Clinical and experimental observations on the inflammatory response following a myocardial infarction

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Sufentanil-medetomidine anaesthesia compared to fentanyl/fluanisone-midazolam is associated with less ventricular arrhythmias and death during experimental myocardial infarction in rats and limits infarct size following reperfusion

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Abstract

To improve infarct healing following myocardial infarction (MI) in humans, therapeutic interventions can be applied during the inflammatory response. Animal models are widely used to study this process. However, induction of MI in rodents is associated with high mortality due to ventricular fibrillation (VF) during coronary artery ligation. The anaesthetic agent used during the procedure appears to influence the frequency of this complication. In the current retrospective study, the effect on ventricular arrhythmia incidence during ligation and infarct size following in vivo reperfusion of two anaesthetic regimens, sufentanil-medetomidine (SM) and fentanyl/fluanisone-midazolam (FFM) was evaluated in rats. Anaesthetics were administered subcutaneously using fentanyl/fluanisone (0.5 ml/kg) with midazolam (5 mg/kg) (FFM group, n=48) or sufentanil (0.05 mg/kg) with medetomidine (0.15 mg/kg) (SM group, n=47). The coronary artery was ligated for 40 min to induce MI. Heart rate and ventricular arrhythmias were recorded during ligation and infarct size was measured via histochemistry after three days of reperfusion. In the SM group, heart rate and VF incidence was lower throughout the experiment as compared to the FFM group (6% versus 30%) (P < 0.01). Fatal VF did not occur in the SM group whereas this occurred in 25% of the animals in the FFM group. Additionally, after three days of reperfusion, the infarcted area following SM anaesthesia was less than half as large as following FFM anaesthesia (8.5 ± 6.4% versus 20.7 ± 5.6%) (P < 0.01). Therefore, to minimize the possibility of complications related to VF and acute death arising during ligation, SM anaesthesia is recommended for experimental MI in rats.
SUFENTANIL-MEDETOMIDINE ANAESTHESIA COMPARED TO FENTANYL/FLUANISONE-MIDAZOLAM IS ASSOCIATED WITH LESS VENTRICULAR ARRHYTHMIAS AND DEATH DURING EXPERIMENTAL MYOCARDIAL INFARCTION IN RATS AND LIMITS INFARCT SIZE FOLLOWING REPERFUSION

Introduction

Ischaemic heart disease is currently the leading cause of death worldwide.\(^1\) Evidence is increasing that targeting the inflammatory response that occurs following cardiac ischaemia/reperfusion (I/R) injury associated with myocardial infarction (MI), contributes to improved cardiac healing.\(^2\)-\(^8\) To study the inflammatory response and putative therapeutic interventions following I/R injury, animal models are of great importance.

The acute inflammatory response triggered by cardiac I/R injury in rats mimics the human response. Therefore, rats are a common choice of use for a MI research model where myocardial ischaemia is induced via temporary coronary artery ligation.\(^9\) Induction of cardiac I/R requires a relative long period of surgical anaesthesia. Although this animal model has been used in numerous studies, induction of myocardial ischaemia is frequently associated with sudden death due to the development of ventricular tachycardia (VT) and eventually ventricular fibrillation (VF).\(^10,\)\(^11\) To avoid unnecessary use of animals, the model requires refinement to minimise fatal loss of animals when ischaemic damage is induced. In general, the anaesthetic effect on the heart rate, the incidence of ventricular arrhythmias during ischaemia and the infarct size can vary widely,\(^11\) emphasizing the importance of the chosen anaesthetic regimen.

Mostly, different agents are combined to provide all of the components of general anaesthesia such as loss of consciousness, analgesia, reflex activity suppression and muscle relaxation.\(^12\) Additionally, using a mixture of agents lowers the required dose per drug thereby diminishing dose-dependent side-effects and can result in a reduction of the total volume of anaesthetic that needs to be injected.\(^12\)

For the current study, the response of the anaesthetic mixtures sufentanil-medetomidine (SM) and fentanyl/fluaniisone-midazolam (FFM) in rats during cardiac I/R surgery was evaluated retrospectively. Both fentanyl or the more potent opioid sufentanil, in combination with the alpha-2 agonist medetomidine has been described to be an effective anaesthetic combination in rats, providing excellent analgesia together with a prolonged sedation time.\(^13,\)\(^14\) Moreover, the effect of SM can be reversed rapidly using butorphanol and atipamazole, which greatly speeds recovery.\(^12\) Fentanyl/fluaniisone is a veterinary anaesthetic combination of the µ-opioid agonist fentanyl, which abolishes pain perception and the neuroleptic fluaniisone, which reduces the undesirable side-effects of fentanyl and provides additional sedative effects.\(^12\) Addition of midazolam to fentanyl/fluaniisone provides muscle relaxation and this combination results in a surgical plane of anaesthesia.\(^15\) Both SM and FFM induce a longer analgesia and sedation time after a single injection as compared to anaesthetics that are most widely used in cardiac I/R studies such as pentobarbital or ketamine-xylazine.\(^13,\)\(^14\) To select the best agent for use in cardiac I/R models in rats, the effect on heart rate and ventricular arrhythmias during 40 min of coronary artery ligation together with the infarct size after three days of reperfusion was evaluated in the current study.

Animals, Materials and Methods

Male Wistar rats \((n = 95,\) aged 6-8 weeks, Harlan Laboratories, Horst, the Netherlands) weighing between 350-420 g were used. Male rats were used in this study because observations
in our laboratory showed a higher lethality in female rats following cardiac I/R (unpublished data). Animals were allowed to acclimatize for at least one week before surgery and were group-housed (3-4) in conventional type IV cages (Tecnilab-BMI, Someren, the Netherlands) placed in one room. Following surgery, rats were placed individually in conventional type III cages for 48 h to allow proper healing of the scar. Cages were bedded with Lignocel (J Rettenmaier and Söhne, Zutphen, the Netherlands) enriched with Enviro-dri® paper fibers (Tecnilab-BMI). A 12h:12h light-dark cycle was maintained and room temperature was kept between 20 and 22°C and humidity at 50 ± 5%. Water and a commercially pelleted diet (2016 Teklad Global 16% Protein Rodent Diet, Harlan Laboratories, Horst, the Netherlands) were provided ad libitum. Animals were tested and shown to be negative for all major rodent pathogens described in the FELASA guidelines.16, 17

Ethical permissions

The studies were approved in 2012 and 2014 by the VU University Amsterdam animal ethics and welfare committee. The VU university Amsterdam is licenced according to the 2010/63/ EU guidelines. The rats involved in the current study were accommodated and cared for conform the guidelines described in appendix A of EST No. 123.

Anaesthesia and analgesia

Rats were anaesthetized with either sufentanil-medetomidine (SM; n=48) or fentanyl/ fluanisone-midazolam, (FFM; n=47). Rats were not randomized and observers were not blinded since the current study assessed ventricular arrhythmia data retrospectively from two separate studies using either SM or FFM anaesthesia. Medetomidine (1.0 mg/ml; Sedastart, AST Farma, Oudewater, the Netherlands) was diluted 1:6⅔ with water for injection (Frensenius Kabi Nederland BV, Den Bosch, the Netherlands) and mixed with sufentanil (0.05 mg/ml, Hameln pharmaceuticals gmbh, Hameln, Germany). The SM mixture was administered subcutaneously (SC) in a volume of 1.5 mL/kg containing 0.05 mg/kg sufentanil and 0.15 mg/kg medetomidine. Fentanyl/fluanisone (0.5 ml/kg of Hypnorn® containing 0.315 mg/ml fentanyl and 10 mg/ml fluanisone, Janssen Pharmaceuticals, Tilburg, the Netherlands) and 5mg/kg midazolam (5 mg/ml; Dormicum®, Roche, the Netherlands) were administered SC using two separate syringes. The rats were weighed prior to injection of the anaesthetics. To minimize stress, the rats were injected next to their cages to which the rats were returned until the anaesthesia had taken effect. Immediately following the surgical procedure, the rats were injected SC with 1 mL 0.9% NaCl to compensate for surgery-related fluid loss. In addition, after completion of the surgical procedure, the rats anaesthetized with SM received 0.2 mg/kg butorphanol (Torbugesic-Vet, Zoetis BV, Capelle aan de IJssel, the Netherlands) freshly mixed with 0.5 mg/kg atipamazole (Atipamazol-HCl, Eurovet, Animal Health, the Netherlands) SC to rapidly antagonise sufentanil and medetomidine respectively. One hour after the administered antagonists, buprenorphine (0.03-0.05 mg/ kg, Temgesic® diluted 1:9 in 0.9% NaCl, Reckitt Benckiser BV, Hoofddorp, the Netherlands) was administered SC to provide a longer period of analgesia than butorphanol18. Although buprenorphine also reverses the effects of sufentanil, its onset of action is relatively slow . Hence, butorphanol was administered primarily as the reversal agent. Rats anaesthetized with
FFM received buprenorphine immediately after completion of the procedure. Buprenorphine administration was continued in both groups every 8-12 h for 48 h following surgery to prolong analgesia. Rats were kept on a heating pad and heart rate and respiratory frequency were closely observed to monitor the rats until recovery of anaesthesia.

**Surgical procedure**

After loss of the pedal withdrawal reflex, rats were intubated with a 16 gauge tube (B Braun Introcan; Oss, the Netherlands) for ventilation. Rats were placed on a heating pad to maintain body temperature at 37°C. Following intubation, rats were ventilated using 40% oxygen (UNO micro ventilator-03, Zevenaar, the Netherlands), fur was removed locally and skin was disinfected with 70% v/v ethanol. All surgeries were performed by the same surgeon during daytime using freshly autoclaved instruments. Cardiac I/R was induced as described previously. In short, a left thoracotomy was performed between the third and the fourth rib. To prevent the lungs collapsing, positive end expiratory pressure (PEEP) of 2 mbar was maintained using a micro ventilator. During surgery, the effects of anaesthesia were monitored by closely observing heart rate, heart rhythm and signs of hypercapnia such as changes in respiratory rate and depth caused by carbon dioxide accumulation. To induce myocardial infarction, the left anterior descending artery was ligated using a 6-0 prolene suture. After 40 min of ischaemia, the suture was removed to allow reperfusion and the thorax was closed using two sutures and maximal lung pressure. On average, total surgery time was 65 min. Welfare of the animals was assessed daily until termination.

**Ventricular arrhythmia assessment**

During the entire period of ligation, the heart rate (HR) as well as sinus rhythm of the rats were monitored using an electro-cardiogram set according to Einthoven I ECG (ADInstruments, Oxford, United Kingdom). HR measurements were noted at three time points, namely 1.) at start of the surgery, 2.) 10 min after ligation and 3.) after 10 min of reperfusion. These time points are representative of the HR during the periods of baseline, ischaemia and reperfusion respectively. The ventricular arrhythmias induced by ischaemia were denoted in accordance to the Lambeth conventions, namely19:

- VT: defined as a sequence of four or more ventricular complexes with a rate faster than the resting sinus rate and with an autonomous return to SR;
- VF: defined as a rate where QRS complexes could not be distinguished individually and return to SR did not occur autonomously; and
- Fatal VF: defined when rats died during ligation as a cause of VF and did not respond to mechanical defibrillation performed manually on the heart.

**Infarct size measurements**

Following three days of reperfusion, a subgroup of the rats were killed humanely by excision of the heart under deep anaesthesia using 5% isoflurane (SM anaesthesia n=9, FFM anaesthesia n=6). The hearts of the other rats were assessed at various times after reperfusion, depending on the design of other studies and the data obtained from these hearts are not
included in the current study. The heart was excised and cut into five equal slices. Three slices were fixed in 4% formalin and embedded in paraffin, two slices were snap frozen in liquid nitrogen. To discriminate viable myocardium from diseased myocardium, histochemical staining with phosphotungstic acid haematoxylin was performed on all five slices as described previously. The stained slides were scanned and the infarcted area and total heart area were marked manually using the Pannoramic viewer programme (version 1.15.4, 3D Histech Ltd, Budapest, Hungary). The average infarct size of all five slices was calculated as a percentage of the complete transverse heart section.

**Statistical analysis**

The incidence of ventricular arrhythmias during the monitoring period was noted as categorical data, presented as percentage (%), and treatment comparisons were made using a chi-squared test. Post hoc analyses were performed to test for associations between two groups using a Fisher’s exact test. HR and infarct size data were distributed normally and differences between the two groups were analysed using a Student’s t-test. HR data is presented with the mean and the standard error of mean (SEM) and infarct size data is presented as box plots with median, 25th-75th percentiles (boxes) and 5-95th percentiles (whiskers). HR at different time points within groups were compared using a paired Student’s t-test. Difference between results were considered statistically significant if the two-sided P-value was ± 0.05. The statistical analysis was performed using Statistical Packages for Social Sciences software (IBM SPSS 22.0.0.0 for Windows, IBM Corp.)

**Results**

**Heart rate**

Rats anaesthetized with SM had a significantly lower HR as compared to FFM anaesthesia at baseline (246 ± 26 versus 323 ± 37 bpm, \(P < 0.001\)), during ischaemia (261 ± 31 versus 301 ± 25 bpm, \(P < 0.01\)) and following reperfusion (264 ± 12 versus 317 ± 36 bpm, \(P < 0.01\)), as shown in Figure 1. Comparison of the HR in rats within the same group at different time points did not show any significant differences.

**Figure 1. Heart rate (HR) in rats during experimental myocardial infarction using SM or FFM anaesthesia.**

HR in rats following anaesthesia administration of either SM or FFM at the start of surgery (Baseline), 20 min after surgery initiation (Ischaemia) and 60 min after surgery initiation (Reperfusion). These time-points are representative for the HR during that period. Coronary artery ligation was initiated at 10 min following surgery initiation. HR data is presented with the mean and the standard error of mean (SEM). ***\(P < 0.001\) **\(P < 0.01\) measured between SM and FFM groups using a Student’s t-test. BPM: beats per minute, SM: sufentanil-medetomidine, FFM: fentanyl/fluanisone-midazolam.
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Incidence of ventricular arrhythmias during 40 min of ischaemia

Figure 2 shows the incidence of ventricular arrhythmias during 40 min of ligation in rats anaesthetized with SM or FFM. In the SM group, 38% (18/48) of the rats maintained sinus rhythm (SR) during ligation, which was significantly higher than the 2% (1/47) of the rats in the FFM group (P < 0.001). The remainder of the rats all experienced either VT or VF during ligation. The occurrence of VT during ischaemia did not differ between the two anaesthesia groups. However, the percentage of rats receiving SM that experienced VF during ligation was significantly lower as compared to the FFM rats (6% (3/48) and 30% (14/47), P < 0.01). Moreover, no rats died during ligation when SM anaesthesia was used whereas 25% (12/47) of the rats anaesthetized with FFM developed fatal VF (P < 0.001).

Infarct size after three days of reperfusion

Figure 3(a) shows the percentages of infarcted area of the heart measured at three days following cardiac I/R surgery in a subgroup of rats using SM and FFM. The infarct area of SM anaesthetized rats (8.5 ± 6.4%) was significantly smaller as compared to the infarct area using FFM anaesthesia (20.7 ± 5.6%) (P < 0.01). Figure 3(b) shows a representative photograph of a smaller infarcted area after cardiac I/R in rats using SM as compared to FFM.

Figure 2 Incidence of ventricular arrhythmia during 40 min of coronary artery ligation.

Incidence of VT, VF or death (in percentage) in rats during 40 min of coronary artery ligation using SM or FFM anaesthesia. ***P < 0.001, **P < 0.01 as compared between groups. VT: ventricular tachycardia, VF: ventricular fibrillation, fatal VF: death during ischaemia, SM: sufentanil-medetomidine, FFM: fentanyl/fluansone-midazolam.

Figure 3 Infarct size in rats following three days of reperfusion.

(a) Infarct size (in percentage) following 40 min of ischaemia and three days of reperfusion in rats using SM or FFM anaesthesia. Infarct size data is presented as box plots with median, 25%-75% percentiles (boxes) and 5-95% percentiles (whiskers) (b) PTAH stain of paraffin embedded hearts showing the pink infarcted area (i) marked with a red line and the viable myocardium (v) in purple. PTAH: phosphotungstic acid haematoxylin, SM: sufentanil-medetomidine, FFM: fentanyl/fluansone-midazolam.
CHAPTER 8

Discussion

To study putative therapeutic interventions during infarct healing following cardiac I/R injury, animal models are of considerable importance. However induction of MI in rodents can be associated with a high mortality mostly, due mostly to the development of VF during coronary artery ligation, resulting in the use of a larger number of animals per study. In this study it is shown for the first time that the use of SM anaesthesia in rats is associated with a lower HR and a reduced incidence of ventricular arrhythmias and deaths during 40 min of ligation compared to FFM anaesthesia, and is not associated with death during VF. In addition, the infarct size was significantly smaller after three days of reperfusion in the SM group compared with the FFM group. Thus VF occurs less, and the survival rate is higher, when rats undergo cardiac I/R surgery under SM anaesthesia in comparison to FFM anaesthesia.

As this was a retrospective study, the experimental design was not randomized and blinded, and this represents a limitation of the study. Rats in the FFM group underwent surgery 1.5 years before the rats in the SM group. However all other variables were kept similar, such as the surgeon performing the procedure, the rat strain, age and sex as well, as the entire surgical methodology, including the instruments used and the operating room. Moreover, the analyses of the ventricular arrhythmias and the infarct size were carried out in an identical manner. Notwithstanding this, the possibility that the lack of randomization could have affected the outcome of the results cannot be completely excluded.

The reduction of ventricular arrhythmias observed using SM anaesthesia could be the result of a smaller infarct size following coronary occlusion. Activated opioid receptors are suggested to provide cardioprotection as they are also described to be involved in ischaemic pre- and postconditioning. Ischaemic preconditioning, first reported in 1986, demonstrates how brief periods of hypoxia prior to prolonged coronary occlusion protects against infarction. In ischaemic postconditioning, brief periods of ischaemia are applied at the start of reperfusion, and this has been shown to be as effective in preventing myocardial injury as preconditioning. In rats, it has been demonstrated that non-selective blocking of the opioid receptor abolishes both the ischaemic preconditioning and post-conditioning protective effect. These results increased the interest in unravelling the mechanism of opioid receptor activation during pre- or post-conditioning of an ischaemic period. It remains debatable which opioid receptor in particular is involved. Several studies showed that the κ- and δ-opioids are involved in cardioprotection whereas μ-opioids, the receptors of sufentanil and fentanyl, are suggested to be less involved. However, it has been demonstrated in rats that pharmacological postconditioning by sufentanil administration early during reperfusion, contributes to infarct size reduction after 2 h of reperfusion. Moreover, pre- and postconditioning with sufentanil prevented hypoxia induced myocardial damage in cultured contracting human arterial trabeculae, a cell culture which mimics the beating human myocardium. These results would be in line with the reduced infarct size as observed following SM anaesthesia in the current study since SM was present during both ligation and early reperfusion, as SM anaesthesia was antagonised after early reperfusion. Nevertheless, only the rats anaesthetized with SM received butorphanol following the surgical procedure which reverses μ-opioid but simultaneously provides analgesia via agonist activity at the κ-opioid receptor. Activation of κ-opioids in
particular are suggested to be involved in attenuating cardiac I/R injury related to pre- or post-conditioning. Conversely, another study showed that activated κ-opioid receptors do not mediate the beneficial ischaemic preconditioning effect. Notwithstanding this, we cannot rule out the possibility that administration of butorphanol could have influenced the infarct size in the SM group. Since blood oxygen saturation levels were not monitored during the current study, the possibility that hypoxia could have been a confounding factor on the incidence of VF during ligation cannot be excluded.

In addition, the presence of the alpha-2 agonist medetomidine in the SM mixture could have suppressed ventricular arrhythmias during ligation. A meta-analysis pointed out that the use of alpha-2 agonists in patients that undergo cardiac surgery can reduce perioperative mortality as a consequence of ventricular arrhythmias and also reduces myocardial infarction. Stimulation of the alpha-2 adrenoceptors in the central nervous system results in a reduction of norepinephrine outflow and thereby directly dampens the sympathetic tone. This can contribute to the development of bradycardia, which is a common side effect of medetomidine, especially when combined with opioids. Moreover, bradycardia has been correlated to suppression of VT and VF.

Dampening of the sympathetic tone to reduce cardiac arrhythmias and mortality following cardiac surgery is used widely in clinical settings by administration of beta blockers, which antagonise beta adrenoceptors. Recently, it has been demonstrated that sympathetic tone suppression early after MI via administration of beta-blockers before reperfusion, reduced infarct size and resulted in an improved cardiac recovery following MI. This suggests that administration of beta-blockers during ischaemia beneficially affects cardiac outcome. However, even though both stimulation of alpha- and inhibition of beta-adrenoceptors results in suppression of the sympathetic tone, whether this effect results in an equal response to ventricular arrhythmia and infarct size following ischaemia remains under debate.

Whether the production of a smaller infarcted area as observed in the current study when using SM anaesthesia would be of preference for an infarct model is depended upon the goals of the specific study. For example where therapeutic interventions that aim to reduce the infarcted area are to be evaluated, a larger infarcted area might produce larger treatment effects. However, the considerable decrease in mortality rate when using SM anaesthesia results in the use of less animals and an alternative approach would be to consider altering the coronary artery ligation method in order to increase the infarcted area, if this was necessary.

Based upon the results in the current study it is recommended that SM anaesthesia should be used for experimental cardiac I/R surgery in rats. Our results show that a change of anaesthetics can consistently influence the infarct size following cardiac I/R and that SM anaesthesia avoids loss of animals by preventing complications related to VF and acute death during coronary artery ligation. This results in a reduction in the amount of animals needed for cardiac I/R studies. To unravel the mechanism of VF suppression by SM anaesthesia is of great interest, not only to for laboratory use, but also for clinical use in patients with coronary artery diseases.
CHAPTER 8

References


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