 was above baseline at T90. The difference between groups in SpO<sub>2</sub> at T30 and T60 was significant ( $P < 0.05$ ). It was observed that SpO<sub>2</sub> in MDS remained below baseline until T60 ( $P < 0.05$ ), while it decreased in MDF until flumazenil application (T25) and increased from T30 ( $P > 0.05$ ). For EtCO<sub>2</sub>, the difference between groups was significant only at T30 and T60 ( $P < 0.05$ ). It was observed to be higher than baseline at T5 and T30 in MDS and in the period

until flumazenil application in MDF (T5 and T25) ( $P < 0.05$ ). Although significant changes in RR, SpO<sub>2</sub>, and EtCO<sub>2</sub> were observed in both groups after midazolam administration, it was assessed that these changes were clinically insignificant and returned to normal with flumazenil administration in MDF.

The difference between the MDS and MDF groups in BT was insignificant ( $P > 0.05$ ). BT decreased significantly in both groups after midazolam administration ( $P < 0.05$ ), but hypothermia was not observed. A new increase ( $P > 0.05$ ) was observed after flumazenil administration (T30) in MDF.

The time-dependent median results of the MDS and MDF groups with respect to arterial blood gas and metabolic variables were summarized in *Table 3*.

With regard to arterial blood gases, although a partial decrease in pH, a statistically significant increase in pCO<sub>2</sub>, and a significant decrease in pO<sub>2</sub> were observed in both groups, these changes were close to the reference ranges. Midazolam was found to cause mild respiratory acidosis in both groups. It was observed that pH, pCO<sub>2</sub>, and pO<sub>2</sub> values returned to the normal range with the increase in RR and SpO<sub>2</sub>  $\geq 95\%$  from T30 on MDF. It was assessed that the decrease in HCO<sub>3</sub><sup>-</sup> and BE was consistent with mild respiratory acidosis, but these changes were not clinically significant.

The difference between and within groups in Hct and Hb was not significant ( $P > 0.05$ ). The difference between MDS and MDF groups in terms of lactate and glucose was significant at T60 and T90 ( $P < 0.05$ ). In MDS, lactate was lower than baseline ( $P < 0.05$ ) and glucose was higher than baseline ( $P < 0.05$ , except T5) up to T90 at a statistically significant level.

The time-dependent median results of the MDS and MDF groups with respect to the electrolyte variables were summarized in *Table 4*.

With respect to Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> levels, the difference between the MDS and MDF groups was insignificant ( $P > 0.05$ ). In both groups, midazolam had no effect on Na<sup>+</sup>, caused a decrease in K<sup>+</sup> until T25 ( $P < 0.05$ ), while Cl<sup>-</sup> remained stable (observed only at T25 in MDS ( $P < 0.05$ )). It was observed that flumazenil had no effect on electrolyte variables in MDF.

## DISCUSSION

Although changes in cardiopulmonary variables and behavioral reflexes caused by IV injection of midazolam in the range of 0.05-0.5 mg/kg in ewes have been studied [14,18,22], the effect of flumazenil antagonism has been reported only on behavioral reflexes [14]. Stegmann et al. [23] reported that benzodiazepine dosages for ewes and goats were similar. In the current study, considering studies in goats,

dose selection was made for midazolam according to the preliminary studies [10,22,24,25] and for flumazenil according to the preliminary study [14]. In light of the literature, IV administration of 0.6 mg/kg midazolam [18,26] and IV administration of 0.02 mg/kg flumazenil [27,28] were considered high doses. This study evaluated changes in mechanical threshold of reflexes in ewes and their effects on cardiopulmonary, arterial blood gas, metabolic, and electrolyte variables with a single high-dose (0.02 mg/kg, IV) injection of flumazenil, a benzodiazepine antagonist, 25 min (T25) after a single high-dose injection of midazolam, whose cardiopulmonary effects were considered minimal.

Flumazenil is well tolerated even in amounts exceeding recommended doses in humans. According to Food and Drug Administration (FDA), the first dose of 0.2 mg of flumazenil should be administered IV within 15 sec. If the desired level of consciousness is not achieved within 60 sec of the first IV administration, a second dose of 0.1 mg may be injected. It is recommended that this be repeated as necessary at 60-sec intervals up to a total dose of 1 mg [29]. Flumazenil has been reported to have virtually no intrinsic pharmacological activity in animals and does not produce tachypnea, CNS agitation, or behavioral changes even in the absence of benzodiazepines [13,14]. In this study, a single IV administration of 0.02 mg/kg flumazenil was sufficient to reverse the effects of midazolam smoothly in a very short time, but there is insufficient information on the pharmacodynamic and pharmacokinetic effects of flumazenil in animals. Because the drug doses and number of ewes used in this study were limited, there is a need to further investigate the different dose rates of midazolam and flumazenil, as well as repeated doses of flumazenil, to uncover the clinical effects of flumazenil.

Simon et al. [22] emphasized that IV administration of midazolam (0.5 mg/kg) resulted in profound sedation in 5 of the 8 ewes within 5 min, profound ataxia was observed in all ewes, and sedation scores were significantly different from baseline in the first 15 min. Upton et al. [18] found that lower doses of midazolam showed minimal sedative effects in ewes, and midazolam produced variable but longer term sedation with increasing dose. Kyles et al. [14] reported that administration of midazolam in ewes resulted in a dose-dependent increase in thermal and mechanical thresholds and that these effects of midazolam, which resulted in dose-dependent sedation and ataxia, could be attenuated by IV administration of flumazenil. Flumazenil has been reported to be used IV, IM, sublingually, endotracheally, or rectally to reverse benzodiazepine-induced sedation, but the most rapid effect may be obtained via IV administration [30]. Because no surgery was performed in the present study, the mechanical stimulus apparatus defined by Carroll et al. [21] was modified and used to determine changes in the mechanical threshold of reflexes. Deep sedation in ewes in both groups within the first 5 min after administration of high-dose midazolam and

recovery from deep sedation shortly after (standing within 53 sec) administration of flumazenil were consistent with the findings of the aforementioned researchers [14,18,22]. As Heniff et al.<sup>[30]</sup> noted; IV administration of both drugs was thought to induce the onset of deep sedation and a rapid response, and it was suggested that this effect might be due to the fact that midazolam, with its lipophilic structure, rapidly crosses the blood-brain barrier and flumazenil has a high affinity for benzodiazepine receptors.

Because flumazenil has a very short half-life and a shorter duration of action than benzodiazepines, resedation may occur if long-acting benzodiazepines were used [28,31,32]. The absence of repeated sedation in any of the ewes in the study was explained by the presence of a short-acting benzodiazepine in its midazolam.

The combination of IV injection of midazolam (0.025-0.05 mg/kg) and ketamine (5-7 mg/kg) is used to induce general anesthesia and enhance muscle relaxation for endotracheal intubation in small ruminants [8,33]. Hypersalivation was observed in 4 of 6 ewes following IV administration of midazolam by Simon et al.<sup>[22]</sup> and in all goats by Stegmann and Bester<sup>[10]</sup>. The main side effect observed in the study was hypersalivation, which was seen in all ewes, consistent with the researchers' [10,22] findings. In ewes put under general anesthesia with midazolam premedication, it should be kept in mind that aspiration of large amounts of saliva can lead to complications if the trachea is left unprotected.

Anesthesia-induced brainstem depression has been reported to cause cardiovascular and respiratory depression, and the extent of depression in the brain is proportional to the depth of sedation, cardiovascular, and respiratory depression [18,34,35]. It has been reported that IV administration of lower doses of midazolam in ewes had minor cardiovascular and respiratory effects compared with baseline, whereas higher doses of midazolam increased HR up to 47% after 10 min, and that cardiovascular depression occurs as a function of dose, but the extent of cardiovascular depression in ewes is not life-threatening [18]. Simon et al.<sup>[22]</sup> discovered that after IV administration of midazolam in ewes, there was an increase of about 22% to HR by the 30<sup>th</sup> min, which was below baseline at the 90<sup>th</sup> min. Other studies performed with benzodiazepines in ewes have reported that diazepam tends to increase HR, that there is an increase in the first 10 min and then a decrease [11], and that remimazolam (CNS 7056), a new benzodiazepine, dose-dependently increases HR up to 53% after 5 min [18]. In the study, high doses of midazolam were observed to induce deep sedation and have profound effects on the cardiovascular system. In line with the findings of the researchers [11,18,22,34], it was observed that the HR increased with deep sedation in the first 30 min of anesthesia and immediately decreased with injection of flumazenil. The longer duration of the increase in HR compared to other studies was attributed to the high dose.

It has been reported that the depth of anesthesia and cardiovascular depression may be directly proportional regardless of the drug causing the sedation, and that low arterial blood pressure, one of the signs of cardiovascular depression, may cause decreased oxygenation of vital organs due to malperfusion of tissues [3,18,36]. Invasive SAP is defined as normotension when the pressure is between 90 and 120 mmHg, hypotension when it is below 90 mmHg, and hypertension when it is above 120 mmHg [3,36]. Upton et al.<sup>[34]</sup> found that midazolam significantly reduced MAP in ewes in the first 35 min (excluding 8<sup>th</sup> and 20<sup>th</sup> min) compared with baseline, but this decrease was only about 8%. Upton et al.<sup>[18]</sup> reported that midazolam causes a dose-dependent decrease of up to 11% in MAP in ewes. In the study, it was remarkable that the baseline median values in both groups were higher than the normotension values reported in the literature [3,36] with respect to SAP and DAP. It was observed that the decrease occurred at SAP and DAP in MDS in the normotensive range and sudden hypertension developed at T30 in MDF. It was considered that the results were compatible with studies conducted with ewes administered IV midazolam [18,34], that hypotension was caused by the central effects of midazolam on vasomotor centers, and that SAP values recorded above 90 mmHg during sedation were high enough to ensure perfusion of vital organs. As noted by Upton et al.<sup>[18]</sup>, the reason for the increase in HR was explained as a compensatory response to the decrease in SAP and DAP.

It has been highlighted that BDZs can reduce RR in animals but rarely affect ventilation and oxygenation, and at high doses can cause dose-dependent respiratory depression or exacerbate existing respiratory depression [37]. Midazolam has been shown to inhibit the central respiratory system in humans, and respiratory depression during conscious sedation has been reported as the most significant adverse effect [38,39], and it has been noted that RR usually returns to normal after BDZs when flumazenil is used [40,41]. It has been reported that there was a transient, dose-dependent reduction in RR of more than 50% after IV administration of midazolam in ewes [18,22], that SpO<sub>2</sub> decreased by approximately 10% at the 10<sup>th</sup> min [34] and that RR and decrease in SpO<sub>2</sub> at the measurement periods and the temporal changes in the EtCO<sub>2</sub> data were compatible [18]. Time to reversal of respiratory depression induced by IV flumazenil and midazolam in dogs was reported to be 120±25 sec [30]. In the study, as reported by the investigators [18,22,34,35], the decrease in RR and decrease in SpO<sub>2</sub> and changes in EtCO<sub>2</sub> were consistent with time. The extent of reduction in SpO<sub>2</sub> with concomitant reduction in paO<sub>2</sub> in both groups did not indicate the need for oxygen therapy. It has been observed that transient and nondramatic changes in the respiratory system disappeared with the use of flumazenil, as noted by Heniff et al.<sup>[30]</sup> and Shalansky et al.<sup>[40]</sup>. It has been suggested that respiratory depression occurred as a result of further brainstem suppression by high doses of midazolam [18], and that the increase in RR

with the use of flumazenil may have been achieved by the disappearance of sedation and the improvement of consciousness<sup>[40]</sup>.

It has been reported that IV administration of BDZs in ewes and goats can result in significant decreases in BT, but these decreases are within the normal range<sup>[10,18,22,35]</sup>. In the current study, it was suggested that the reduction in BT, which occurred in relatively normal ranges in agreement with results in the literature and was not considered clinically significant, might be related to the vasodilation that developed after midazolam administration, as noted by Upton et al.<sup>[18]</sup>.

The values of arterial blood gasses and metabolic variables obtained in this study differed, although only partially, from the literature on conscious values in ewes<sup>[42-44]</sup>. Midazolam has been reported to cause changes in blood gas levels and small fluctuations in HCO<sub>3</sub><sup>-</sup> and BE in goats while these changes were minimal<sup>[24,25]</sup>, whereas there was a significant decrease in paO<sub>2</sub> at 10<sup>th</sup> min in ewes<sup>[34]</sup>. Upton et al.<sup>[18]</sup> indicated that it caused a decrease in pH, an increase in paCO<sub>2</sub>, and a decrease in paO<sub>2</sub> concurrently cause mild but significant respiratory acidosis in ewes, and these effects were more significant with remimazolam (CNS 7056) compared to with midazolam. Other studies conducted with benzodiazepines in ewes reported that diazepam decreased paO<sub>2</sub> at the onset of sedation (at the 2<sup>nd</sup> min) but remained close to the control group after the 15th min, but had no significant effect on paCO<sub>2</sub> and acid-base variables<sup>[11]</sup>. Midazolam caused mild respiratory acidosis in the present study, which was not considered clinically significant, with a decrease in pH and paO<sub>2</sub> and an increase in paCO<sub>2</sub>. It improved in a short time with the increase in RR and SpO<sub>2</sub> after the administration of flumazenil in MDF. In line with the findings of the aforementioned investigators<sup>[11,18,24,25,34]</sup>, high dose midazolam was observed to cause a clinically acceptable level of transient respiratory depression in healthy ewes.

De Carvalho et al.<sup>[45]</sup> reported that although significant changes occurred in HR and arterial blood gases, no significant electrolyte abnormalities were noted. While mild hypoventilation and decreases in HCO<sub>3</sub><sup>-</sup> and BE, which were associated with hypoxemia, resulted in findings supporting respiratory acidosis, the changes in metabolic and electrolyte variables were clinically insignificant. As noted by De Carvalho et al.<sup>[45]</sup>, the mechanism of such changes in ewes remains unclear.

In conclusion, single and slow administration of 0.6 mg/kg midazolam via the IV route in ewes resulted in beneficial effects on sedation depth and reflex scores, but transient and clinically acceptable adverse effects on cardiopulmonary, blood gas and metabolic variables. A single and slow administration of 0.02 mg/kg flumazenil via the IV route was sufficient to shorten the recovery time and reverse the adverse effects within 5 min. It

was concluded that further studies at different doses are needed to better understand the potential role of flumazenil as a clinical antagonist in ewes, and that midazolam and flumazenil at these doses can be used in ewes that are expected to recover from sedation in a short time.

## AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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## CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

## AUTHOR'S CONTRIBUTIONS

Ü.Y.: Experimental design, data collections, data analysis, manuscript writing. K.Y. and A.Ş.: Data collections, data analysis.

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