COMPARISON OF THE EFFECTS OF TWO ANAESTHETIC COMBINATIONS IN RABBITS ON SOME NEUTROPHIL FUNCTIONS IN VITRO

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ABSTRACT: Tissue injury during surgery as well as anaesthesia can lead to modulation of neutrophil function. The aim of this study was to assess how two types of anaesthesia, i.e. combination of ketamine/midazolam/propofol (Group I) and ketamine/propofol (Group II) influenced neutrophil function during and 24 h after operation in rabbits. Blood samples were taken prior to anaesthesia, at 30 and 60 min, and 24 h after starting the experiment. At these time points, degranulation and free radical generation were assessed in both groups. After 30 min of anaesthesia, we observed a decrease in elastase release from 50.96±0.71% to 26.52±4.85% in Group I and from 51.00±0.7% to 41.00±5.48% in Group II, respectively. In subsequent measurements, the elastase level increased to values lower than before starting anaesthesia. The myeloperoxidase (MPO) release decreased significantly after 30 min of anaesthesia in Group I, then increased to the value similar to the pre-anaesthetic level after 60 min. MPO level in Group II also decreased after 30 min but to a lesser degree, and then after 1 h MPO slightly exceeded the pre-anaesthetic level. In both groups, 24 h after the start anaesthesia the values obtained decreased, but remained at a level higher than at the time before anaesthesia. We observed a decrease in alkaline phosphatase (ALKP) levels in both studied groups after 30 min of anaesthesia (from 24.77±5.9% to 15.7±2.1% and from 23.6±1.14% to 10.6±0.89%, in Groups I and II, respectively), with a subsequent increase after 60 min. In Group I, we observed a significant (P<0.01) decrease in NO production 30 min after the start of anaesthesia. After increasing to the pre-anaesthetic level, the NO level was almost constant in both subsequent measurements. In Group II, NO level decreased 30 min after the onset of anaesthesia and then increased, reaching a slightly higher level than at the start of anaesthesia. The superoxide generation by neutrophils from rabbits of both groups decreased after 30 min of anaesthesia and then rose. Because the anaesthetic combinations used alter neutrophil function only transiently, both of them would be applied in surgery in rabbits without the risk of disturbance to the healing process.

Key Words: Neutrophil function, elastase, anaesthesia, propofol, degranulation.

INTRODUCTION

Accidental or surgical tissue injury evokes an inflammatory response, which is essential for the reconstruction process. The peri-operative balance between immunostimulation and immunosuppression may be affected by multiple factors. Dysregulation of the inflammatory process may provoke postoperative
complications such as wound healing disturbances or infections leading to sepsis and multiple organ failure causing even death (Schneemilch et al., 2004).

Neutrophils are the leading cells in the first response to trauma. It is known that initial resistance to bacterial infection is mediated by them (Davidson et al., 1995). Activated neutrophils can degranulate and release some enzymes such as elastase, myeloperoxidase (MPO), alkaline phosphatase (ALKP) and free radicals; moreover, they would be more resistant to apoptosis (Lentz et al., 2007, Wessely-Szponder, 2008). MPO is a specific enzyme of primary granules of neutrophils and is a marker of in vivo neutrophil activation (Hans et al., 1991). ALKP score, in turn, reflects the neutrophil activity during surgery (Tabuchi et al., 1989). While local neutrophil migration to the site of tissue damage is important for wound healing and protection against invading organisms, systemic activation can lead to sequestration in organs and can cause tissue damage by released enzymes and free radicals (Lentz et al., 2007, Wessely-Szponder, 2008). On the other hand, inhibition of neutrophil activation to protect cells against deleterious effects may appear as a double-edged sword because activated neutrophils also play a key role in innate immunity and a disorder of any of their functions may allow bacterial infection to develop (Davidson et al., 1995, Thiry et al., 2004). Therefore, partial and transient restriction of neutrophil action would be beneficial to the healing process by neutralisation of excessive enzyme and free radical release, but without inhibition of the bactericidal function of neutrophils (Thiry et al., 2004). It was reported that some immune functions are depressed during anaesthesia and surgery, but the contributory role of anaesthetic agents in this process is poorly understood (Davidson et al., 1995).

Rabbits are commonly anaesthetised animals, however, their peri-anaesthetic mortality is considerably high (1 in 601) (Grint and Murison, 2008). They are generally regarded as animals in which the anaesthetic risk is relatively high comparing to other species. Possible reasons included high surface area: volume ratio and therefore susceptibility to hypothermia, high metabolic rate, difficulties in placing venous catheter and endotracheal intubation, as well as some anatomical conditions (Henke et al., 2005, Grint and Murison, 2008, Rozanska, 2009). The anaesthesiological schemes used in this experiment were tested previously in our clinic during surgical procedures in rabbits. In both combinations, changes in haemodynamic parameters remained within acceptable range and these schemes allowed for uneventful recovery (results not published).

Propofol (2,6-diisopropylphenol) is an intravenous short acting anaesthetic agent widely used for inducing and maintaining anaesthesia and sedation. This drug has minimal side effects, a controllable anaesthetic state, quick onset and rapid recovery from general anaesthesia. Apart from multiple anaesthetic advantages, propofol demonstrates a number of non-anaesthetic effects including immunomodulatory potential. It has the ability to enhance cytotoxic T lymphocyte activity. It is also a potent antioxidant, which may protect the organs against ischemic or hypoxic injury through its effects on KATP channels (Vasileiou et al., 2009). However, some authors postulated that some suppressive effects of propofol may be caused by the solvent intralipid (Cheng, 2005).

Water soluble benzodiazepine - midazolam produces good muscle relaxation and potentitates the effect of anaesthetics. Intramuscular or intravenous midazolam administration is recommended as a routine short-acting sedative (Harcourt-Brown, 2004). Both midazolam and ketamine have been used extensively for anaesthesia and/or sedation in rabbits. It has been proved that high concentrations of these anaesthetics inhibit human neutrophil functions (Nishina et al., 1998). Moreover, midazolam inhibited mouse mast cell proliferation and mediated by immunoglobulin E release of TNFα from mast cells (Helmy and Al-Attiyah, 2001).

The aim of this study was to compare ketamine/midazolam/propofol and ketamine/propofol anaesthesia in rabbits undergoing surgery, in the light of neutrophil function during and 24 h after surgery.
MATERIALS AND METHODS

Animals and study design
The study protocol was approved by the Local Ethics Committee. The aim of the surgery was the implantation of biomaterial into the tibial defect. The study was carried out on 10 healthy New Zealand White (NZW) rabbits, males with mean body weight of 4000±100 g (mean±standard deviation). Rabbits were allocated randomly into two groups of five individuals. Physical examination of each rabbit was performed before the surgical procedure. After induction of anaesthesia with xylazine (Sedazin; Biowet, Pulawy, Poland) 5 mg/kg and ketamine (VetaKetam; Vetagro, Lublin, Poland) 30 mg/kg intramuscularly, the marginal ear vein was catheterised with a 22-gauge sterile catheter for administration of anaesthesia. Group I received ketamine, midazolam, propofol (ketamine/midazolam/propofol group) and Group II received anaesthetic mixture containing ketamine and propofol (ketamine/propofol group). For animals in Group I, ketamine 0.25 mg/kg/min, midazolam (Midanium; Polfa S.A., Warsaw, Poland) 0.2 mg/kg/min and propofol (Propofol 1%, Fresenius Kabi, Poland) 0.2 mg/kg/min as anaesthetic mixture were administered by intravenous continuous rate infusion (diluted in 5% glucose). In Group II, ketamine (0.35 mg/kg/min) and propofol (0.25 mg/kg/min) were administered intravenously as continuous rate infusion diluted in 5% of glucose. Mean arterial blood pressure, heart rate, temperature, and blood gas were monitored every 20 min during infusion. The mean duration of anaesthesia was 65 min (60-70 min).

Blood was taken into tubes with 3.8% sodium citrate (anticoagulant) at a ratio of 1:10. Four blood samples were obtained at four time points: before induction of anaesthesia, 30 min after start of anaesthesia, 60 min after start of anaesthesia, and 24 h after surgery.

Isolation of neutrophils
Neutrophils were isolated from peripheral blood according to the method described previously (Wessely-Szponder, 2008). Briefly, after initial centrifugation for 15 min at 1000 g (4°C), the plasma layer and buffy coat were discarded. Red blood cells were lysed by adding double-distilled water and gently mixing. After restoration of the isotonicity by adding 2.7% NaCl and mixing, the suspension of neutrophils was centrifuged again in the same conditions. The remaining pellet was washed with phosphate-buffered saline (PBS-Biomed, Lublin, Poland). After isolation, viability of neutrophils was determined by trypan blue exclusion and cells were then adjusted to a final concentration of 2×10⁶ cells/mL.

Degranulation assay
Neutrophil degranulation was assessed by elastase, MPO, and ALKP release. 100% enzyme content was estimated by incubating cells in the presence of 0.5% CTAB (hexadecyltrimethylammonium bromide-Sigma), since CTAB results in complete cell lysis and release of all granule enzymes. Elastase activity was measured with azocasein (Sigma) as a substrate after 10 min incubation at room temperature. MPO release was measured spectrometrically after 10 min incubation at room temperature with equal volume of o-phenyldiamine (OPD-Sigma). ALKP level was estimated after 10 min incubation with equal volume of 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP-Sigma). The elastase, MPO, and ALKP reactions were stopped by the addition of TCA, H₂SO₄ and NaOH, respectively. Absorbance was measured on ALAB-PLATE READER ELISA at 492 nm for elastase and MPO, and at 405 nm for ALKP. All samples were assayed in duplicate (Wessely-Szponder, 2008).

Nitric oxide production
Nitric oxide level was determined by Griess reaction: 50 μL of supernatant were mixed with 200 μL of Griess reagent (1% sulphanilamide, 0.1% naphthylendiamine dihydrochloride and 2.5% H₃PO₄). All tests
were done in duplicate. Absorbance at 545 nm was measured after 10 min incubation with Griess reagent and compared with a standard. Obtained values were expressed as a concentration of nitrite, the stable product of NO, which accumulates in medium (Rindour et al., 2000).

**Superoxide generation**

Superoxide anion production was measured by the method described by Confer and Simons (1986). Neutrophils were incubated with 0.1% nitroblue tetrazolium (NBT-Sigma) solution at room temperature for 10 min and then absorbance was read at 545 nm. All tests were done in duplicate. Nanomoles of superoxide produced over the incubation period were calculated using the extinction coefficient 21.1 nmol (Galligan and Coomber, 2000).

**Apoptosis assay**

Samples from neutrophil preparations containing 1×10⁶ cells were stained with May-Gruenwald-Giemsa solution. Apoptosis morphology (chromatin condensation, cytoplasm vacuolisation) was evaluated by observation of slides with light microscopy at the magnification of x 1000. In each preparation 200 cells were counted and the percentage of apoptotic cells was determined as described by Kakuta et al. (2006).

**Statistical analysis**

Statistical analysis was performed using the computer program STATISTICA 5.0 (StatSoft, Poland). Examined values were expressed as mean (standard deviation) and were compared with using analysis of variance and Student’s t-test and differences were considered as significant at \( P<0.05 \) and at \( P<0.01 \).

**RESULTS**

**Neutrophil elastase release**

After 30 min of anaesthesia, we observed a decrease in elastase release in both of our experimental groups (from 50.96±0.71 to 26.52±4.85% in Group I and 51.00±0.7 to 41.00±5.48% in Group II). In further measurements, the elastase level increased in both groups. The higher increase was seen in the Group II. The last measured value (after 24 h from start of anaesthesia) was lower than pre-anaesthetic level in Group I and was near to pre-anaesthetic level in Group II (Figure 1).

**Neutrophil MPO level**

In our study, the MPO release decreased significantly after 30 min from start of anaesthesia in Group I (\( P<0.01 \)), then increased to the value approximate to pre-anaesthesia level after 60 min. In Group II, the MPO level also decreased significantly (\( P<0.05 \)) after 30 min from start of anaesthesia, but to a lesser degree, and then after 1 h increased to the value 43.35±3.12%. After 24 h in Group I, obtained values remained at a slightly higher level than before the start of anaesthesia, whereas in Group II these values were decreased but remained at the level higher than before anaesthetic administration (Figure 2).

**ALKP release**

A simultaneous significant (\( P<0.05 \)) decrease in ALKP level in both studied groups after 30 min of anaesthesia was observed (from 24.77±5.9 to 15.7±2.1% and from 23.6±1.14 to 10.6±0.89% in Groups I and II, respectively), with a subsequent increase after 60 min. Then, in final measurements, the obtained values in both groups were close to these after 60 min but lower than were seen before anaesthetic administration (Figure 3).
Nitric oxide production

In Group I, we observed a significant \( (P<0.01) \) decrease of NO production after 30 min of anaesthesia and then the NO level was nearly constant and similar to pre-anaesthesia in both subsequent measurements (at t 2 and t 3). In Group II, NO level decreased (but without statistical significance) after 30 min of experiment and then increased at two further measurements reaching a slightly higher level than before administration of anaesthetic agents (Figure 4).

Superoxide generation

The superoxide generation by neutrophils from rabbits from both groups decreased after 30 min of anaesthesia (with statistical significance in Group I, \( P<0.05 \)), and then rose to a higher degree in Group II (3.44±0.4 nM/10⁶ cells). In the last measurement, these values in both groups were slightly higher than the value of pre-anaesthesia (2.52±0.53 and 3.18±0.35 nM/10⁶ cells, respectively)(Figure 5).

Assay of apoptosis

There were no significant differences between all studied groups and measurements (data not shown).

DISCUSSION

The inflammatory response to surgical injury is essential for tissue repair and to enhance the organism’s healing potential (Toft and Tonnesen, 2008). In humans, during general anaesthesia immunomodulatory effects of anaesthetic and sedative agents may interfere with immune function, but their action may be reversible because of the short administration period (Helmy and Al-Attiyah, 2001).

We observed a decrease in elastase release in both of our experimental groups after 30 min from start of anaesthesia; in subsequent measurements, these values increased, although after 24 h from start of anaesthesia they were near to pre-anaesthetic level.
Midazolam is known to inhibit certain aspects of the immune function. It was suggested that benzodiazepines bind to specific receptors on macrophages and inhibit their capacity to produce IL-1, IL-6 and TNF. Both propofol and midazolam altered IL-8 secretion from isolated neutrophils (Helmy and Al-Attiyah, 2001). Galley et al. (1998) observed decreased extracellular IL-8 under the influence of midazolam and propofol. The decrease in neutrophil elastase release observed in our study may therefore be explained by lower IL-8 release in response to both midazolam and propofol in Group I or to propofol alone in Group II.

In our study, the MPO release decreased significantly 30 min after start of anaesthesia in Group I, then gradually increased through time to the value approximate to pre-anaesthetic level. In contrast, in Group II the MPO level decreased (to a lesser degree) 30 min after start of anaesthesia.

According to Muhling et al. (2002) propofol in concentrations of 5 and 50 μg/mL in Hank’s balanced salt solution significantly decreased MPO released from neutrophils isolated from human blood after 60 min of incubation or longer. On the contrary Hans et al. (1991) observed that MPO level measured 1 h after surgery was significantly higher compared with previous levels and the authors assumed activation of neutrophils after surgery.

We observed an almost simultaneous decrease of ALKP level in both groups after 30 min of anaesthesia with a subsequent increase at 60 min after start of anaesthesia. Then, the values observed were lower than before anaesthesia.

Tabuchi et al. (1989) discovered that ALKP level lowered transiently during anaesthesia and then increased 1.6 times more than pre-anaesthetic level on 1st postoperative day.

In Group I, we observed a significant decrease in NO production 30 min after start of anaesthesia and in both further measurements the NO level was nearly constant and similar to the pre-anaesthetic level. In Group II, NO level decreased insignificantly 30 min after start of anaesthesia then increased at two further measurements and remained slightly higher than at start of experiment.
Effects of anaesthesia on neutrophil function

NO synthesis requires the participation of nitric oxide synthase (NOS), present in two isoforms: constitutive (cNOS) and inducible (iNOS). It was reported that propofol at a dose of 75 μM has a direct inhibitory effect on iNOS especially when production was induced by lipopolysaccharide (LPS), and inhibits inducible NO production. The potential regulatory mechanism may be involved with the alteration of the production of inflammatory mediators that upregulate the expression of inducible enzyme activity. Several studies revealed a reduction of pro-inflammatory mediators and no change in anti-inflammatory mediators in surgical patients treated with propofol (Vasileiou et al., 2009). On the other hand, Gonzales-Correa et al. (2008) showed that incubation with increasing concentrations of propofol intensified NO production and cNOS activity in neutrophils, and reduced iNOS activity in a concentration-dependent manner. Ketamine, in turn, is known as an N-methyl-D-aspartate (NMDA) antagonist, indirectly reducing NOS activity (Galley et al., 1995).

Our study revealed that the superoxide generation by neutrophils from both groups decreased (statistical significance only in Group I) and then rose in both groups, but to the highest degree in Group II (3.44±0.4 nM/106cells). In the last measurement, these values in both groups decreased but were higher than the pre-anaesthesia values.

According to Muhling et al. (2002), propofol significantly decreased superoxide generation after 60 min of incubation or longer. Huetteman et al. (2006) also observed inhibition of respiratory burst by propofol. However, it was stated that neutrophil functions are influenced by multiple factors not only by anaesthetics (Zilberstein et al., 2002). Therefore, transient increase of superoxide production would be explained by neutrophil activation and Hans et al. (1991) suggested that neutrophil activation would be caused by surgery.

There are some discrepancies about the role of other anaesthetics used in our study in modulation of neutrophil function. According to Galley et al. (1998) and Frolish et al. (1999), midazolam had no effect on neutrophil respiratory burst at clinically relevant concentrations. Another previous findings showed that midazolam and ketamine did not scaveng the reactive oxygen species (ROS) generated but rather inhibited the ability of neutrophils to produce ROS (Nishina et al., 1998). Chang et al. (2005), in turn, revealed that ketamine showed immunomodulating effect on neutrophils by suppression of oxidant production and chemotactic activity. Krumholtz et al. (1995) stated that ketamine in clinically relevant concentrations inhibited both phagocytosis and killing of bacteria in vitro. In a study conducted by Zilberstein et al. (2002), ketamine decreased superoxide production by neutrophils in humans. It also inhibits respiratory burst activity in endotoxaemic rats (Zhou et al., 2007). According to Nishina et al. (1999) xylazine at clinically relevant concentrations had no effect on studied neutrophil functions.

Study carried out by Hans et al. (1991) revealed that MPO values increased significantly 1 h after surgery due to post-operative neutrophil activation independently of the anaesthetic protocols. We observed the
similar effect not only on MPO release but also on superoxide after 1 h, when in case of superoxide the level of response was higher in ketamine/propofol group. Moreover, in terms of NO generation by neutrophils, the increase was observed after 24 h. On the other hand, elastase release after 24 h returned to pre-anaesthesia level and ALKP release was lower than pre-anaesthetic value.

Propofol reduces apoptosis of endothelial cells by suppressing caspase-3 activity (Wang et al., 2007). We have not seen the similar effect in neutrophils during experiment. However, studies conducted by Goto et al. (2003) on humans revealed that midazolam in commonly administered doses inhibits neutrophil apoptosis in culture. In our experiment, we did not see this effect on rabbit neutrophils ex vivo. Moreover, Galley et al. (1998) stated that midazolam and propofol had no effect on neutrophil viability.

**CONCLUSIONS**

In our study, both anaesthesiological regimes transiently decreased neutrophil action to abolish possible tissue injury during neutrophil activation, but without persistent loss of immune function. Neutrophil activity returned to almost pre-anaesthesia level 24 h after surgery to supply proper immune function.

**REFERENCES**


