Three combinations of clonidine in association with tiletamine-zolazepam for anaesthesia induction in rats: evaluation of reflexes and pain sensibility

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ABSTRACT: The aim of this study was to assess the combination of tiletamine-zolazepam (Zoletil 20®) with three different doses of clonidine for general anaesthesia induction in rats submitted to vascular microsurgery. The evaluation of anaesthetic and analgesic effects was performed in 30 Wistar rats randomly divided into three groups and induced with Zoletil 20 [90 mg/kg Intraperitoneal (IP)] associated with three different doses of clonidine (60–90–120 µg/kg IP). Four clinical parameters were evaluated after induction: loss of righting reflex, voluntary movement, the pedal withdrawal response, and pain sensitivity tested by pinching the tail. The combination of Zoletil with 90 and 120 µg/kg of clonidine provided a surgical anaesthesia; however, 90 µg/kg of clonidine provided the most rapid anaesthesia induction, as confirmed by data obtained by clinical evaluation of the loss of the pedal withdrawal response and the absence of the tail pinch response. The increase in dose of clonidine did not lead to a more rapid action of the α2 agonist, probably due to achievement of a dose-dependent plateau.

Keywords: anaesthesia induction; clonidine; reflex test, tiletamine-zolazepam, Winstar rats

Several studies have been realized in order to optimise anaesthetic protocols and to improve the efficacy and safety of conventional anaesthetics in rats. In the past, Tribromoethanol has been extensively used in rat anaesthesia; however, specialized literature has reported a high mortality, development of peritonitis and other intestinal complications due to local irritation after intraperitoneal (IP) injection (Flecknell et al. 2007). Traditionally, some barbiturates such as pentobarbital or thiobatubarbital, have been used as anaesthetics of choice both in small or farm animals and in rats; however, they are characterised by poor analgesic effects, hypothermia, prolonged respiratory depression and need preferentially, intravenous administration, not easily achievable in rats (Flecknell et al. 2007).

Nevertheless, injection (usually intraperitoneal) rather than inhalation anaesthesia is the method of choice in small rodents (Arras et al. 2001), and its specific intent is to provide rapid induction of surgical anaesthesia, good analgesia, to be reversible and to have minimal side effects with a wide margin of safety. Arras et al. 2001 showed that in mice these criteria were largely met by several different combinations, such as ketamine-xylazine-acepromazine, ketamine-xylazine, ketamine-xylazine-azaperone and tiletamine-zolazepam-xylazine. A combination of dissociative anaesthetic agents and α2 agonists were also investigated in rats (Branson and Booth 1999; Arras et al. 2001; Ferrari et al. 2005; Saha et al. 2007). Previous studies reported that tiletamine-zolazepam is an effective and safe agent for minor surgical procedures in rats (Saha et al. 2007), with several pharmacological advantages such as rapid induction time, excellent muscle relaxation and smooth recovery (Ferrari et al. 2005). However, the

disadvantage of dissociative anaesthetics is that the degree of analgesia appears to be great for somatic pain while poor for visceral pain (Flecknell et al. 2007). For this last reason, to obtain a balanced anaesthesia for vascular microsurgery in rats, we devised a protocol with a combination of tiletamine-zolazepam and the α2 agonist clonidine.

Clonidine is an α2 adrenergic drug originally used as an anti-hypertensive, and has recently been studied in animal research (Ossipov et al. 1997; Yamazato et al. 2001; Kaczynska and Szereda-Przestaszewska 2006; Gil et al. 2009). Clonidine has greater selectivity for the α2 adrenergic receptors than for α1 receptors; the ratio of α2/α1 selectivity is lower (220 : 1) than other α2 agonists such as medetomidine and dexmedetomidine (1600 : 1 and 1620 : 1, respectively), but higher than xylazine (160 : 1) (Barash et al. 2009).

The aim of this work was to clinically evaluate three different doses of clonidine in combination with tiletamine-zolazepam for intraperitoneal induction of general anaesthesia in rats.

Our investigation was based on the evaluation of four recognised parameters in small rodents: loss of righting reflex, absence of voluntary movements, loss of pedal withdrawal response and reaction to tail pinching (Arras et al. 2001; Ferrari et al. 2005; Flecknell et al. 2007).

MATERIAL AND METHODS

Thirty male outbred Wistar rats, 10 weeks of age, were involved in this ethically approved microsurgery procedure. Rats were housed for 15 days in quarantine at the Animal Facility in Catanzaro University (Italy). After quarantine, rats were housed in groups of two in solid floored caging (332 × 150 × 130 mm) containing Legnocel litter (Harlan). The temperature in the room was controlled at 22 ± 2 °C and 50–65% humidity. Animals were maintained with an "8 h light–8 h dusk–8 h dark" cycle. A commercial pelleted diet (Teklad 20/8) and water *ad libitum* were provided. In all, rats were submitted to 25 days of acclimatisation with the aim of ensuring adequate well-being and to exclude the possibility of stress due to the change in accommodation.

Anaesthesia protocols

The thirty rats were randomly divided into three groups and at the time of induction did not receive any oxygen supplementation. At the beginning of the procedure, each rat was restrained by an operator in dorsal recumbency. Anaesthesia was induced with three different protocols, in which a mixture of tiletamine-zolazepam (Zoletil 20, Virbac, Milan Italy) was administered at a dose of 90 mg/kg in association with three different doses of clonidine (Catapresan 150 mcg/ml, Boehringer Ingelheim Italy) [60 μg/kg (Protocol A), 90 μg/kg (Protocol B) and 120 μg/kg (Protocol C)] (Table 1).

An operator injected a combination of Zoletil and clonidine (60 μg/kg or 90 μg/kg or 120 μg/kg) intraperitoneally in the left posterior abdominal quadrant.

Another operator recorded the time of starting the procedure (when the rat was taken out of the cage) and the time of drug administration which corresponded to the removal of the syringe from the site of inoculation (T0).

An experienced veterinary anaesthetist who was blind to the treatment checked the depth of anaesthesia and analgesia in the rats. The rat was placed in the cage until the loss of the righting reflex (T1) and the loss of voluntary movements (T2) were observed. Both reflexes were assessed, timed from the moment of induction (T0), and recorded. Any attempt to regain the standing position and any voluntary movement were considered as evidence of the persistence of the two parameters. Reflex assessments were performed every 15–30 s.

Table 1. Doses of Clonidine and weight distribution in rat groups – different doses of clonidine in association with Zoletil 20 (90 mg/kg) in the three protocols are in the second column. All information about the weights of rats in the three groups is included in order to show the normal distribution

Anaesthetic protocols	Dose of clonidine (μg/kg)	Number of rats	Mean weight (g)	Median (g)	Standard deviation	Lowest value (g)	Maximal value (g)
A	60	10	339.1	342.0	13.23	316	357
B	90	10	340.1	348.5	17.16	310	360
C	120	10	336.2	336.5	14.46	317	359

For the pedal withdrawal reflex, the animal's hind limb was extended slightly and the interdigital webbing of the foot firmly pinched. For the tail pinch reflex, the operator used a forceps to pinch the rat's tail in the third distal quarter of the length of the tail for a few seconds. Both reflexes were evaluated according to previously described procedures (Ferrari et al. 2005) and following their absence, the rat was submitted to experimental vascular microsurgery, approved by the Italian Health Ministry.

To exclude the possibility that anaesthetic combination effects were affected by the time of administration (morning or afternoon), the combination was administered either in the morning (9.00 a.m.; in five rats of each group) or afternoon (2.00 p.m.; in the other five rats for each group).

After the surgical procedure, all rats were submitted to euthanasia (Tanax, Intervet, Italy) according to Italian Health Ministry recommendations.

Statistical analysis

Statistical tests were used to evaluate the influence of weight and clonidine dose on the time required for the loss of the righting reflex (T1), voluntary movement (T2), the pedal withdrawal response (T3) and pinching of the tail (T4).

The recorded data were analysed using the following methods: ANOVA (analysis of variance) was used to compare means when homoscedasticity and normality could be assumed; the Bartlett test was used to verify the homoscedasticity; and the Shapiro-Wilk test to verify the normal distribution of data and residuals, both necessary conditions for ANOVA. When homoscedasticity was rejected, the Kruskal-Wallis test was used instead to make the comparisons between central tendencies. The significance level was set at $P < 0.05$ in all tests.

RESULTS

Weight

To exclude the influence of rat weight on the final result a comparison was made using an ANOVA method, which did not reveal any statistically significant differences between the three groups ($P = 0.665$). The weight of rats in the three groups had a homogeneous distribution, with no statistically significant difference. The average weight was 339.1 g in the first group (Protocol A), 340.1 g in the second group (Protocol B) and 336.5 g in the third group (Protocol C). The medians of the three groups were 342 g, 348.5 g and 336.5 g, respectively, while the largest standard deviation was found in the second group (17.16) in which the minimum value recorded was 310 g and the maximum was 360 g (Table 1).

Loss of righting reflex (T1)

The loss of the righting reflex was not affected either by weight ($P = 0.31$) or by different doses of clonidine ($P = 0.405$) (Figure 1).

The Bartlett test was used to confirm data homogeneity; no significant differences were observed ($P = 0.28$). The presence of outliers meant that the residuals did not follow a normal distribution (Shapiro-Wilk test, $P = 0.0048$), but the homogeneity of the variances allowed us to confidently accept the validity of the results.

Accepting that the weight did not affect the T1 according to the Kruskal-Wallis test ($P = 0.545$) we were able to affirm that the protocol had no effect on this reflex. The loss of the righting reflex (T1) occurred faster in group C with a mean value of 2:38 min.

Absence of voluntary movements (T2)

The weight of the rats and the different doses of clonidine did not influence the time required for the absence of voluntary movements (Figure 1). As in the previous case, homoscedasticity was observed (Bartlett test $P = 0.36$), but no normal distribution (Shapiro-Wilk test $P = 0.03$). The lack of normality was again due to the presence of an outlier. If this is omitted from the analysis, the same result holds and normality can be accepted (shapiro test P-value 0.97). If we do not omit the outlier and consider no effect of weight, the Kruskal-Wallis test confirmed the absence of effect of the doses of clonidine ($P = 0.61$).

T2 was lowest in group C, in which the average and median values and the standard deviation were 3:28 min, 2:01 min and 2:49 min, respectively.

Loss of pedal withdrawal response (T3)

Assessment of the loss of the limb retraction reflex did not include data from rats in group A,

because only two patients showed the loss of this reflex.

Comparison of the data obtained using Protocols B (clonidine 90 μg/kg) and C (clonidine 120 μg/kg) showed no statistically significant differences (P = 0.098) (Figure 1).

The most rapid loss of this reflex was observed in patients of Protocol B. The normal distribution of the data was confirmed by the Shapiro-Wilk test (P = 0.15) and homoscedasticity by the Bartlett test (P = 0.21).

Pain sensibility tested by pinching the tail (T4)

The comparison was made between group B and C, because rats in group A did not lose this reflex. Homoscedasticity (P = 0.22) and the normal distribution of residuals (P = 0.45) were confirmed by the Bartlett test and Shapiro-Wilk test, respectively.

No reaction to tail pinching was observed in group B after an average time of 21:43 min, while in those of group C the same reflex was lost after an average time of 52:41 min. A significant difference was observed between groups (P < 0.001) (Figure 1), although one rat in group B was excluded from the study due to the presence of a mild and abnormal reaction to the pain test.

Other data

The average length of surgery was 1:32:02 h in the group of Protocol B, and 2:09:06 h in the group induced with Protocol C, but these data were not subjected to any statistical analysis because they were directly influenced by the capacity and the ability of the different surgeons.

An additional anaesthetic bolus of Zoletil was administered to nine subjects, five of which were

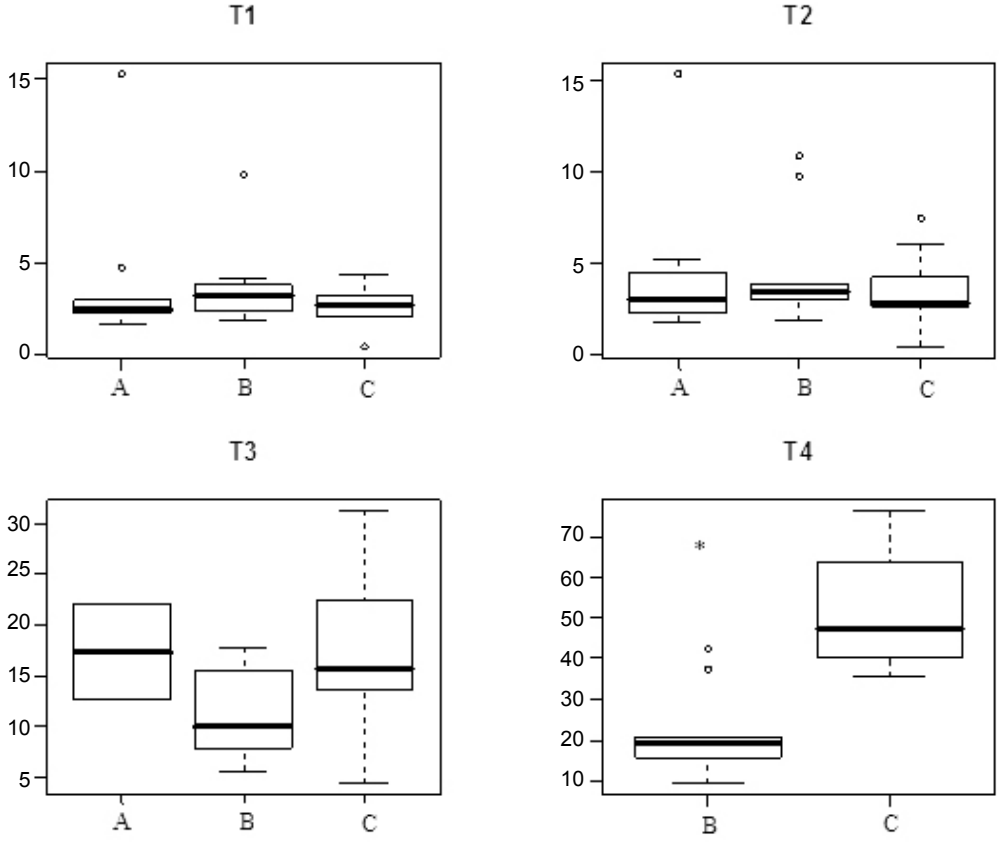

Figure 1. Temporal boxplots of the different clinical evaluations (T1 = loss of righting reflex; T2 = absence of voluntary movements; T3 = loss of pedal withdrawal response; T4 = pinching the tail) in the three groups of study. Time in minutes is on the y-axis and the different protocols (Protocol A; Protocol B; Protocol C) are on the x-axis were reported. In the T3 boxplot only two rats of Protocol A showed the loss of pedal withdrawal response and they were not considered for the statistical analysis; while no rats from this same group showed the loss of reaction to the tail pinching. Significant differences (*P < 0.05) was detected relative to T4 evaluation between B and C; ° = outliers

induced by Protocol B (clonidine 90 μg/kg) and four by Protocol C (clonidine 120 μg/kg).

DISCUSSION

The aim of this study was to evaluate three different doses of clonidine in association with Zoletil for general anaesthesia induction in rats. The choice of dose for each drug was carefully calculated after close evaluation of the specialised literature. In particular, a dose of 90 mg/kg of Zoletil 20 is justified by previous studies, in which lower doses have not ensured an adequate plane of surgical anaesthesia (Arras et al. 2001). Indeed, Arras et al. (2001) showed that 80 mg/kg of tiletamine/zolazepam (Telazol) in association with xylazine (20 mg/kg) were associated with a moderate safety margin; and afterwards Saha et al. (2007) observed that a single intramuscular dose of 60 mg/kg of tiletamine/zolazepam (Telazol) required a supplementary dose of 30 mg/kg.

The three different doses (60–90–120 of μg/kg) of Clonidine were selected with close reference to the study performed in rats by Ossipov et al. (1997). Ossipov et al. (1997) observed that intrathecal administration of clonidine showed the maximal possible effect (MPE) at a dose of 100–200 μg; this value was interpreted by the authors as the clonidine plateau for pain control. Moreover, previous studies established an anti-allodynic action of clonidine at lower doses (20–60 μg) (Nichols et al. 1995; Yaksh et al. 1995).

The four parameters evaluated in our study to investigate the plane of surgical anaesthesia are widely accepted by the scientific community for clinically evaluating anaesthesia and analgesia status in rats (Ferrari et al. 2005; Flecknell et al. 2007). In particular, the importance of the absence of voluntary movements (T2) was underlined by Flecknell et al. (2007), because together with the T1 it denotes the successful induction of an unconsciousness plane. In contrast to other studies (Taylor et al. 2000; Janssen et al. 2004; Welberg et al. 2006), we decided to evaluate both parameters because T1 signifies the achievement of the first stage of general anaesthesia or stage of "voluntary movement", defined as lasting from initial administration to loss of consciousness; while T2 denotes the overcoming of the second stage or stage of delirium or involuntary movement, in which patients lose any voluntary control (Flecknell et al. 2007).

Moreover, anaesthesia depth and analgesia were evaluated by assessing the loss of the pedal withdrawal response and pinching of the tail in accordance with to other procedures previously described (Ferrari et al. 2005).

After we had demonstrated that the three groups were homogeneous in weight we observed that Protocols A, B and C did not significantly affect T1 and T2; the same is true for T3 values in B and C Protocols. However, it is our opinion that the results obtained for T3 assessment should be re-evaluated using a larger number of rats because it might be useful to demonstrate the statistical effect of different doses of clonidine. Ferrari et al. (2005) suggested that the pedal withdrawal was one of the most sensitive indicators for surgical plane in rats.

The only variable that was significantly influenced by the different protocols was T4 "the reaction to tail pinching" (correlated with loss of pain sensibility), tested by applying a painful stimulus to the tail. It disappeared more rapidly in rats induced by Protocol B (Zoletil + clonidine 90 μg/kg). Even if B and C Protocols gave a surgical anaesthesia, the increased clonidine dosage did not allow faster reaching of a painless level. A limited dose-response effect was previously noted by Ossipov et al. (1997), who showed that the plateau in the dose-effect curve of clonidine was obtained at a dose of 100–200 μg/kg after intrathecal injection. It is the authors' opinion that in our study the plateau of the curve was probably reached when the α2A receptors in the spinal cord were fully saturated, preventing the possibility of an increase in the analgesic effect. Previous studies have demonstrated that the α2A receptor mediates the anti-nociception induced by systemic application of α2 agonists, including clonidine and dexmedetomidine (Philipp et al. 2002), and that clonidine could suppress the generation of a series of action potentials in tonically-firing neurones (TFNs), neurones of the dorsal horn considered to be a key element in the nociceptive processing system (Wolff et al. 2007).

Moreover, the results obtained by Gil et al. (2009) proved the negative influence of α1 receptor agonist activity on α2 agonist-mediated analgesia, and if we consider that clonidine is not a highly selective α2 agonist (Chiari et al. 1999; Kaczynska and Szereda-Przestaszewska 2006; Gil et al. 2009), and is not selective for the different sub-types of the α2 receptors (Philipp et al. 2002), we could suppose that the clonidine agonist activity of α1A and α1B receptors reduces its analgesic efficacy.

It was also reported that α2 agonists were unable to exert their action in stressed animals, because they released higher amounts of endogenous catecholamines (Flecknell et al. 2007).

In this regard, rats induced the last day of the experiment could be exposed to higher levels of stress by environmental stimuli, such as the management of the cages for induction of anaesthesia in the other rats. For this reason, handling and anaesthesia induction never took place in the housing room.

Moreover, the rats were accustomed to the manoeuvres of the operator, who cleaned the cages and administered food and water daily.

In conclusion, the induction of anaesthesia with Protocol C provided adequate surgical anaesthesia in rats submitted to vascular microsurgery. However, the most rapid analgesic coverage was observed in the group of rats anesthetised using Protocol B. Increasing the clonidine dose to 120 μg/kg did not lead to a more rapid action of the α2 agonist, probably due to achievement of a dose-dependent plateau, as found in previous studies (Ossipov et al., 1997). Moreover, saturation of α2 receptors and the subsequent activation of α1 should not be ruled out when clonidine is administered as an analgesic-sedative drug (Gil et al. 2009).

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