

Analyses of anaesthesia with ketamine combined with different sedatives in rats

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ABSTRACT: The goal of this study was to establish an easy-to-perform, reliable, and safe protocol for intraperitoneal injection anaesthesia in rats, as well as an easy method to assess anaesthesia depth under routine clinical conditions. Seventy Wistar rats (35 males and 35 females) were used to evaluate intraperitoneal anaesthesia with ketamine (75 mg/kg) combined with one of the following central nervous system depressors: acepromazine (2.5 mg/kg), diazepam (5 mg/kg), medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and xylazine (2.5 mg/kg). Significant differences were found between the combinations in ataxia, recovery and righting parameters, as well as respiratory and heart rates and haemoglobin saturation by oxygen, whereas significant differences between sexes for the parameters of ataxia, hypnosis, recovery, righting and exploratory behaviour were observed upon waking. The best results for parameters such as induction, and maintenance and recovery from anaesthesia (more regular and stable) were observed with α_2 -agonists: ketamine/xylazine in males and ketamine/medetomidine in females.

Keywords: anaesthesia; rats; ketamine; sedatives

The daily tasks of clinical veterinarians are diverse and complex, and they must possess broad knowledge in diverse disciplines of veterinary medicine as well as the ability to implement a wide variety of procedures successfully. Owing to the diversity of species that are presented at veterinary clinics – with increasing frequency rodents and exotic animals – clinical veterinarians must be cognisant of the peculiarities of a large number of species in order to treat each case appropriately. Among rodents, there is a current tendency towards rats (*Rattus norvegicus*) as pets, and therefore rats are increasing in frequency as patients in veterinary clinics. Like any animal, rats have pathologies specific to their species, and certain characteristics that the veterinarian should be aware of in order to properly handle this species. Anaesthesia, therefore, is essential to enable not only surgical approaches but also many types of manipulations that require immobilisation of the animal with drugs (Crowell-Davis and Murray 2006).

Further, laboratory animals, in particular rodents, play an important role in research, and the rat es-

pecially is one of the most important animal models in biomedical research. Ensuring the welfare of laboratory animals and avoiding unnecessary pain and suffering is one of the main considerations in animal experimentation, and is strictly regulated by the law. To allow the conducting of painless procedures on rodents in biomedical research, anaesthesia is usually required in experimental protocols (Flecknell 2009). Well-controlled anaesthesia is the basis for minimising the potential for intraoperative complications and ensures reproducible success of surgical interventions. For that reason, short-acting drugs are used, especially in animals that are difficult to intubate and in which venous access is difficult to obtain (Henke et al. 2004). Any adverse effects have to be acceptable from the standpoint of animal welfare; in particular, the anaesthetics should not be tissue irritants or otherwise pain-inducing, and mortality should be low. Furthermore, any new protocol has to fulfil practical considerations: the anaesthetic substances should be easily available and simple to prepare. Injection (usually via the intraperitoneal route [i.p.]) is one of the

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methods of choice in rodents. Among drugs which are used parenterally, ketamine combined with a number of other compounds, such as benzodiazepines, phenothiazines and α_2 agonists, has been used as a chemical restraint in different laboratory animals, allowing a dose-dependent response (Arras et al. 2001; Welberg et al. 2006).

The procedures described below utilised mixtures of two components, because no single substance can satisfy all the requirements of anaesthesia (e.g. analgesia, hypnosis, muscular relaxation) on its own. As a result, we put together combinations of ketamine (dissociative anaesthetics) with another single drug from each of the following groups: α_2 -agonists (xylazine or medetomidine), benzodiazepines (diazepam or midazolam) or phenothiazine (acepromazine), using published protocols and appropriate dose ratios. A total of five protocols were selected for detailed comparison.

These compounds and each anaesthetic protocol were evaluated according to different criteria, including availability (commercially available, legal restrictions) and ease of handling (availability as a sterile solution, ease of dilution, chemical stability), toxicity (mortality and adverse effects) and efficacy (quality of anaesthesia).

The goal of this study was to establish an easy-to-perform, reliable, and safe anaesthetic protocol for rats, as well as a method that is easy to adapt to the needs of a wide variety of veterinary clinics and research laboratories. To this end, we evaluated injectable anaesthesia by the intraperitoneal route using ketamine combined with different depressors in Wistar rats.

MATERIAL AND METHODS

Seventy Wistar rats (*Rattus norvegicus*), 35 males and 35 females aged from 12 to 16 weeks, were used. Rats were obtained from the breeding colony of the Central Service of Animal Experimentation of the University of Cordoba (Spain). Each sex group was randomly divided into five groups of seven animals. Each group was housed in plastic cages at 20–24 °C, 55% \pm 10% relative humidity and with a 12-h light/dark cycle and *ad libitum* access to drinking water and a standard food-pellet diet (Panlab®, Barcelona, Spain). Each group of male animals was injected with a mixture of ketamine and a sedative. The same protocol was carried out for female groups.

The research procedures were carried out after approval by the animal care committee of the University of Cordoba (Spain) and in concordance with the European Directive for the Protection of Experimental Animals (Directive 2010/63/UE).

Drug procedures. Drugs were prepared as a mixture of ketamine-QN01AX03¹-(Imalgene 1000®- Merial, 75 mg/kg), with one of the following sedatives: acepromazine-QN05AA04-(Calmo Neosan®-Pfizer, 2.5 mg/kg), diazepam-QN05BA01-(Valium®-Roche, 5 mg/kg), midazolam-QN05CD08-(Dormicum®-Roche, 5 mg/kg), medetomidine-QN05CM91-(Domtor®-Pfizer, 0.5 mg/kg) or xylazine-QN05CM92-(Rompum®-Bayer, 2.5 mg/kg). Atipamezole (QV03AB90), as atipamezole hydrochloride (Antisedan®, Pfizer, Madrid, Spain), at a 0.5 mg/kg b.w. dose was used for reversal 90 min after injection of ketamine + medetomidine.

Experimental procedure. Rats were weighed (Sartorius BP210 D electronic balance) and marked on the tail with an indelible pen. An appropriate volume of the drug mixture was injected by the intraperitoneal route, lateral to the midline next to the umbilicus, as a single dose and introduced in an individual box. Another researcher, that was blinded to the mixture injected, tested and assessed variables and selected times. To exclude any effects of circadian rhythm, the experiments were always performed during the daytime from 9.00 AM to noon.

Time-related parameters of anaesthesia. After administration (recorded as injection time) the animal, in an individual cage, was observed for incoordination and ataxia (recorded as “ataxia time”) until it lost its righting reflex (recorded as “hypnosis time”). Then it was laid in dorsal recumbency without fixation on a homeothermic blanket to maintain body temperature. The animals breathed room air for the duration of the procedure. In order to assess the depth and extent of anaesthesia, the pedal withdrawal reflex (PWR) was tested every 5 min after the animal was placed on the homeothermic blanket, alternating between the left and the right limbs, until withdrawal was positive (Alves et al. 2007). The indicators were adopted from published protocols (Smith 1993; Whelan and Flecknell 1994; Reuelto et al. 2004; Flecknell 2009). In order to reduce sources of variation in response to the stimuli, all reflex tests were carried out and assessed by the same operator.

¹ATCvet code. http://www.whocc.no/atcvet/atcvet_index

A series of simple tests were carried out on each rat to evaluate the intensity and duration of the anaesthesia (Smith 1993; Flecknell 2009). To reduce sources of variation in response to the stimuli, all tests were conducted and assessed by the same operator. The intensity of the anaesthesia was assessed by recording the presence or absence of the pedal reflex as mentioned above. The point at which the PWR was negative was recorded as “surgical time”. The point at which the PWR gave a weak response was recorded as “recovery time”. The next point was the righting reflex recovery, and was recorded as “righting time”. Next, the animals were returned to their individual cages until exploratory reflex recovery (recorded as “exploratory time”) and observed for the next 2 hours for any abnormal behaviour.

Cardiorespiratory measurements. The cardiorespiratory parameters evaluated were respiratory rate (breaths/min), heart rate (beats/min) and haemoglobin saturation by oxygen (percentage). The respiratory rate (RR) was monitored at 10, 20, 40 and 60 min; the heart rate (HR) and haemoglobin saturation by oxygen (SpO₂) were monitored at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75 and 90 min from administration of the anaesthetic combination. All measures were taken when the rat was placed in dorsal recumbence on the homeothermic blanket until the righting reflex recovery. Respiratory rate was assessed by the same opera-

tor by evaluating movements of the thorax during sleep. Heart rate and haemoglobin saturation by oxygen were evaluated by a veterinary pulse-oxymeter (2000T veterinary transreflectance sensor). The pulse-oxymeter probe was placed over the base of the tail when the animal was laid in dorsal recumbency.

Data and statistical analysis. Data were recorded in a Microsoft-Excel macro sheet and are shown as mean, number of measurements (n) and standard deviation (s.d.). Statistical analysis was made using STATGRAPHICS: Chi-square “ χ^2 ” and Shapiro-Wilks statistic “W” were used for checking the fit of normal (Gauss) distribution. The normal distribution of data was always refused. Subsequently, a non-parametric Kruskal-Wallis test for comparison of multiple samples to assess possible differences between sexes for each specific parameter taking data from all drug combinations, but not each association separately, was performed. A Kruskal-Wallis test was also used to assess differences between specific parameters, but not separately for sexes. The Mann-Whitney test (Wilcoxon) for comparison between samples from two consecutive time points and consecutive time points under anaesthesia was carried out. Parametric (Pearson product moment) and non-parametric (Spearman rank) correlations were performed. The minimum significant value for a statistical hypothesis was $P < 0.05$.

Table 1. Statistic values of ataxia, hypnosis, loss and recovery of the pedal reflex, recovery, righting exploratory reflex from anaesthesia with ketamine + central nervous system depressor (CNSD) in rats (units: minutes); data expressed as mean \pm S.D.

| CNSD | Induction | | Anaesthesia | | Recovery | | |
|----------------|-----------------|-----------------|------------------|-------------------|--------------------|--------------------|--------------------|
| | ataxia | hypnosis | pedal reflex | | recovery | righting | exploratory |
| | | | loss | recovery | | | |
| Males | | | | | | | |
| Acepro-mazine | 0.85 \pm 0.44 | 2.70 \pm 2.48 | | | 52.17 \pm 21.80 | 84.5 \pm 20.15 | 90.33 \pm 25.81 |
| Diazepam | 0.32 \pm 0.08 | 1.22 \pm 0.41 | 6.16 \pm 2.41 | 28.83 \pm 4.35 | 45.5 \pm 5.36 | 66.17 \pm 9.06 | 67.33 \pm 8.41 |
| Medeto-midine | 0.78 \pm 0.24 | 1.44 \pm 0.34 | 2.11 \pm 0.26 | 92.25 \pm 0.5 | 92.5 \pm 0.58 | 114.75 \pm 22.95 | 120.75 \pm 27.94 |
| Midazolam | 2.09 \pm 1.29 | 2.15 \pm 1.17 | | | 45.6 \pm 10.50 | 67.8 \pm 24.08 | 68.8 \pm 24.08 |
| Xylazine | 1.03 \pm 0.30 | 1.24 \pm 1.09 | 7.73 \pm 1.99 | 29.06 \pm 3.38 | 46.67 \pm 28.15 | 61.17 \pm 28.08 | 63.5 \pm 29.15 |
| Females | | | | | | | |
| Acepro-mazine | 1.45 \pm 0.38 | 3.11 \pm 0.35 | | | 107.80 \pm 25.29 | 138.20 \pm 19.27 | 165.75 \pm 17.00 |
| Diazepam | 1.56 \pm 0.67 | 3.07 \pm 0.92 | 11.50 \pm 2.38 | 43.75 \pm 20.69 | 77.80 \pm 15.96 | 142.00 \pm 44.80 | 153.80 \pm 40.78 |
| Medeto-midine | 1.48 \pm 0.29 | 2.80 \pm 0.34 | 4.50 \pm 1.05 | 95.00 \pm 3.52 | 99.67 \pm 5.75 | 151.17 \pm 43.25 | |
| Midazolam | 2.12 \pm 1.32 | 4.03 \pm 2.53 | | | 90.40 \pm 13.01 | 137.20 \pm 14.03 | 144.00 \pm 12.11 |
| Xylazine | 1.39 \pm 0.35 | 3.77 \pm 1.28 | 9.00 \pm 1.41 | 36.00 \pm 22.63 | 50.60 \pm 15.19 | 75.00 \pm 13.73 | 95.40 \pm 14.98 |

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Table 2. Non-parametric statistical analysis (Kruskall-Wallis H test) for comparison between sexes and combinations for ataxia, hypnosis, loss and recovery of the pedal reflex, recovery, righting exploratory reflex

| CNSD | Induction | | Anaesthesia | | Recovery | | |
|---|-----------------|-----------------|--------------|----------|-----------------|-----------------|-----------------|
| | ataxia | hypnosis | pedal reflex | | recovery | righting | explora-tory |
| | <i>H</i> -value | <i>H</i> -value | loss | recovery | <i>H</i> -value | <i>H</i> -value | <i>H</i> -value |
| Between sexes (<i>P</i> -value)* | 15.7032* | 20.3744* | 2.89053 | 3.21651 | 16.9149* | 20.7567* | 20.3128* |
| Between combinations (<i>P</i> -value)* | 13.2306* | 6.97272 | 16.673* | 18.7699* | 16.7319* | 15.363* | 6.19343 |

*significant differences (*P* < 0.05)

RESULTS

Time-course of different anaesthetic stages and planes

Time-related parameters of anaesthesia for male rats are listed in Table 1, which shows mean values and standard deviations of each parameter for each sedative combined with ketamine. In the same way, time-related parameters of anaesthesia for female rats are listed in Table 1. The male global mean values are lower than those for females. It is important to note that the pedal withdrawal reflex was observed in both sexes in response to diazepam, medetomidine and xylazine, whereas it was not observed for acepromazine or midazolam. Non-parametric Spearman rank correlation test indicated that loss of PWR was not coincident with chronological recuperation ($r = 0.092$; $P > 0.05$). Other time-related parameters of induction of anaesthesia (ataxia and hypnosis), as well as recovery from anaesthesia (recovery, righting and exploratory behaviour), were obtained for all combinations. All animals injected with the medetomidine com-

bination were also injected 90 min subsequently with atipamezol; yet, two males still died under anaesthesia. Non-parametric ANOVA (Kruskal-Wallis test) was used to compare between sexes and between combinations (Table 2) because data were not normally distributed (Chi-squared and Shapiro-Wilks statistic test).

Physiological parameters

Respiratory rate (RR). The respiratory rate data were not normally distributed ($W = 0.894$; $P < 0.01$). The mean values for this physiological indicator are shown in Figure 1. The data and the evolution of the indicator between sexes were different: the combination with midazolam showed higher values in males, while the combination with diazepam was highest in females. The Kruskal-Wallis test showed significant differences ($P < 0.05$) between sexes and between anaesthetic combinations (ketamine combined with a sedative drug). Two males exhibited a respiratory depression with less deep breaths, leading to apnoea and finally death.

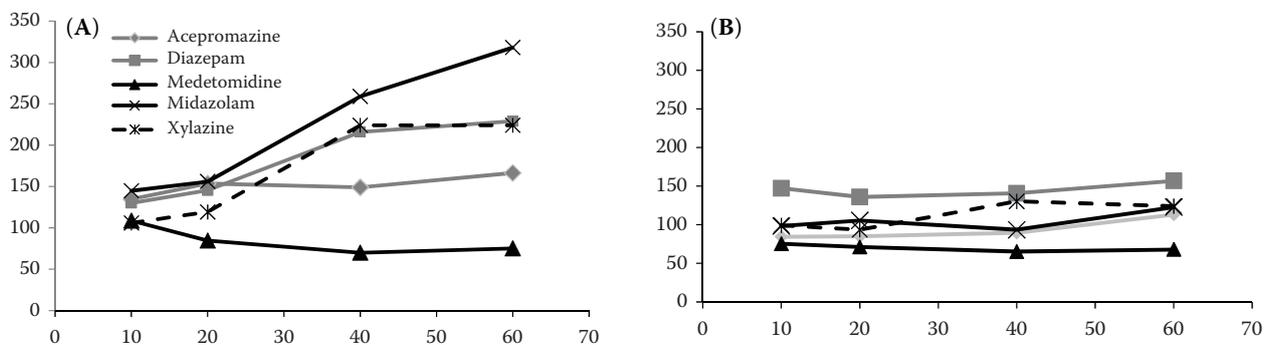


Figure 1. Respiratory rate in males (A) and in females (B), for the combinations of ketamine with a central nervous system depressor.

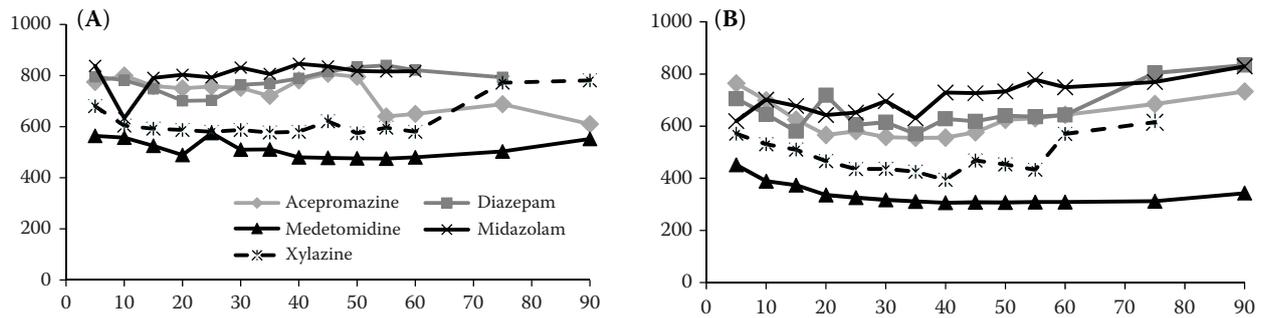


Figure 2. Heart rate: mean values of males (A) and females (B) at 5, 10, 15, 20, 25, 30, 35, 40, 45, 40, 55, 60, 75 and 90 min

Heart rate (HR). The mean HR values are shown in Figure 2. The HR data were not normally distributed ($W = 0.895$; $P < 0.01$) and were different between sexes and between times. The Kruskal-Wallis test showed that there were significant differences between sexes and between anaesthetic combinations ($P < 0.05$). The heart rhythm was irregular with all the combinations in females, while in males this tendency was not as high. Males had higher mean values than females at different times with different combinations (Figure 2). The medetomidine combination induced a low and more regular heart rhythm in both sexes and the lowest data were found for females.

The Mann-Whitney (Wilcoxon) test showed that there were significant differences between all the sedative results ($P < 0.05$), but comparison between HR values for acepromazine and for diazepam were statistically similar and differences were not found ($P > 0.05$).

Haemoglobin saturation by oxygen (SpO_2). The mean global values of percentage haemoglobin saturation by oxygen for the two sexes and at different times monitored are shown in Figure 3, for males (a) and for females (b). Similar to the physiological parameters described above, the data were not normally distributed ($W = 0.903$; $P < 0.01$). The

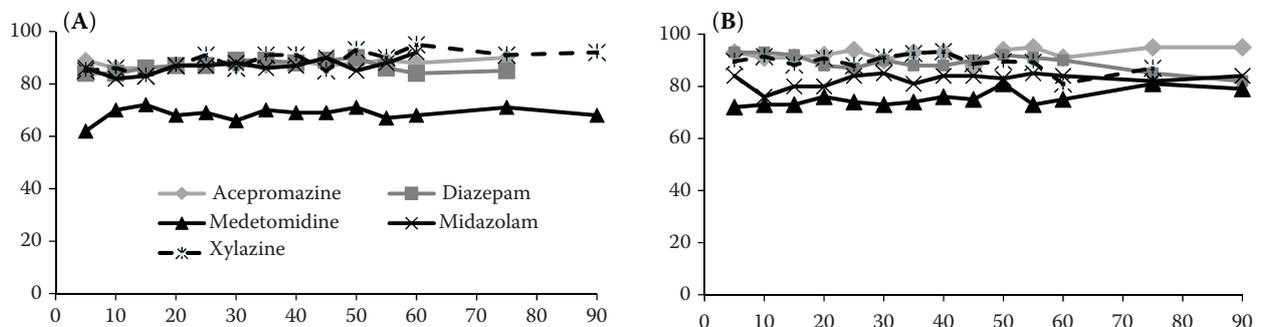


Figure 3. Haemoglobin saturation by oxygen percentage in males (A) and females (B) at 5, 10, 15, 20, 25, 30, 35, 40, 45, 40, 55, 60, 75 and 90 min

Kruskal-Wallis test showed significant differences between combinations ($P < 0.05$) and between sexes ($P < 0.05$). The lowest observed values were for the medetomidine combination and two males eventually died as a result.

We found that there was a good correlation for all values of SpO_2 versus all values of RR ($r = 0.658$; $P < 0.001$) and a low correlation in the case of SpO_2 versus HR values ($r = -0.140$; $P > 0.05$). These results suggest that respiratory rate could be an important factor for SpO_2 , and we established the following equation to relate both variables by linear regression: $SpO_2 = 63.3761 + 0.1263 \times RR$.

DISCUSSION

Regulatory bodies place particular emphasis (European Union: Directive 2010/63/CE and Spanish: "R.D. 53/2013") on reducing unnecessary pain, stress and suffering, as well as the use of large numbers of animals. For these reasons, we used only 35 male and 35 female rats, and each animal received one anaesthetic combination.

The objective of the present study was to analyse anaesthetic combinations of ketamine and

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one of several central nervous system depressors. In veterinary medicine, ketamine is one of the most widely used anaesthetic agents in all animal species. Ketamine is a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor, and elicits depressed activity of the thalamocortical and limbic system, and depression of nuclei in the reticular activating system (Posner and Burns 2009). The drug induces a state of dissociative sedation and analgesia. Ketamine also exerts adverse effects in rodents, such as respiratory depression at high doses, increased salivary secretions, poor muscle relaxation, prolonged induction time and a very short sleep time (Dittmar et al. 2004; Welberg et al. 2006; Struck et al. 2011). Ketamine was selected as an anaesthetic drug at 75 mg/kg b.w., because in rats the dose can range from 30 to 200 mg/kg b.w. and can be administered by several routes (Hawk and Leary 1999; Flecknell 2009; Struck et al. 2011; Redfors et al. 2014).

Ketamine alone is not a good anaesthetic, and it is usually combined with other drugs (Hawk and Leary 1999; Arras et al. 2001; Orr et al. 2005; Flecknell 2009; Wellington et al. 2013). We used acepromazine, diazepam, medetomidine, midazolam and xylazine in order to establish which combination could best establish a short (but sufficient) anaesthetic period, as well as recovery, spontaneously or after the use of the specific antagonist atipamezol, in the case of the medetomidine combination. The sedatives for each anaesthetic combination with ketamine were selected according to commercial availability (injectable medicine), chemical stability, ease of handling and dilution, and legal restrictions, as well as efficacy (quality of anaesthesia) and toxicity (mortality and adverse effects) criteria (Arras et al. 2001). Doses of the sedatives used in this study were in accordance with doses recommended by previous investigations (Wamber et al. 1996; Hawk and Leary 1999; Flecknell 2009). An intraperitoneal anaesthesia provides a shorter time to recumbency, a longer total sleep time, and a more consistent loss of toe-pinch response (Struck et al. 2011).

Preliminary studies by different authors have reported that medetomidine induces a long period of sedation with a severe decrease in the respiratory rate and of arterial haemoglobin saturation by oxygen; to keep the beneficial effects, we used medetomidine as has been recommended (Henke et al. 2004; Hahn et al. 2005). Baker et al. (2011) reported that the administration of atipamezole over a short

period of time may lead to a “re-sedation effect”. We established a time of 90 min after the medetomidine injection for the administration of the antagonist.

When ataxia and hypnosis were evaluated, we observed that, in general, males were in ataxia and hypnosis before females, with significant differences between sexes. These differences were also manifest between combinations in the case of ataxia, but not with respect to the appearance of hypnosis. The diazepam combination produced ataxia and hypnosis earlier in males, whereas in females, ataxia was earlier with xylazine and the onset of hypnosis occurred quicker with medetomidine. Midazolam produced ataxia and hypnosis later in females, whereas in males, midazolam was the combination that produced ataxia at the slowest rate, and acepromazine was the slowest inducer of hypnosis. Once animals underwent hypnosis, they were placed on a homeothermic blanket to avoid loss of body temperature because hypothermia can be one of the most frequent causes of mortality in animals undergoing anaesthesia (Flecknell 2009).

In general, all males treated with the α_2 -agonist/ketamine combination lost PWR and this occurred earlier than in females. These observations are in contrast to data obtained in mice by Serrano et al. (2013) who reported that female mice lost PWR earlier than males in response to treatment with α_2 -agonists. However, the rats did not lose the pedal reflex after treatment with the acepromazine and midazolam combinations. Therefore, the α_2 -agonists as well as diazepam combinations with ketamine produced a relatively good anaesthetic level. However, it should be noted that once the PWR returned in diazepam-treated females, these rats needed approximately 30 min to recover. This long period of sleep precluded the performing of any painful manipulations. In the case of the medetomidine combination in males, in two rats there was an intense respiratory depression and low haemoglobin saturation by oxygen (SpO_2). This required administration of atipamezol in order to reverse these signs, but in spite of this, the two rats died. The relatively good results, in both males and females, for α_2 -agonist combinations (xylazine in males, and medetomidine in females), may be related to the described analgesic action of this group of drugs, which is why these combinations have been recommended by some authors (Cruz et al. 1998; Arras et al. 2001; Orr et al. 2005). Consequently, only the combination of α_2 -agonists and ketamine seems to be adequate at these doses for use as an anaesthetic because it reduces pain

perception, as previously reported by other authors (Cruz et al. 1998; Arras et al. 2001). Similar to other authors, we did not observe an increase of diuresis (side-effect of α_2 -agonists), nor salivary secretion (Cruz et al. 1998; Serrano-Caballero et al. 2013).

In agreement with Cruz et al. (1998), we also found that females recovered later after treatment with the ketamine/medetomidine combination than males, and with respect to different combinations, medetomidine was the drug that induced the longest period of anaesthesia in both sexes (Grint and Murison 2008). However, similar mean durations of anaesthesia with combinations of midazolam and medetomidine with ketamine at different doses have been reported in rabbits by Grint and Murison (2008). These observations differ from our data in which midazolam did not induce anaesthesia.

In most of the parameters evaluated, there were significant variations related to sex ($P < 0.05$), which indicates that the sex of the rat being anaesthetised must be taken into account, as it may become relevant from a clinical point of view (Zambricki and D'Alecy 2004).

The small respiratory depression elicited by the acepromazine combination is in agreement with data reported by Flecknell (2009). Medetomidine led to a more depressed respiratory rate and haemoglobin saturation by oxygen level as a consequence of its well-known sympathetic neuro-vegetative depression at the presynaptic level, and, thus, it might be suitable to include oxygen as a supplement to the room air that is breathed for the duration of the procedure. The lowest HR was observed with medetomidine and xylazine, similar to that reported by Redfors et al. (2014) who, using the ketamine/xylazine combination (30/5 mg/kg), observed a HR even lower than seen in our study with the same anaesthetic combination but at a higher concentration (75/5 mg/kg). It has been reported that the effect of combinations of ketamine and xylazine depends on the quantities of ketamine and xylazine (Arras et al. 2001; Kawai et al. 2011). The other combinations produced a small reduction in HR (Arras et al. 2001; Orr et al. 2005), with a lower reduction in males than in females.

CONCLUSIONS

Our results demonstrate that combinations of ketamine with acepromazine or midazolam do not produce anaesthesia in rats (males and females) and

so they are not recommended, at least at the doses reported here. The diazepam combination at the dose employed here produced a good anaesthesia in both sexes, although taking into account our results, its use would be more advisable in males than in females. For short periods of anaesthesia, combinations with α_2 -agonists led to the best results; in females this was best achieved with medetomidine and in males the most effective agent was xylazine. However, sympathetic neurovegetative depression is an important consideration and must be counteracted by other drugs and perhaps with oxygen supplementation.

Our results show significant differences between sexes with respect to the different anaesthetic combinations. Thus, in the case of rats, it is necessary to take into account the sex of the animal before choosing the anaesthetic protocol, in order to ensure the safety of the combination anaesthetic administered.

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