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EFFECTS OF DIFFERENT ANESTHETIC AGENTS ON GM-CSF, MCP1, IL1α AND TNFα LEVELS IN RAT SEPSIS MODEL

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Anesthetic agents could alter the course and outcome of physical trauma, as well as experimentally or naturally occuring severe infections, by regulating several immune response mechanisms. The aim of our study was to investigate the influence of several commercially used anesthetic agents (ketamine, propofole, pentylentetrazole – PTZ) on cytokine concentrations, animal survival and pathohistological changes in the model of rat sepsis. In adult, male Wistar rats after different anesthetic traeatment and induction of sepsis by cecal ligation and punction we estimated serum levels of IL1α, TNFα, GM-CSF and MCP-1 at 12h intervals. After 48h of sepsis induction, the largest number of animals survived in the group treated with PTZ (47%), while the lowest survival rate was in the propofole treatment group (24%). Contrary to survival rate, the most abundant pathohistological changes were seen on preparations from PTZ and than in ketamine/PTZ treated groups, without any significant changes in the CNS of propofole treated animals. In the propofole treated group there was a prominent increament of GM-CSF values at 12h and 24h, followed by a significant decreament at 36h. These changes were negatively correlated to the survival rate in this group. This group had the lowest levels of MCP1 at all evaluated time intervals. After high initial levels, IL1α and TNFα levels fell to undetectable concentrations and at 24h increased to a high level. In PTZ as well as ketamine groups, at 12 h interval, GM-CSF levels were lower than in the propofole treated group. Contrary, MCP-1 levels were higher in these groups comparing to propofole group. After a high initial peak, IL1α levels decreased to low but detectable levels, followed by an intensive rise in ketamine treated, but with further decrement in pentazole treated groups. TNFα levels were low through all evaluated intervals in both these groups. Our results indicate that induction of anaesthesia of animals with sepsis with variuos anesthetic agents is connected to different pathohistological

CNS changes, distinct serum cytokine profiles and diverse survival rates.

Key words: anesthetic agents, GM-CSF, IL1α, ketamine, MCP1, rat, pentylenetetrazole, propofole, sepsis, TNFα

INTRODUCTION

Anesthetic agents could alter the course and outcome of physical trauma, as well as experimentally or naturally occuring severe infections, by regulating several immune response mechanisms (Schneemilch *et al.,* 2005). Results of studies in the '90s showed that propofole, tiopenton, midazolame and ketamine in clinically relevant concentrations have effects on phagocytosis, oxygen radical production and intracellular cytolysis (Davidson *et al.,* 1995). These effects could be harmful in patients that have a low PMN number and function, as it is the case in sepsis and chronic inflammatory diseases. Studies with i.v. anesthetics showed that propofole did not significantly increase intensity of programmed cell death in the lymphocyte population (Song *et al.,* 2004). This propofole effect is considered beneficial, as early cell mediated cytotoxic activity is essential in response to infection. Mitogen stimulation of the healthy mononuclear cell population (MNC) in the presence of different concentrations of tipoentol, phentanile, suphentanile, sevoflourane, NO and combination of these anesthetics resulted in a lowered production of key T lymphocyte growth factors (IL2 i sIL2-R) only as a response to tiopental and NO. Contrary, servoflourane exerted stimulatory effects upon MNC (Schneemilch *et al.,* 2005). Propofole, either before or simultanously given with endotoxin, exerts a protective effect on endotoxin induced lung damage in this animal model (Gao *et al.,* 2004). Results of this study pointed out that propofole reduced mortality by interfering in endotoxin disturbance of endothelial barrier and by decreasing production of TNFα and NO. On the other hand, propofole represses the PMN bactericidal ability (measured by intensity of stimulated respiratory burst) without changes in phagycitosis capacity (Heine *et al.,* 2000). Ketamine exerts anti inflammatory effects on LPS stimulated macrophages, both *in vivo* and *in vitro* conditions. Administration of ketamine in different cell cultures, on LPS stimulated glial cells, astrocytes and microglial cultures resulted in decreased TNFα nad PGE_2 production (Shibakawa *et al.,* 2005). In the same experimental system, propofole did not exert significant effects on inflammatory cytokine and prostaglandin production. Ketamine increased survival rate in experimentally induced severe sepsis with viable *E. Coli* in rats (Shaked *et al.,* 2004), as well as increased survival of experimental animals exposed first to thermal injury and than to induction of sepsis (Gurfinkel *et al.,* 2006). It alters the immune response by acting on several levels of regulation. Ketamine changes cytokine production by decreasing endotoxin induced production and secretion of IL6. Further, ketamine alters adhesive molecules on PMN (Weigand *et al.,* 2000), inhibits early activation signals in MNC, resulting in reduced TNFα production (Yu *et al.,* 2002) and inhibits MNC proinflammatory cytokine

production (Kawasaki *et al.,* 1999; Shaked *et al.,* 2004). Ketamine can reduce tissue damage induced by bacterial products by regulation of NO, one of the crucial endotoxin effector molecules, by reducing iRNK level for inducible NO syntase in endotoxemia conditions (Helmer *et al.,* 2003). As measured in tissues of experimental animals, ketamine reduced NO production in the jejunum, ileum, colon, kidneys, liver and spleen. Even in the CNS, ketamine induced an increase in the number of hyppocampal neurones, together with reduction of neuronal inducible NO synthase (Keilhoff *et al.,* 2004). Ketamine indirectly exerts potent antinflammatory effects on the neurotransmitter level, behaving as agonist of A2A receptors, modulating regulatory functions on leukocyte chemotaxis, TNFα and IL6 production (Blackwell *et al.,* 1996).

MATERIALS AND METHODS

Animals and treatments

Adult male Wistar rats, weighting 200-250 g, were used for the experiments. They had free access to food and water and were kept under humane and environmentally controlled conditions (a temperature of 23±2°C and a light: dark cycle of 13 : 11 hours). Sepsis was induced in 100 animals, 20 animals per group, by cecal ligation and punction, according to Wichterman rat sepsis model. The first group was treated with pentylenetetrazol (65 mg/kg), second with propofole (10 mg/kg) administered immediately after PTZ, third with ketamine (50 mg/kg) administered immediately after PTZ, fourth with ketamine (50 mg/kg), and fifth with propofole (10 mg/kg). Blood samples were taken at the beginning (0h) and at 12h intervals, from the retrobulbar venous plexus. After coagulation, samples were centrifuged, the serum decanted, aliquoted, frozen and kept at -70°C until testing. The experiment lasted 48h, and after that the animals were sacrificed.

Cytokine determination

Cytokine concentrations (TNFα, IL1α, MCP1, GM-CSF) were measured with commercial flow cytometric test (Bender MedSystems, USA, BMS825FF Rat Cytokine 6 plex). Frozen samples kept at -70° C, 25 μL volume. Samples were added to reaction beds specific for investigated cytokines. After incubation and washing the PE solution was added and incubated under the same conditions. After further washing and fixation, samples were analyzed on a flowcytometer (Beckman Coulter XL-MCL). Data files that contained mean fluorescence intensity for every particular cytokine in the analysed samples were recorded and analyzed with commercial sowtvare (FlowCytomix Pro Software BMS8401FF). Concentrations of all cytokines are given as pg/mL.

Pathohistological analyses

Brain samples (measuring 2x1x0.5cm) were fixated in 10% formalin, paraffin cast in automatic tissue processor (Leica TP 1020). After cutting (Leica RM 2145) tissue samples were further stained (Maier Hematoxilin) and analyzed on a light microscope (Olympus BX-41).

Statistics

Differences in frequencies were tested by Chi-square test. Values of cytokines were given as the median or mean ± SD and were tested by the Mann Whitney U test. Statistical significance was assumed at $p<0.05$.

RESULTS

Survival rate

Only few hours after surgical induction of sepsis, 14% of propofole/pentylentetrazole treated animals died (Table 1). After 12h in all groups increased death rate, ranging from 5% in ketamine/pentylenetetrazole group to 30% in ketamine group. At 24h interval the most prominent death rate increase was reported in the propofole and propofole/pentylenetetrazole group, while the smallest was in the pentayole group. The same observation was made after 12h, i.e. at 36h of monitoring. Finally, the largest death rate was recorded in propofole and propofole/pentylenetetrazole groups, followed by ketamine and ketamine/pentylenetetrazole groups, with the smallest number of deaths in the pentylenetetrazole group.

Table 1. Survival rate in investigated groups

Time	Survival (%) in treated groups					
	K	PTZ	P	KE	P + PTZ	KE + PTZ
0h	100	100	100	100	86	100
12h	100	86	78	70	77	95
24h	100	50	22	30	32	43
36h	100	50	22	30	24	38
48h	100	0	0	0	0	0

Pathohistological findings

Pathohistological findings were estimated in several basic structures of the brain tissue, that is the vascular compartment, neurones and glial tissue (Table 2). The vascular compartment was analyzed according to the presence or absence of structural changes manifested as hyperemia, oedema, thrombosis, hemorrhagia, oedema of blood vessel endothel, and hyalinisation of blood vessels. The most intensive changes, with the highest score were in the pentylenetetrazole group, uniformly expressed on all examined structures. Thrombosis and hyalinisation were mostly reported at the meninge of blood vessels in the hippocampus region. Changes of the same type were present on the brain blood vessels, with a wide perivascular space as the result of oedema liquid collection. Smaller blood vessels, as well as capillaries revealed endothelial oedema, which resulted in incomplete or complete obstruction. Similar changes, but without thrombosis and oedema of endothelial cells, but far less intensive, were seen at CNS preparations from ketamine/pentylenetetrazole and ketamine

treated animals. There were also neuronal degenerations together with the picture of neuronal number decrement. Again, all these changes were most intensive in the pentylenetetrazole treated group and less abundant in ketamine treated groups. Contrary to survival rate, which was lowest for the propofole treated group, changes in the CNS of these animals were comparable to the controls.

Table 2. Pathohistological findings in brain samples

	K	PTZ	P+PTZ	KE+PTZ	KE	P
Vascular changes						
Hyperemia	0	3	0	1	1	0
Oedema	0	3	1	1	1	0
Haemorrhagia	0	3	1	3	1	0
Thrombosis	0	3	0	0	0	0
Endothelial oedema	0	3	0	2	0	0
Blood vessel hyalinisation	0	3	0	0	0	0
Neurones						
Neuronal degeneration	0	3	0	2	2	0
Glia						
Gliosis	0	3	0	1	1	0

Concentrations of cytokines

Concentrations of TNFα, IL1α, MCP1, GM-CSF, IFNγ and IL4 were estimated in samples of sera taken every 12h of monitoring time.

At the initial time interval, IL1 alpha was detectable in most samples, with the highest detectability (92%) in the pentylenetetrazole group and lowest in the ketamine/pentylenetetrazole treated group (31%). Next two intervals, at 12h and 24h are characterized with a gradual decrease of IL1α detectability in all investigated groups, followed by a rapid increment at 36h, again in all investigated groups except pentylenetetrazole treated animals. Basically, a similar scheme is revealed in the determination of IL1α concentrations. High initial IL1α values were detected in all groups, with the highest value in the pentylenetetrazole treated group. This was significantly higher than the lowest detected concentration in ketamine/pentylenetetrazole treated group ($p=0.0155$, MW test). At 12h time interval there was a decrease in IL1α concentrations in all groups. This trend continued further on and at 24h in all groups except in the ketamine treated, in which we detected an increase 3 times bigger than initially measured. From 24h until 36h, there was an increase in IL1α values in all groups excluding the pentylenetetrazole treated (Figure 1). At 36h, levels of IL1α in the pentylenetetrazole treated group were significantly lower than those measured in ketamine, ketamine/pentylenetetrazole or propofole/ pentylenetetrazole groups ($p=0.0180$, $p= 0.0025$, $p=0.0373$, respectively, MW test).

Analogously to IL1alpha levels, TNFα detectability was initially high, ranging from 15% in ketamine/pentylenetetrazole to 50% in the pentylenetetrazole treated group. At 12h there was a dissociation in detectability between ketamine and pentylenetetrazole on one side, and all the other groups on the other side. Namely, detectability was at the lowest level in the ketamine and pentylenetetrazole treated groups, and higher than initially in other groups. This inverse relation continued at 24h and 36h time intervals, with a rise and fall of TNFα detectability in ketamine and pentylenetetrazole treated groups. Concentrations of TNFα were initially high, with the highest level in the ketamine treated group. At 12h and 24h time intervals TNFα was absent from samples of all investigated groups, but, at 36h there was an intensive increase of TNFα concentration in the propofole treated group (Figure 1). These differences did not reach statistical significance.

Monocyte chemotactic protein 1 (MCP-1) was initially present in all tested samples. In the propofole treated group there was a significant detectability

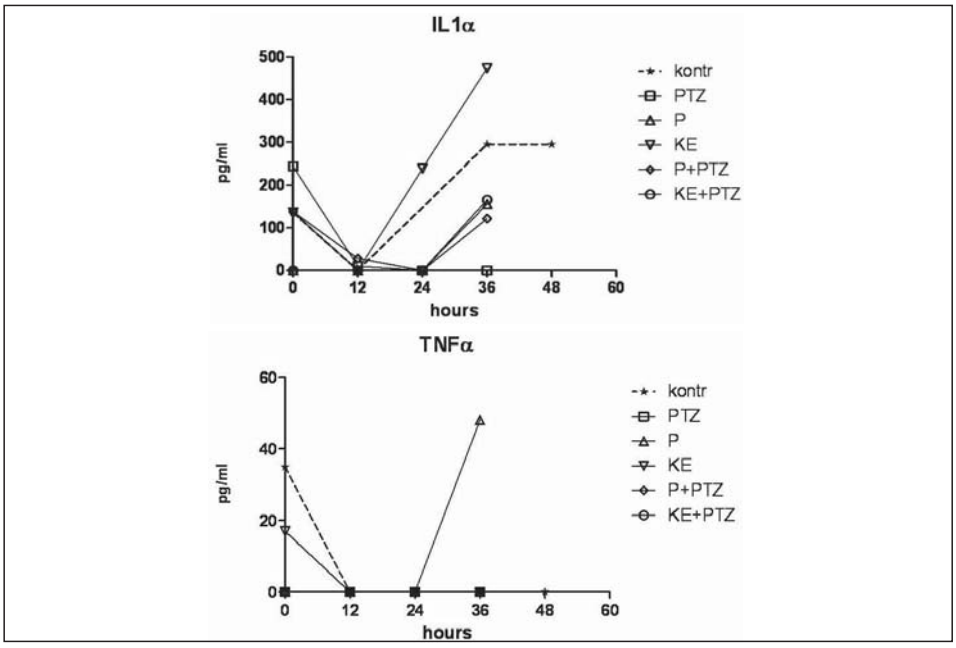

Figure 1. IL1 alpha and TNF alpha concentrations in investigated groups (median values, pg/mL)

decrement at 12h, more pronounced at 24h time point. In the pentylenetetrazole treated group (without other agents or in combination with ketamine and propofole) there was a transient decrease in detectability at 24h, which reached the initial level at 36h. At the first time point, at 0h, MCP-1 concentrations were similar in all investigated groups (Figure 2). After 12h a significant increase was

detected in all groups with the exception of propofole treated group, in which were detected significantly lower MCP-1 concentrations than those in ketamine, ketamine/pentylenetetrazole or propofole/pentylenetetrazole treated groups ($p=0.0285$, $p= 0.0110$, $p=0.0080$, respectively, MW test). After 24h there was a relative fall of MCP-1 levels in all investigated groups, followed by an increase at 36h at levels comparable to those reached after the first 12h.

Detectability of granulocyte monocyte colony stimulating factor (GM-CSF) was very high initially, ranging from 80% in the ketamine group up to 100% in the propofole treated group. In the following time points there were generally three types of detectability trends. GM-CSF detectability in propofole and pentylenetetrazole groups showed a gradual but significant fall, reaching 0% detectability for PTZ and 40% for the ketamine group. From initial 0h time point to 36h, GM-CSF detectability was between 60-80% in the groups treated with ketamine and propofole in combination with pentylenetetrazole, and 100% in the ketamine treated group. Initially, the concentration of GM-CSF was highest in the pentylenetetrazole treated group, significantly higher than the groups treated with the same agent in combination with ketamine or propofole ($p=0.0159$, $p= 0.0404$, respectively, MW test). 12h time interval was characterized with GM-CSF levels increment which was moderate in the ketamine and PTZ treated groups, but very intensive in others (Figure 2). After 24h the rise was continued only in the propofole/PTZ group, with decreasing GM-CSF levels in others, most prominent in propofole and ketamine / PTZ groups. Finally, at 36h, GM-CSF concentrations

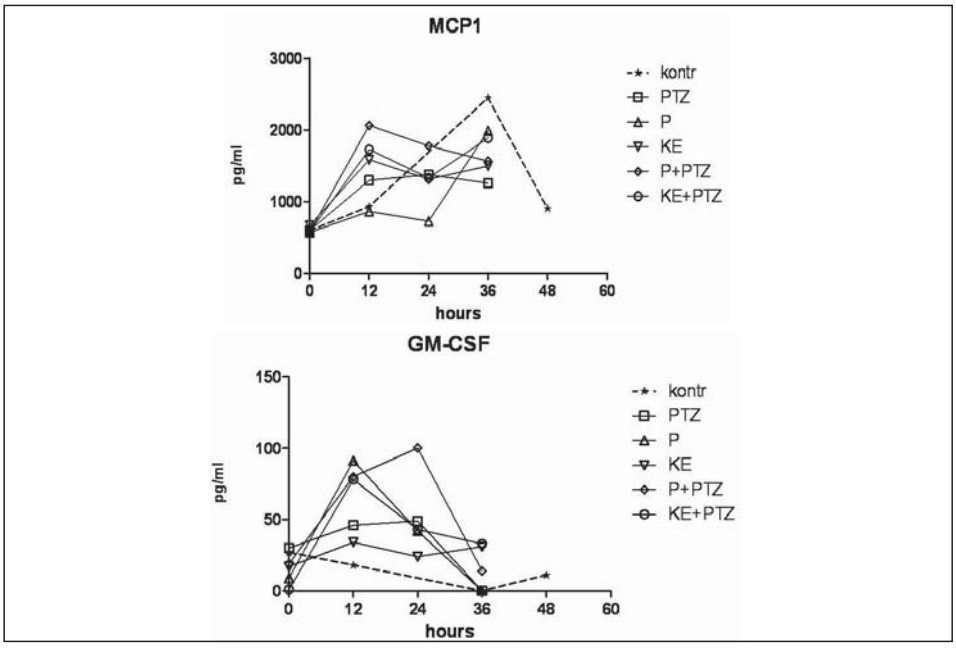

Figure 2. MCP-1 and GM-CSF concentrations in investigated groups (median values, pg/mL)

were undetectable in PTZ and propofole treated groups, while others had levels comparable to initial values.

DISCUSSION

Ketamine exerts numerous immunomodulatory effects which are dose dependent. *In vitro* conditions, in anesthetic concentrations it reduces LPS and LPS/LPS-BP complex ligation to cells, which strongly influences the monocyte/ macrophage functions (Yu *et al.,* 2002), resulting in reduced IL1α and TNFα production upon LPS stimulation (Chen *et al.,* 2009; Neder *et al.,* 2004; Sun *et al.,* 2004). These results indicated a possible indirect anti inflammatory ketamine feature, reflected in lowered LPS influence at macrophage system stimulation, that could reduce damaging uncontrolled inflammatory response to infection. Our results are in concordance with this view on ketamine actions. Early in sepsis, at 12h time point, when the endotoxin concentration, is at its highest, in ketamine treated animals, low IL1α levels colud be the result of its decreased production caused by decreased LPS ligation to target receptors of macrophages. Later, metabolic removal of ketamine from the organism make LPS stimulation of macrophages possible and complete, which results in a late rise of inflammatory cytokine production.

Contrary to earlier findings, whose results showed that ketamine antiinflammatory effects are partly mediated by reducing TNFα concentration (Chen *et al.,* 2009; Neder *et al.,* 2004; Sun *et al.,* 2004; Chang *et al.,* 2010), in our study TNFα levels detected in the ketamine treated group did not differ significantly from control animals. On the other hand, TNFα detectability was highest in samples of those animals who died immediately after anesthetic treatment followed by cecum ligation and punction, with the possibility that they developed endotoxic shock.

MCP-1 is a potent chemoatractant acting as a regulator of numerous monocyte functions in several inflammatory processes. Animal studies on pharmacological inhibition of MCP-1 with bindarite, showed that high MCP-1 levels are directly connected with intensive monocyte accumulation in the visceral organs (Ramnath *et al.,* 2008). Recombinant MCP-1 given intraperitoneally potected mice from endotoxin induced sepsis, which resulted in increased serum IL10 together with decreased IL12 and TNFá concentrations (Zisman *et al.,* 1997). So, in the endotoxin induced sepsis model, MCP-1 exerts a protective role, altering cytokine network balance in an antiinflammatory direction. Inhibition of MCP-1 function increases production of pro-inflammatory cytokines on the periphery, a lower number and intensity of inflammatory brain lesions and is associated with decreased serum corticosterone levels (Thompson *et al.,* 2008). All this gives the basis to conclude that MCP-1 could be the crucial mediator that transfers inflammatory signals from the periphery to the CNS in this model of sepsis. In our results, ketamine induced the lowest initial MCP-1 levels, lower even than in control animals. On the other side, the ketamine treated group had the highest MCP-1 levels at 12h and 24h time points, higher than pentylenetetrazole

treated group that had the highest survival rate. High MCP-1 levels in the inital interval and at 12h time point, in the pentylenetetrazole treated group could be connected with most prominent pathohistological CNS changes seen in this group, reflected in intensive hiperemy, oedema, haemoraghic and thrombotic lesions, together with neuronal degeneration and gliosis. High serum levels could mediate monocyte and PMN accumulation, together with upregulation of their functions and as a consequence, inflammatory cytokine production from microglia.

Inflammation on the periphery causes numerous physiological and behavioral CNS changes. Responsible mechanisms, main mediators and signal paths which are part of this phenomenon are far from understood. Induction of rat intestinal inflammation with TNBS was connected with significant, although reversible inflammatory hypocampal response, featured by microglial activation and TNFα production. Administration of pentylenetetrazole or intraventricular TNFα injection resulted in convulsions and significant damage in other CNS structures (Riazi *et al.,* 2008). Authors concluded that CNS excitability increament is based on TNFα production from microglia and represents one of the mechanisms that connect neurological and behevioral changes with acute or chronic inflammation. In an other study, Cyclosporine A (CyA) administration resulted in inhibition of pentylenetetrazole induced convulsions. Anticonvulsive effects of CyA were explained by inhibition on macrophage production of inflammatory cytokines, chemotactic mediators and NO (Homayoun *et al.,* 2002).

GM-CSF is considered as extremely important in sepsis, as a factor who could regain, reverse reduced, inhibited potentials of monocytes (Meisel *et al.,* 2009; Bilgin *et al.,* 2001). *In vivo* administration of GM-CSF significantly increases survival rate, affecting the severity of symptoms and absence of other complications in sepsis. In the conditions of heavy reduction of PMN number caused by severe infections, mobilisation of PMN together with upregulation of all functional activities could begin and restore the protective response of host to infection, as well as inflammation control. Our results showed the highest detectability of GM-CSF at 36h in the ketamine treated group. The highest GM-CSF concentrations were detected at 12h, in propofole and ketamine/PTZ or propofole/PTZ treated groups. Dramatic GM-CSF decrease from 12h to 24h in propofole and propofole/PTZ group could be connected with significantly higher death rate recorded at this time interval in these groups, again implying a possible protective role of this cytokine. We did not find any data that connects the influence of administrated anesthetics to endogenous, unmodified GM-CSF values.

On the basis of these results, we could speculate that certain anesthetic agents could directly influence the functions of the immune resopnse in conditions of severe infections, making them important factors in the outcome of acute and chronic inflammatory diseases.

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EFEKTI RAZLIČITIH ANESTETIKA NA NIVOE GM-CSF, MCP1, IL1α I TNFα NA PACOVSKOM MODELU SEPSE

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SADRŽAJ

Anestetici mogu izmeniti tok i ishod uticaja fizičke traume kao i eksperimentalno ili prirodno nastalih teških infekcija, regulacijom nekoliko mehanizama imunskog odgovora. Cilj naše studije je bio da ispitamo uticaj nekoliko komercijalno korišćenih anestetika (ketamina, propofola i pentzlentetrazola) na koncentraciju citokina, preživljavanje životinja i patohistološke promene u modelu sepse indukovane u pacova. U odraslih pacova, muškog pola, po primeni različitih anestetika i indukcije sepse podvezivanjem i punkcijom cekuma, utvrđivali smo nivoe IL1α, TNFα, IL4, IFNγ, GM-CSF i MCP-1 u intervalima od 12 sati. Nakon 48 sati od indukcije sepse, najveći broj životinja preživeo je u grupi tretiranoj pentylenetetrazolom (50%), potom u ketamin/pentylenetetrazol tretiranoj grupi, dok je najmanja stopa preživljavanja bila u grupi tretiranoj propofolom (22%). Nasuprot stopi preživljavanja, najizraženije patohistološke promene su utvrđene na preparatima grupa tratiranih pentazolom i ketamin/pentylenetetrazol, bez značajnih promena u CNS u grupi životinja tretiranih propofolom. U uzorcima grupe tretirane propofolom detektovali smo intezivan porast vrednosti GM-CSF 12h i 24h, praćen

značajnim padom posle 36h. Ove promene su negativno korelirale sa stopom preživljavanja u ovoj grupi. Ova grupa je imala i najniže vrednosti MCP-1 u svim praćenim vremenskim intervalima. Posle visokih inicijalnih nivoa, vrednosti IL1α i TNFα pale su na nedetektabilne a u terminu od 24h porasle su na visok nivo. U grupama tretiranim pentazolom i ketaminom, 12. sata, vrednosti GM-CSF bile su niže od grupe tretirane propofolom. Nasuprot tome, vrednosti MCP-1 su bile veće u ovim grupama u odnosu na propofolom tretiranu grupu. Posle visokog inicijalnog skoka, vrednosti IL1α smanjuju se na niske ali detektabilne vrednosti, a zatim sledi intezivan rast u ketaminom tretiranoj grupi i dalji pad vrednosti u pentilenetetrazol tretiranoj grupi. Vrednosti TNFα su u ove dve grupe bile niske u svim ispitivanim intervalima. Vrednosti IL4 i IFNγ su bile praktično nedetektabilne u svim ispitivanim grupama. Naši resultati ukazuju da je indukcija anestezije u životinja sa sepsom različitim anesteticima povezana sa različitim patohistološkim nalazima u CNS, različitim serumskim profilom citokina i različitom stopom preživljavanja.