Comparison of systemic effects
of midazolam, ketamine, and isoflurane anaesthesia
in rabbits

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Abstract

Introduction: Clinical doses of anaesthetic agents were administered to rabbits and effects on the brain, heart, and liver were investigated biochemically and histopathologically. **Material and Methods:** The rabbits were randomly divided into three main groups (16 rabbits each) and each group into study (n = 8) and control (n = 8) groups. All study group rabbits received 3 mg/kg of midazolam (M) intramuscularly. Group 1.1 (M) received nothing further, group 2.1 (MK) also received 25 mg/kg of ketamine, and group 3.1 (MKI) besides ketamine was also given 2% isoflurane to induce anaesthesia for 30 min. NaCl solution in the same volume as midazolam and ketamine was injected into the controls. **Results:** In clinical evaluation significant differences were detected in respiratory and heart rates. In blood gas analysis the PO_2 and PCO_2 values showed statistical differences in anaesthesia intervals. Significant biochemical value changes were recorded in creatine kinase-Mb, glucose, and total protein. Histopathological liver examinations revealed higher total apoptotic and normal cell numbers in the MK than in the M and MKI groups. Apoptotic cell numbers were statistically significant in M and MK groups. **Conclusion:** Anaesthetic agents may increase programmed apoptosis. The MKI anaesthetics combination was found to cause less cell destruction in general than the other study groups. It was indicated that MKI was the safer anaesthetic combination in rabbits.

Keywords: isoflurane, ketamine, midazolam, systemic effects, histopathology.

Introduction

The anaesthetic agents (xylazine, midazolam, detomidine, and ketamine) have adverse side effects on the cardiorespiratory, cardiovascular, and nervous systems. It is desirable that ideal anaesthesia protocols have the least harmful effects on these systems and their organs and are as safe as possible. Therefore, studies investigating the toxic effects of different anaesthetics or their combinations on body systems do not abate.

The most commonly used anaesthetic agents in veterinary medicine are midazolam, ketamine, and isoflurane. However, side effects of these drugs such as hypotension, bradycardia, and apnea, and some toxic effects in tissue have been reported (14, 16, 17).

Benzodiazepines such as midazolam are potentially sedative-hypnotic and are used as injectable anaesthetics. When used in clinical doses, respiratory and heart rates, tidal volume, blood pH, blood gases, cardiac output, and mean arterial blood pressure do not change (6, 19, 22).

On the other hand, some harmful effects in individuals with liver and kidney diseases have been proven and reported to accumulate in the body with repeated doses (6). Neurotoxic effects of midazolam have been determined. Furthermore, vascular lesions have been shown in *in vivo* studies. It has also been

reported that apoptosis by anaesthetics at high doses may lead to necrosis in various tissues (7, 12, 26).

Ketamine is one of the most commonly used anaesthetic agents in experimental animals (19). Contrary to most anaesthetic agents, it does not reduce heart rate nor cause myocardial depression (9). Inhaled anaesthetic agents are used for both safe induction and continuation of anaesthesia. At higher concentrations than the minimum alveolar concentration, isoflurane increases intracranial pressure, affects liver functions minimally, and reduces kidney blood flow. However, it does not result in functional or pathological renal impairment due to the minimum level of inorganic fluorine and urea exchange considered harmful to the organism being higher than that caused by isoflurane (11, 14).

In recent years it has been shown that anaesthetic agents may induce apoptosis at different doses (7, 12, 26). However, studies are still underway to determine whether the agents induce apoptosis precisely, and it has also been reported that the rate of apoptosis is higher in those who experience more potent effects than others from the same volume dose of anaesthetic agent. Studies have also reported that apoptosis caused by a combination of synergistic anaesthetic agents is higher than that caused by single agents (21). As a corroborative example, Braun *et al.* (5) investigated apoptosis induced by ketamine in human cells and reported that ketamine leads to apoptosis in neural and non-neural cells even at millimolar doses. In contrast, Fredrikson *et al.* (13) reported that ketamine, an NMDA receptor antagonist, does not cause apoptosis, even in neonatal brain cells.

In the present study, the application of the anaesthesia protocol to rabbits at normal clinical doses was to investigate whether they cause apoptosis, especially in the brain, heart, and liver. Additionally, other harmful effects were also investigated and their possible effects were substantiated by biochemical parameters and histopathological studies. All the results were reflected in the field of veterinary anaesthesiology.

Material and Methods

Animals. A total of 48 New Zealand rabbits (24 female and 24 male), four months old and weighing 2.24 ± 0.57 kg were used. Each of the rabbits was kept in a separate cage in the Erciyes University, Experimental Research, Application, and Research Center until the study began. *Ad libitum* feeding was allowed. The average room temperature and lighting were set at $21 \pm 1°C$ and 12 h light and 12 h dark. The rabbits were randomly divided into three main groups and each had 16 rabbits. Each group was also divided into two subgroups: a study (n = 8) and a control (n = 8).

Study groups and anaesthesia protocol. Anaesthetic-differentiated study groups were designed as below:

Group 1.1 (n = 8): midazolam group (M) administered 3 mg/kg of midazolam intramuscularly (IM);

Group 1.2 (n = 8): midazolam control (MC) administered NaCl solution equal to midazolam volume IM;

Group 2.1 (n = 8): midazolam-ketamine (MK) administered 3 mg/kg of midazolam and 25 mg/kg of ketamine (10 min after midazolam administration) IM;

Group 2.2 (n = 8): midazolam-ketamine control (MKC) administered NaCl solution equal to volume of midazolam and ketamine IM;

Group 3.1 (n = 8): midazolam-ketamine-isoflurane (MKI) administered 3 mg/kg of midazolam and 25 mg/kg of ketamine (10 min later) IM, intubated 10 min later and maintained under anaesthesia for 30 min with 2% isoflurane and 1% O_2;

Group 3.2 (n = 8): midazolam-ketamine-isoflurane control (MKIC): administered NaCl solution IM twice in the same volume of midazolam and ketamine as in the group 3.1.

The intubation of group 3.1 was through 2.5mm cuffed tube and was achieved by visualising the tracheal passage with the aid of a laryngoscope (Üzümcü, Turkey). Anaesthesia was carried out with a Classic 2000 anaesthesia machine (SMS Medical Company, Turkey).

Sampling periods and analysis. A blood sample (1.5 mL) was drained from the *vena auricularis* for blood gas and serum biochemical analysis before anaesthesia (0 min) and at 10, 30, 60, 180, and 300 min after midazolam and midazolam-ketamine administration, and isoflurane anaesthesia. Each rabbit was decapitated at 300 min of anaesthesia.

Blood LDH samples were centrifuged for 15 min at 3,000 rpm in a Rotafix 32A machine (Hettich, USA), and serum samples were obtained. They were stored at −20 °C until analysis. Biochemical analysis of serum was carried out in a fully automatic Cobas Integra auto analyser (Roche Diagnostics GmbH, Germany). The values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and creatine kinase-Mb (Ck-Mb) activities, albumin, total protein (TP), creatine (Cr), blood urea nitrogen (BUN), cardiac troponin I (cTnI), and glucose (Glu) levels were measured with a Cobas E 411 analyser (Roche Diagnostics GmbH, Germany) using their commercial kits.

Venous blood gas analyses. Samples were analysed by CC cartridge in an IRMA True (LifeHealth, USA) device. Measurement of pH, PCO_2 mmHg, PO_2 mmHg, Htc %, Na mM/L, K mM/L, Ca mM/L, HCO_3 meq/L, tCO_2 mM/L, BE mM/L, BEecf mM/L, $O_2SAT\%$, and total Hb (tHb) values were determined before anaesthesia and during

midazolam, midazolam-ketamine, and isoflurane anaesthesia intervals for the control and study groups.

Clinical evaluations. Heart (HR) and respiratory rates (RR), body temperature (BT), anaesthesia depth, and encountered complications were recorded before and during anaesthesia intervals for the control and study groups. Hypothermia, bradycardia, and hypoventilation were monitored during anaesthesia.

Histopathological analyses. A systemic necropsy was performed on each rabbit (8). Tissue samples of the liver, heart, brain, lungs, spleen, stomach, intestine, pancreas, and adrenal gland were fixed in 10% formalin. Paraffin sections of 5 μm thickness were stained with haematoxylin and examined under a BX51 light microscope (Olympus, Japan). Some sections from the liver, heart muscle, and brain were prepared on adhesive slides in order to determine apoptotic and necrotised cells. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to derive the apoptotic index in the form of an In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Germany).

Statistical analysis. Comparisons within and between groups were made using SPSS software version 22 in Windows 7 (IBM, USA). Arithmetic mean (M) ± standard deviation (SD) values of the data were considered. Friedman analysis and the Kruskal-Wallis test were used for the repeated measurement, and a chi-square test was carried out for histopathological findings. $P < 0.05$ and $P < 0.001$ were considered significant. Control group values were similar to each other and were also not statistically significant. The mean values of all control groups (MC, MKC, and MKIC values) were used as one set of control group values (Tables 1, 2, 3, and 4).

Results

Clinical findings. BT had decreased significantly at all sampling times compared to 0 time in all study groups (Table 1). There were significant differences in RR between the baseline (0 time) and 10, 30, 60, and 180 min points in all study groups. RR decreased starting from 10 min after anaesthesia and continuing to 180 min, although it tended to increase at 300 min after anaesthesia. A significant difference was determined in HR between the baseline (0 time) and 10, 30, and 60 min points after anaesthesia in the MK group. Furthermore, a significant difference was also recorded between the baseline value and values at 10 and 30 min after anaesthesia in the MKI group. The lowest and highest values of HR were at 30 and 300 min, respectively, in the MKI group.

Venous blood gas analyses. When venous blood pH values were compared within groups, there was a significant difference between the baseline and 10, 30, 60, 180, and 300 min points after anaesthesia in the MKI group (Table 2). Significant differences in pH were also found at 10, 30, 60, and 180 min after
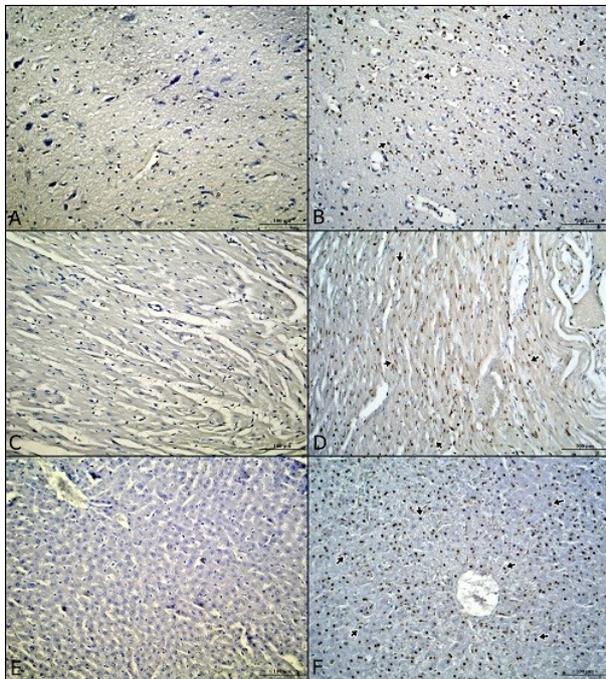
anaesthesia when a comparison was made between the control and study groups. Differences were also recorded of PCO_2 in the M, MK, and MKI groups between the baseline and anaesthesia intervals. These differences were significant between control and study groups. Statistical difference in relation to PO_2 was significant between the baseline and 180 min point in the M group, between the baseline and 30, 180, and 300 min points in the MK group, and between the baseline and 60, 180, and 300 min points in the MKI group. PO_2 values were also statistically different when study and control groups were compared. Differences were recorded in HCO_3, tCO_2, BE, BEecf, and O_2SAT values in study and control groups. Statistically significant differences of HCO_3 were detected at 0, 60, and 180 min of anaesthesia in the MKI group. The MK group $tCO2$ values disclosed significant differences at 0, 30, and 60 min, and the MKI group did at 0, 10, and 30 min. The increases and decreases of BE, BEecf, and O_2SAT values were in reference ranges in study and control groups.

Biochemical findings. The results of biochemical analysis in all groups are given in Table 3. AST values at 30 and 60 min were higher in study groups than control groups but they were in reference ranges. The difference in BUN value in the M group was significant between the baseline and 10, 60, and 180 min points after anaesthesia. Some variations in BUN values were also determined for the other groups but they were within reference values. There were statistical differences for Ck-Mb values between the baseline and 10, 30, 60, and 300 min points in the MKI group. Mean Cr values changed in all groups but they were in reference ranges. Glucose increased significantly in study groups between the baseline and 10, 30, 60, 180, and 300 min points, with the highest values recorded 60 min after anaesthesia. Similarly, the increase was also significant between the control and study groups. A significant difference was determined in LDH values between the baseline and 60, 180 and 300 min points in the MK group, with the highest value being at 180 min. The LDH value also increased in the MKI group at 30, 60, 180, and 300 min. There were significant differences in TP measurement in study groups between the baseline and the other anaesthesia intervals. TP values decreased after 10 min and continued to do so until 300 min of anaesthesia. TP values were also different between the study and control groups but were in reference range. No significant differences were obtained for cTnI values between the baseline and anaesthesia intervals or between study and control groups.
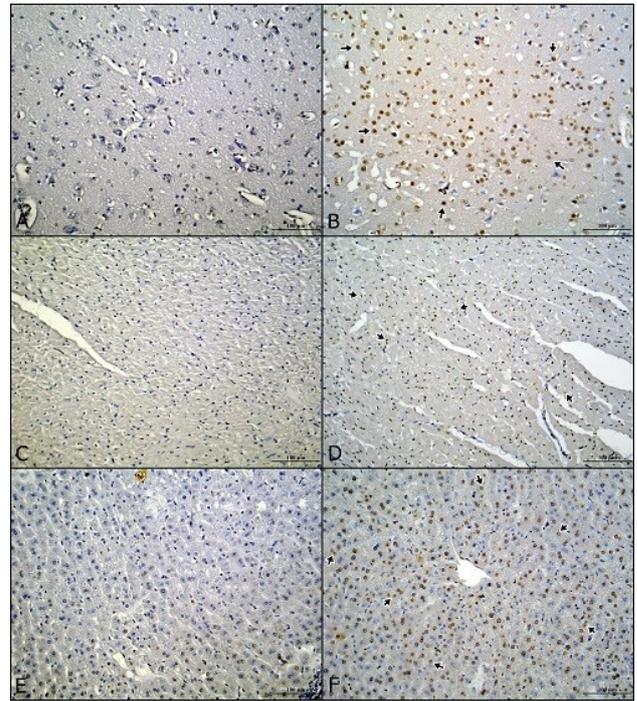
Histopathological findings. Normal and apoptopic total cell numbers were examined in the liver, brain, and heart and comparisons were made between the groups (Figs 1, 2, and 3). Normal and apoptopic cell numbers determined by the TUNEL method are given in Table 4.
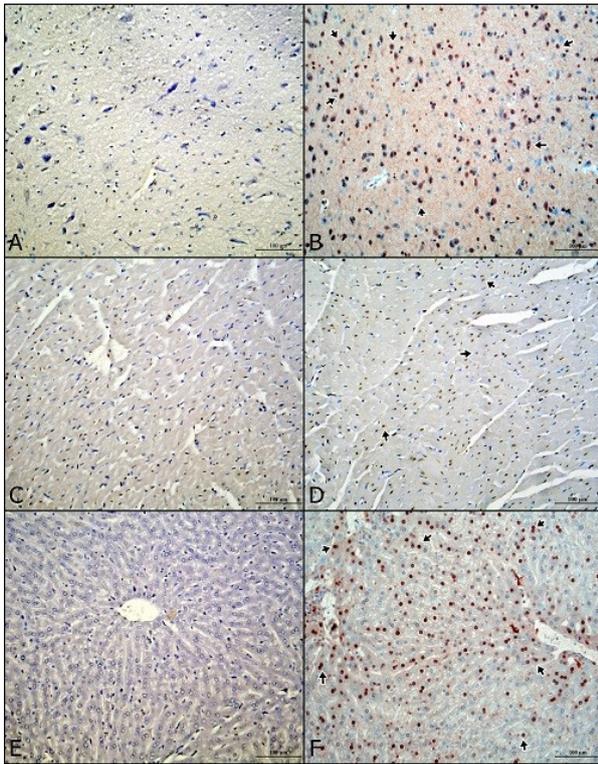
Fig. 1. Immunohistochemical findings of M and control groups
A – control, brain, B – M group, glia and neuron cells (arrows), C – control, myocardium D – M group, myocardium and myocardial cells (arrows), E – control, liver, F – M group, liver, hepatocytes (arrows), Bar – 100 μm

Fig. 3. Immunohistochemical findings of MKI and control groups
A – control, brain, B – MKI group, glia and neuron cells (arrows), C – control, myocardium, D – MKI group, myocardium and myocardial cells (arrows), E – control, liver, F – MKI group, liver, hepatocytes (arrows), Bar – 100 μm

Fig. 2. Immunohistochemical findings of MK and control groups
A – control, brain, B – MK group, glia and neuron cells (arrows), C – control, myocardium, D – MK group, myocardium and myocardial cells (arrows), E – control, liver, F – MK group, liver, hepatocytes (arrows), Bar – 100 μm

Histopathological examinations of the liver revealed significant differences between the M and MK groups in apoptopic and total cell numbers. Apoptopic cell numbers were higher in the MK group than in the M group. Apoptotic brain tissue cell numbers were significantly different between the MK, MKI and control groups. Also apoptotic cell numbers were higher in study groups than control groups.

There were statistical differences in apoptopic heart muscle cell numbers between the M and MKI groups and between the MK and MKI groups. Apoptopic cell numbers were the lowest in the MKI group. Significant difference was also present between the M, MK and control groups. Evident vascularisation with hyperaemia was determined by macroscopic and microscopic examinations in the M group in the heart muscle, liver, and particularly in the central nervous system. No abnormal findings were encountered for the other organs examined which were the stomach, intestine, spleen, lungs, adrenal gland, kidneys, pancreas, and lymph nodes.

Histological examination of the organs of study group rabbits revealed focal haemorrhages in a few areas and hyperaemia of the vessels of the liver, heart muscle, and particularly the central nervous system. No significant abnormal lesions were found in the tissues of control group rabbits.

Table 1. Descriptive statistics for clinical findings (M ± SD) over the time intervals of the groups

Values	Group	0 min	10 min	30 min	60 min	180 min	300 min
BT (°C)	M	39.03 ± 0.48	38.41 ± 0.92*	38.03 ± 1.04*	38.10 ± 0.99*	37.82 ± 1.60*	38.25 ± 0.77*
	MK	38.37 ± 0.75	37.85 ± 0.70*1	37.25 ± 0.96*,*1	37.12 ± 0.92*,*1	37.00 ± 0.95*,*1	37.22 ± 0.74*,*1
	MKI	38.83 ± 0.90	36.63 ± 0.98*,*1	36.13 ± 1.13*,*1	35.52 ± 1.60*,*1	36.27 ± 1.70*,*1	36.88 ± 1.57*,*1
	Control	38.70 ± 0.57	38.80 ± 0.15	38.37 ± 0.20	38.22 ± 0.27	38.25 ± 0.23	37.90 ± 0.25
RR (min)	M	122.25 ± 34.39	79.75 ± 23.38*,*1	71.62 ± 15.90*,*1	76.75 ± 25.38*,*1	86.00 ± 29.33*	97.75 ± 39.62*
	MK	142.75 ± 27.81	87.50 ± 42.15*,*1	57.00 ± 24.49*,*1	57.00 ± 13.52*,*1	64.87 ± 23.64*,*1	87.50 ± 44.88*
	MKI	117.75 ± 13.66	39.62 ± 16.51*,*1	43.75 ± 14.75*,*1	57.50 ± 22.72*,*1	80.75 ± 21.08*	94.5 ± 23.99*
	Control	136.00 ± 18.14	123.50 ± 18.03	101.50 ± 20.47	95.00 ± 17.13	100.00 ± 16.90	108.5 ± 24.30
Heart rate (beat/min)	M	259.30 ± 38.9	236.40 ± 35.40	273.10 ± 23.70	255.50 ± 30.40	272.50 ± 24.30	268.5 ± 35.80
	MK	258.60 ± 55.4	260.60 ± 23.60	252.40 ± 39.70	206.50 ± 23.30*	267.50 ± 35.40	251.5 ± 31.60
	MKI	276.90 ± 49.5	253.60 ± 47.20	232.80 ± 42.30*	264.00 ± 38.60	284.50 ± 21.90	298.0 ± 37.10
	Control	235.60 ± 44.4	301.80 ± 11.90	290.40 ± 31.80	294.00 ± 42.00	285.80 ± 20.00	283.5 ± 29.80

* Comparison between anaesthetic intervals at the same line (P < 0.05). *1 – statistical significance between study group and control group in the same column (P < 0.05).
BT – body temperature, RR – respiration rate, HR – heart rate, M – midazolam, MK – midazolam-ketamine, MKI – midazolam-ketamine-isoflurane

Table 2. Blood gas values of each group and their statistical significance (M ± SD)

Values	Group	0 min	10 min	30 min	60 min	180 min	300 min
pH	M	7.39 ± 0.06	7.38 ± 0.06	7.39 ± 0.10*1	7.33 ± 0.08*1	7.39 ± 0.07	7.40 ± 0.08
	MK	7.41 ± 0.08	7.39 ± 0.08	7.40 ± 0.06	7.35 ± 0.04*1	7.35 ± 0.07	7.36 ± 0.07
	MKI	7.43 ± 0.08	7.24 ± 0.13*,*1	7.32 ± 0.11*,*1	7.24 ± 0.11*,*1	7.32 ± 0.08*,*1	7.34 ± 0.08*
	Control	7.41 ± 0.09	7.38 ± 0.07	7.48 ± 0.06	7.49 ± 0.06	7.45 ± 0.07	7.43 ± 0.06
PCO_2 (mm/Hg)	M	32.25 ± 8.72	33.13 ± 8.67*,*1	34.70 ± 7.90*,*1	35.1 ± 7.02*,*1	34.39 ± 8.30*	35.56 ± 6.51*,*1
	MK	29.69 ± 8.38	30.00 ± 5.64*	39.29 ± 5.44*,*1	41.10 ± 5.50*1	35.74 ± 8.52*	34.55 ± 8.64*
	MKI	17.96 ± 2.05	38.28 ± 10.52*,*1	34.48 ± 10.07*,*1	29.11 ± 6.32*,*1	24.15 ± 6.62*	21.31 ± 8.12*1
	Control	21.81 ± 5.07	21.58 ± 4.83	19.48 ± 2.86	18.65 ± 3.01	17.06 ± 3.85	14.68 ± 2.65
PO_2 (mm/Hg)	M	76.74 ± 17.88*1	74.01 ± 11.93*1	72.94 ± 12.83*1	69.58 ± 13.25*1	61.31 ± 14.47*,*1	68.04 ± 21.59*1
	MK	68.41 ± 21.53*1	65.91 ± 18.01*1	54.99 ± 9.63*,*1	64.18 ± 11.82*1	50.48 ± 10.06*,*1	48.64 ± 11.96*,*1
	MKI	73.25 ± 10.69*1	88.75 ± 13.23*1	63.75 ± 24.30*1	46.13 ± 13.49*,*1	46.88 ± 10.86*,*1	41.88 ± 13.14*,*1
	Control	48.63 ± 6.25	46.88 ± 5.05	46.13 ± 7.06	44.25 ± 6.73	48.50 ± 9.32	49.50 ± 8.97
HCO_3 Meq/L	M	18.93 ± 3.75	19.70 ± 3.61	20.54 ± 3.06	20.49 ± 3.39	20.38 ± 4.92	21.54 ± 3.78*1
	MK	20.61 ± 5.44	20.51 ± 4.22	23.86 ± 2.39	23.39 ± 1.83*1	21.83 ± 2.78*1	20.29 ± 2.00*1
	MKI	17.08 ± 3.29	23.34 ± 6.06*1	17.68 ± 5.72	14.09 ± 3.83*	13.63 ± 1.71*	13.19 ± 2.35*
	Control	17.53 ± 2.83	16.35 ± 2.12	19.05 ± 2.65	18.99 ± 2.67	15.43 ± 2.98	13.99 ± 1.47
tCO_2 mM L /	M	19.8 ± 3.99*1	20.73 ± 3.68*1	21.61 ± 3.21	20.63 ± 5.12*1	21.66 ± 4.34*1	22.55 ± 3.93*1
	MK	19.3 ± 6.64*1	20.4 ± 5.55*1	26.15 ± 3.09*,*1	25.84 ± 2.19*,*1	21.96 ± 4.55*1	20.84 ± 2.73*1
	MKI	12.38 ± 3.11	29.0 ± 9.00*	17.75 ± 8.27*	13.86 ± 4.96	11.0 ± 3.93	9.0 ± 3.12
	Control	14.0 ± 3.46	12.99 ± 2.72	15.05 ± 2.96	14.76 ± 2.86	11.14 ± 4.25	8.19 ± 2.59
BE mM/L	M	−4.13 ± 3.34	−3.49 ± 3.78*1	−3.01 ± 3.66	−4.50 ± 5.39	−2.74 ± 4.55*1	−2.13 ± 4.44*1
	MK	−3.2 ± 7.40	−4.35 ± 4.99*1	0.03 ± 3.38*1	−1.94 ± 1.73*1	−4.41 ± 3.44*1	−5.43 ± 2.79*1
	MKI	−9.35 ± 4.29	−1.65 ± 6.73*,*1	−8.55 ± 7.49*1	−13.01 ± 5.03*1	−12.93 ± 2.69	−14.16 ± 3.30*
	Control	−8.45 ± 3.93	−10.08 ± 3.02	−6.39 ± 3.61	−6.46 ± 3.61	−9.86 ± 4.49	−12.91 ± 2.38
BEecf mM/L	M	−5.48 ± 3.85	−4.66 ± 4.05*1	−3.93 ± 3.71	−5.91 ± 5.68	−3.86 ± 4.12*1	−2.89 ± 4.63*1
	MK	−5.31 ± 8.03	−3.70 ± 6.19*1	−0.11 ± 3.70	−1.71 ± 2.12*1	−4.16 ± 3.95*1	−6.26 ± 3.37*1
	MKI	−12.09 ± 4.50	−0.56 ± 8.10*,*1	−9.49 ± 8.61	−14.31 ± 5.54	−15.10 ± 3.35	−16.70 ± 3.37
	Control	−10.76 ± 4.11	−11.71 ± 3.75	−8.79 ± 3.79	−8.86 ± 3.80	−12.96 ± 4.71	16.18 ± 2.47
O_2SAT %	M	90.98 ± 9.94	92.65 ± 3.13	93.56 ± 5.25*1	89.95 ± 6.90*1	87.83 ± 8.98	88.41 ± 14.24
	MK	88.09 ± 15.82	89.09 ± 9.85	85.04 ± 10.48	90.33 ± 2.85	85.18 ± 6.89	78.45 ± 14.90
	MKI	93.38 ± 3.66	97.75 ± 3.49*1	78.38 ± 29.51*	74.75 ± 20.28*	77.63 ± 15.95*	74.00 ± 18.44*
	Control	82.38 ± 7.34	80.63 ± 6.69	82.63 ± 6.11	81.25 ± 5.92	83.38 ± 5.92	84.25 ± 6.67

* Comparison between anaesthetic intervals at the same line (P < 0.05). *1 – statistical significance between study group and control group in the same column (P < 0.05).
pH – venous blood pH, PCO_2 – partial venous blood carbon dioxide, HCO_3 – bicarbonate, tCO_2 – total carbon dioxide, BE – base excess, BEecf – extra cellular fluid, O_2SAT – oxygen saturation, M – midazolam, MK – midazolam-ketamine, MKI – midazolam-ketamine-isoflurane

Table 3. Serum biochemical values of each group and their statistical significance (M ± SD)

Values	Group	0 min	10 min	30 min	60 min	180 min	300 min
ALP (I/U)	M	5.57 ± 0.62	5.16 ± 0.62[*,*1]	5.14 ± 0.62[*]	5.12 ± 0.52[*]	4.84 ± 0.62[*]	4.65 ± 0.78[*,*1]
	MK	5.92 ± 0.21	5.54 ± 0.50[*]	5.25 ± 1.04[*,*1]	5.39 ± 0.80[*]	5.33 ± 0.67[*]	5.59 ± 0.42[*]
	MKI	5.54 ± 0.23	4.75 ± 0.62[*,*1]	4.45 ± 0.70[*,*1]	4.5 ± 0.59[*,*1]	4.8 ± 0.62[*]	4.67 ± 1.19[*]
	Control	5.98 ± 0.03	5.96 ± 0.09	5.90 ± 0.10	5.66 ± 0.20	5.03 ± 0.36	5.15 ± 0.38
ALT (I/U)	M	41.5 ± 51.92[*1]	44.25 ± 33.45[*1]	35.75 ± 33.91[*1]	36.37 ± 30.99	31.12 ± 23.33[*]	28.25 ± 22.92[*]
	MK	21.62 ± 7.50	19.5 ± 7.17	21.13 ± 1.24	24.63 ± 12.14	22.25 ± 9.57	23.75 ± 10.60
	MKI	22.75 ± 5.12	18.0 ± 4.27	19.25 ± 7.77	21.37 ± 6.50	24.63 ± 9.92	34.13 ± 16.98[*,*1]
	Control	15.63 ± 8.61	15.38 ± 7.48	13.88 ± 5.48	16.38 ± 6.23	15.13 ± 5.05	17.5 ± 5.48
AST (I/U)	M	32.13 ± 24.52	47.38 ± 27.07	58.38 ± 30.61[*,*1]	63.13 ± 30.42[*,*1]	77.0 ± 31.74[*,*1]	96.75 ± 49.55[*,*1]
	MK	24.13 ± 9.06	32.63 ± 9.02	37.38 ± 14.7	46.63 ± 18.42[*]	55.13 ± 21.67[*,*1]	51.63 ± 16.69[*]
	MKI	22.38 ± 3.81	39.0 ± 16.37	38.5 ± 14.45	62.63 ± 22.3[*,*1]	84.5 ± 38.92[*,*1]	100.38 ± 67.51[*,*1]
	Control	14.88 ± 4.01	17.0 ± 4.53	18.75 ± 4.59	23.5 ± 7.17	22.88 ± 6.05	35.88 ± 9.77
BUN (mg/d*l*)	M	18.19 ± 4.13	21.30 ± 4.13[*]	19.39 ± 4.09	20.81 ± 4.56	21.31 ± 4.92[*]	19.86 ± 3.35
	MK	18.14 ± 3.61	18.88 ± 4.02	18.96 ± 5.21	20.09 ± 5.66	22.61 ± 3.03[*]	24.55 ± 3.69[*,*1]
	MKI	16.04 ± 5.76	13.44 ± 2.79[*,*1]	13.18 ± 2.89[*]	13.29 ± 2.89[*,*1]	14.21 ± 3.50[*1]	15.94 ± 4.60[*1]
	Control	19.24 ± 4.60	18.58 ± 4.70	20.54 ± 4.73	20.35 ± 3.88	19.11 ± 1.07	19.29 ± 1.64
Ck-Mb (I/U)	M	480.15 ± 196.42	353.72 ± 107.62	424.5 ± 69.14	436.5 ± 69.57	387.02 ± 156.50	319.41 ± 141.05
	MK	645.80 ± 640.59	655.87 ± 695.48	646.91 ± 743.31	626.88 ± 687.33	660.12 ± 511.89	458.96 ± 202.05
	MKI	804.98 ± 664.33[*1]	325.53 ± 136.25[*]	348.73 ± 133.76[*]	612.86 ± 203.60[*]	645.60 ± 207.90[*1]	421.97 ± 150.94[*]
	Control	376.06 ± 99.0	387.16 ± 106.11	321.61 ± 77.29	446.67 ± 46.5	527.4 ± 66.91	479.96 ± 27.63
Cr (mg/dL)	M	1.05 ± 0.21	1.02 ± 0.24	1.12 ± 0.13	1.02 ± 0.22	1.0 ± 0.34	1.0 ± 0.25
	MK	1.08 ± 0.18	1.01 ± 0.16	0.93 ± 0.26[*]	0.93 ± 0.14[*]	0.98 ± 0.06	0.98 ± 0.17
	MKI	1.3 ± 0.40	1.14 ± 0.16[*]	1.09 ± 0.16[*]	1.05 ± 0.20[*]	1.05 ± 0.18[*,*1]	1.09 ± 0.34[*]
	Control	1.02 ± 0.13	1.06 ± 0.16	1.16 ± 0.16	0.93 ± 0.14	0.79 ± 0.08	0.88 ± 0.08
Glu (mg/dL)	M	141.88 ± 23.86	244.63 ± 109.60[*,*1]	264.88 ± 66.63[*,*1]	292.63 ± 62.58[*,*1]	233.00 ± 112.56[*,*1]	22.63 ± 137.03[*,*1]
	MK	128.13 ± 45.36	183.25 ± 46.75[*]	238.75 ± 72.81[*,*1]	267.00 ± 77.05[*,*1]	220.38 ± 115.30[*,*1]	208.13 ± 149.34[*,*1]
	MKI	81.75 ± 32.81[*]	187.38 ± 4.70[1*]	170.75 ± 39.47[*]	232.00 ± 51.61[*,*1]	219.13 ± 53.73[*,*1]	138.88 ± 43.87[*]
	Control	125.25 ± 34.62	156.88 ± 68.92	168.88 ± 63.33	138.88 ± 13.10	132.00 ± 14.59	125.63 ± 16.06
LDH (I/U)	M	393.13 ± 167.68	355.38 ± 121.88	535.38 ± 252.56	474.25 ± 169.53	378.75 ± 133.05	321.13 ± 129.96
	MK	395.00 ± 217.94	395.25 ± 158.46	473.00 ± 225.60[*1]	592.88 ± 204.35[*,*1]	865.25 ± 352.50[*,*1]	644.25 ± 247.82[*]
	MKI	404.38 ± 120.31	365.25 ± 151.04	469.50 ± 175.09[*,*1]	866.25 ± 346.09[*,*1]	1263.13 ± 895.82[*,*1]	1389.50 ± 106.49[*,*1]
	Control	105.88 ± 52.74	129.13 ± 64.27	136.63 ± 76.81	280.00 ± 98.97	297.88 ± 74.27	367.38 ± 109.85
TP (g/dL)	M	7.25 ± 1.28	6.60 ± 1.52[*]	6.65 ± 1.42[*]	6.61 ± 1.05[*]	6.16 ± 1.59[*]	6.10 ± 1.61[*]
	MK	6.85 ± 0.62	6.41 ± 0.64[*,*1]	5.94 ± 1.17[*,*1]	6.0 ± 1.16[*]	6.28 ± 0.70	6.13 ± 0.76[*]
	MKI	7.0 ± 0.76	5.75 ± 0.48[*]	5.46 ± 0.79[*]	5.56 ± 0.52[*]	5.66 ± 0.68[*]	5.41 ± 1.25[*]
	Control	7.45 ± 0.68	7.26 ± 0.54	7.11 ± 0.67	6.31 ± 0.47	5.99 ± 0.35	5.71 ± 0.32
cTnI (µg/dL)	M	0.1 ± 0.07	0.07 ± 0.00	0.10 ± 0.05	0.16 ± 0.07	0.23 ± 0.12	0.27 ± 0.09
	MK	0.07 ± 0.00	0.07 ± 0.01	0.07 ± 0.00	0.08 ± 0.02	0.11 ± 0.12	0.13 ± 0.08
	MKI	0.09 ± 0.04	0.21 ± 0.31	0.39 ± 0.84	0.47 ± 0.96	0.76 ± 1.32[*1]	1.26 ± 2.29[*,*1]
	Control	0.07 ± 0.00	0.07 ± 0.01	0.1 ± 0.05	0.12 ± 0.10	0.11 ± 0.06	0.11 ± 0.07

[*] Comparison between anaesthetic intervals at the same line ($P < 0.05$). [*1] – statistical significance between study group and control group in the same column ($P < 0.05$). ALP – alkaline phosphatase, ALT – alanine aminotransferase, AST – aspartate aminotransferase, BUN – blood urea nitrogen, Ck-Mb – creatine kinase-Mb, Cr – creatine, Glu – glucose, LDH – lactate dehydrogenase, TP – total protein, cTnI – cardiac troponin, M – midazolam, MK – midazolam-ketamine, MKI – midazolam-ketamine-isoflurane

Table 4. Histopathological evaluation of apoptotic and normal cell numbers of each group (median (min-max))

Tissue	Group	Apoptopic cell	Normal cell
Liver	M	306.0 (90–430)*	341.0 (257–545)
	MK	470.0 (422–572)*	323.0 (297–342)
	MKI	398.0 (141–520)	278.0 (156–463)
	Control	313.0 (176–512)	405.0 (327–510)
Brain	M	232.5 (151–343)	249.0 (179–282)
	MK	246.5 (221–441)**	200.5 (178–283)
	MKI	259.0 (120–412)**	130.5 (120–198)
	Control	151.0 (120–210)	247.0 (163–305)
Myocardium	M	354.0 (266–372)**,*	213.0 (197–220)
	MK	306.0 (265–420)**,*	282.5 (225–332)
	MKI	197.0 (135–248)	302.5 (203–329)
	Control	184.5 (152–224)	279.0 (196–354)

** Difference between the groups in the same column is statistically significant (P < 0.001), * Difference between the experiment and control group is statistically significant (P < 0.05). M – midazolam, MK – midazolam-ketamine, MKI – midazolam-ketamine-isoflurane

Discussion

It was determined that midazolam, ketamine, and isoflurane anaesthesia caused changes in rabbit organs. In the clinical analysis, after anaesthesia, the low BT and RR were found to be significant statistically (P < 0.05) (Table 1). The drop in body temperature observed in the experimental groups 10 min after anaesthetic application is the result of suppression of the thermoregulation centre by the anaesthetics. Environmental temperature and progressing vasodilatation contributed to the development of hypothermia (15, 16, 18).

When venous blood gases were evaluated, the determined changes were within references ranges (2). Especially in the M and MK groups at 30 and 60 min, and in the MKI group except for at 300 min, pH values decreased significantly at the other time points, which was compatible with the increase in PCO2 at the same times and this condition supported a suspicion of moderate respiratory acidosis. In the study groups, changes were particularly pronounced at 30 and 60 min. Respiratory acidosis was present as reported in the literature which seems to occur as a result of suppression of the respiratory system by anaesthetics (24, 25).

The Ck-Mb and cTnI values are indicators for degeneration of the heart muscle as a result of arrhythmia occurring during anaesthesia (27). In the present study, Ck-Mb and cTnI values in each study group were different at different sampling times. Differences observed in the Ck-Mb and cTnI values are believed to be the result of a fall in the heart rate caused by the anaesthetic agents and this occurred due to hypoxia, which was determined by blood gas analysis. Blood gas analysis showed that in experimental groups, starting from 10 min, PCO2 generally increased and

PO2 decreased and these were the evidence of hypoxia (Table 2). But in the MKI group, after 30 min, PCO2 values did not increase due to O2 reinforcement. Furthermore, Glu and LDH values were also higher than their reference ranges and these increases also supported hypoxia (Table 3).

During anaesthesia, due to anaesthetic agents used, respiratory and cardio vascular system depression occurs and this reduces tissue perfusion. After deterioration of tissue perfusion and slowing circulation, the anaerobic metabolism starts to produce lactate. Increased lactate causes an increase in blood glucose level. In this situation, the liver produces glucose more than normal and LDH values rise (10). Furthermore, the reason for an intensification in LDH and AST activity is an increase in the concentrations of anaesthetic agents in the liver and subsequently developing uplift in the cellular enzyme secretion.

In general, the presence of high enzyme values compared to control values proved the effects of anaesthetic agents on Glu, LDH, and AST. On the other hand, apart from Glu, LDH, and AST values, biochemical parameters were within reference ranges (24). In the MKI group, LDH values (1,263.1 ± 895.8 IU/L and 1,389.5 ± 1,060.5 IU/L) obtained at 180 and 300 min, respectively were higher than those of the same group at 0 min and other groups at the same time and this shows the important effect of the triple combination (the MKI group) on LDH. Although LDH values in other groups were in the reference ranges, when the values were compared to their baseline values they were higher. It can be speculated that these increases could be due to organ hyperaemia occurred in the study groups. When the triple combination was used, it is believed to have had a more toxic effect at organ level on LDH value than other groups. Several anaesthetic agents are metabolised in the liver and removed from the body through the kidneys. Therefore, anaesthetic agents have more toxic effects on the liver and kidneys (17). Changes caused by midazolam and ketamine as a result of their metabolism in the liver were compatible with the literature data consulted (13, 25).

In the present study, apoptotic brain tissue cell numbers were different in MK, MKI, and control groups. The number of apoptotic cells in the MKI group was higher than in the M, MK, and control groups. However, the differences were only statistically significant between the MK and MKI groups (P < 0.05). In rats, intratracheal usage of 5% ketamine caused neurological changes (1). But in humans, the usage of ketamine caused none (4). The neurodegenerative changes and apoptosis in nerve tissue determined in the present study were in agreement with the results obtained from rodents. Toxic effects of ketamine on the nervous system in these animals may manifest even at normal parenteral doses. Furthermore, in rabbits receiving midazolam and preservative-free midazolam, evident histopathological

changes were also observed under light and fluorescence microscopy (12). Midazolam has neurotoxic properties when administered neuroaxially *in vivo*. It also induces neurodegeneration with other general anaesthetics in neonatal animals. Especially in studies on apoptosis, anaesthetic agents used at different concentrations inhibit mitochondrial activity and induce apoptosis (26).

In the present study, it has also been shown that ketamine, midazolam, and triple combination anaesthesia may cause apoptosis, suggested by the lesser extent of apoptosis in the controls, which supports the above studies. But, in different organs, there are different effects. In the MK group the apoptotic effects on the liver cells were especially more pronounced than those of the midazolam study group. A greater number of apoptotic cells in the liver, especially in the MK group, the potential to be due to metabolism of midazolam and ketamine in the liver, participation of inflammatory mediators in portal circulation together with a greater amount of catecholamine, and attribution to an increase in synergistic effects of these two anaesthetic agents, all these circumstances showed compatibility with the literature data (5, 13). In addition, increases in the enzyme values obtained in the present study support histopathological apoptotic changes in the liver.

Oxygen saturation may be reduced if the combination of anaesthetic agents is appropriate to provoke this. Diminished oxygen saturation results in hypoxia and hypothermia leading to neuronal cell death in the brain (20). In addition, anaesthetic agents that have water solubility such as midazolam, and anaesthetics that can pass easily into fatty tissue, other tissues, and cross the blood brain barrier have high affinities to GABA and NMDA receptors in CNS, so may cause increase in apoptosis.

The increase in apoptosis in brain cells in this study was in agreement with this explanation and the literature data (5, 11, 23). Changes in oxygen saturations and body temperatures, which are affected by the anaesthetic agents used, probably increase the rate of apoptosis and apoptotic cell count. However, the highest number of apoptotic cells being in the MKI group supported the theory that it was the effect of oxygen saturation. Furthermore, ketamine and isoflurane support cerebral blood flow and increased intracranial pressure, supporting increased cerebral metabolism with increased blood flow. Their combination has also been reported to block the calcium channels, reduce the intracellular calcium flux, and prevent the release of neurotransmitter substances on glutamate NMDA receptors, thus, it shows a neuroprotective effect in the brain (11, 28). The fact that the apoptotic cell number in the brain differs from that of control groups, especially in the MK and MKI groups, suggests that ketamine and isoflurane may cause apoptosis in brain cells. In histopathological examinations in the course of the present study,

hyperaemia and focal bleeding in the cerebral vessels of experimental groups were in agreement with the literature data insofar as they were from increasing intracranial pressure and blood flow (11, 28). Furthermore, the histopathological findings obtained by Auroy *et al.* (3) investigating the neurotoxic effects of midazolam were compatible with the histopathological findings of the present study.

Isoflurane inhalation anaesthesia inflicted a lower apoptotic cell number in the myocardium than in the M and MK groups. The rise in apoptotic cell numbers detected in the heart supported the assumption that anaesthetic agents exert such effects on the cardiovascular system.

The increased number of apoptotic cells in the M and MK groups led to a more pronounced negative chronotropic effect on the cardiovascular system of midazolam and ketamine, whereas in group MKI, the presence of lower numbers of apoptotic cells compared to groups M and MK was thought to be due to oxygen support during anaesthesia.

In conclusion, the anaesthetic agents used may normally increase the occurrence of programmed apoptosis in body systems. The MKI anaesthesia combination used in the study group was found to cause less cell destruction in general than in MK group and anaesthetic agents used in the other experimental groups. Therefore, when the advantages such as safe entry to anaesthesia, prolongation of anaesthesia, and oxygen support are taken into account, it was observed that MKI combination anaesthesia caused less toxic effect in rabbits than midazolam and midazolam-ketamine, and provided safer anaesthesia. Considering this situation, it is suggested that the selection of anaesthesia combinations is important and the studies on this subject are inadequate, and it is needful to continue them.

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