The influence of intravenous anaesthetics on polymorphonuclear leukocyte function

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Polymorphonuclear leukocytes (PMNL) play a vital role in the defence against invading bacteria. It is known that some anaesthetics inhibit PMNL function and, thus, possibly enhance perioperative infection. We investigated the effect of methohexitone, flunitrazepam, and droperidol on three bactericidal PMNL functions, i.e., superoxide anion production, hydrogen peroxide generation, and activity of released myeloperoxidase, in vitro. Approved photometrical assays were used. Superoxide anion was measured by the reduction of cytochrome C, hydrogen peroxide by the horse radish peroxidase catalysed oxidation of phenol red, and myeloperoxidase by the turnover of 2,2'-azino-di(3-ethylbenzthiazoline) sulfonic acid. Methohexitone ($P \leq 0.001$) and flunitrazepam ($P \leq 0.01$) inhibited superoxide anion production, and methohexitone ($P \leq 0.01$) reduced hydrogen peroxide generation but only at concentrations beyond clinical relevance. Droperidol did not cause any alteration of the PMNL functions tested. Consequently, it seems unlikely that the usual doses of methohexitone, flunitrazepam, or droperidol promote bacterial infections in vivo by impairing the activity of myeloperoxidase or by inhibiting the generation of superoxide anion or hydrogen peroxide.

Les leucocytes polynucléaires jouent un rôle essentiel de défense contre l'invasion bactérienne. On sait que certains anesthésiques inhibent cette fonction et ainsi pourraient favoriser l'infection périopératoire. Nous avons recherché in vitro les effets du méthohéxitone, du flunitrazépam et du dropéridol sur trois fonctions des polynucléaires, i.e. la production de l'anion superoxyde, la génération du peroxyde d'hydrogène et l'activité de la myéloperoxydase. Des épreuves photométriques reconnues ont été utilisées. L'anion superoxyde a été mesuré par la réduction du cytochrome C, le peroxyde d'hydrogène par l'oxydation catalytique du rouge phénol par la peroxydase du raifort et la myéloperoxydase par la production de l'acide sulfonique 2,2'-azino-di(3-éthylbenzthiazoline). Le méthohexitone ($P \leq 0,001$) et le flunitrazépam ($P \leq 0,01$) inhibent la production de l'anion superoxyde, et le méthohexitone diminue la génération du peroxyde d'hydrogène mais seulement à des concentrations qui dépassent l'usage clinique. Le dropéridol ne cause pas d'altérations des fonctions polynucléaires. Par conséquent, selon toute probabilité, les doses usuelles de méthohexitone, de flunitrazépam ou de dropéridol ne favorisent pas l'infection bactérienne in vivo par altération de l'activité de la myéloperoxydase ou par l'inhibition de la génération de l'anion superoxyde ou du peroxyde d'hydrogène.

Key words

ANAESTHETICS, INTRAVENOUS: droperidol, flunitrazepam, methohexitone;
BLOOD: leukocytes.

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In spite of the availability of potent antibiotics perioperative infection is still an important clinical problem. In humans, bacteria are confronted with a complex defence system which, above all, consists of polymorphonuclear leukocytes (PMNL). Some anaesthetics may disturb PMNL function. The mechanism responsible for this effect is still unknown. At the beginning of this century Graham[1] described the depression of phagocytosis caused by ether. Since that time researches have been conducted and reviews of the most important results in this field were published by Stevenson *et al.*[2] and by Kress and Eberlein.[3] Despite this, many questions concerning the influence of anaesthetics on PMNL functions remain unanswered. We studied the action of methohexitone, flunitrazepam, and droperidol on superoxide anion formation, hydrogen peroxide production, and activity of released myeloperoxidase *in vitro*. Superoxide anion, hydrogen peroxide, and myeloperoxidase are of interest because they are important components of the oxygen-dependent bactericidal system with which PMNL are equipped.

Methods

The permission to conduct the study was obtained from the ethical committee of the medical faculty of Justus-Liebig-University Giessen.

Blood samples

Heparinised venous blood samples (10 u heparin-sodium ml^{-1}; Sigma, Deisenhofen) were obtained from ten healthy male volunteers (age: 29 ± 4 yr; body weight: 75 ± 8 kg; height: 1.80 ± 0.05 m (mean and SD).

Isolation of PMNL

The PMNL were isolated with a modification of a method described by Hjorth *et al.*[4] Percoll 55% and Percoll 74% were produced by diluting isotonic Percoll (Sigma, Deisenhofen) with normal saline solution. Four ml Percoll 55% were poured into a polystyrene tube and underlayered with 4 ml Percoll 74% and overlayered with 4 ml heparinised blood and centrifuged at 350 × g for 20 min at 20°C. The PMNL band was then carefully removed and suspended in 10 ml phosphate buffered saline (PBS; Gibco, Karlsruhe). After centrifugation at 350 × g for 10 min at 20°C the supernatant fluid was decanted. The few erythrocytes contaminating the pellet were lysed by adding 2 ml double-distilled water. After 20 sec, isotonia was restored with 1 ml saline 2.7% (Merck, Darmstadt). After administering 7 ml PBS the tube was centrifuged at 350 × g for 10 min at 20°C and the supernatant was decanted. The purity of the PMNL yield was microscopically evaluated, and viability was confirmed as 95% by the trypan blue exclusion test.[5]

Superoxide anion formation

Superoxide anion production was measured photometrically by the reduction of cytochrome C using a modification of the method described by Stangel *et al.*[6] which is similar to that of Weening *et al.*[7] Cytochrome C, 100 mg (type VI; Sigma, Deisenhofen) were dissolved in 30 ml PBS containing 0.99 g glucose per 100 ml (Merck, Darmstadt). The solution was portioned and frozen at −20°C. Opsonized zymosan was used to stimulate PMNL and was produced by incubating 100 mg zymosan (Sigma, Deisenhofen) with 6 ml AB pool serum for 30 min at 37°C. After washing with saline and centrifugation at 350 × g for 10 min opsonized zymosan was resuspended in 10 ml PBS glucose buffer, portioned, and frozen at −20°C. Isolated PMNL ($0.8 \times 10^6 ml^{-1}$) were incubated with the anaesthetic to be tested for 15 min at 37°C. Then 500 µl zymosan, 150 µl AB pool serum, 250 µl cytochrome C, and 500 µl PMNL suspension were poured into a test tube. A preparation containing 500 µl buffer instead of zymosan was used as zero adjustment. After incubation for 15 min at 37°C the reaction was stopped by putting the test tube into ice water. After centrifugation, at 350 × g for 5 min at 4°C, extinction of the supernatant was measured photometrically at 546 nm (digitalphotometer 6114 S; Eppendorf, Hamburg). The amount of superoxide anion produced resulted from the extinction coefficient of cytochrome C ($21\ mMol^{-1} \cdot cm^{-1}$) according to the law of Lambert, Beer, and Bouguer.[6] Preparations containing no anaesthetic were simultaneously measured. The assay was able to detect 0.001 nmol of superoxide anion.

Hydrogen peroxide formation

Hydrogen peroxide production was determined photometrically using a method described by Stangel *et al.*[6] which is a modification of the system mentioned by Pick and Keisari.[9] The process is based on horse radish peroxidase catalysed oxidation of phenol red by hydrogen peroxide. Phenol red (Sigma, Deisenhofen) was dissolved in double-distilled water ($10\ mg \cdot ml^{-1}$) and horse radish peroxidase (type II: Sigma, Deisenhofen) in PBS glucose buffer ($5\ mg \cdot ml^{-1}$). The PMNL were isolated as described before and were stimulated by opsonized zymosan. The final test preparation consisted of 500 µl zymosan, 125 µl AB pool serum, 12.5 µl horse radish peroxidase, 12.5 µl phenol red, 12.5 µl sodium azide (200 mM; Merck, Darmstadt), 500 µl PMNL suspension ($2 \times 10^6\ ml^{-1}$), and the anaesthetic to be tested. After incubation for 30 min at 37°C the preparation was centrifuged at 350 × g for 10 min at 4°C. After adding 25 µl sodium hydroxide solution (1.0 normal; Merck, Darmstadt) extinction of the supernatant was measured photometrically at 623 nm. The hydrogen peroxide concentration was determined using a calibration curve previously established. The assay was able to detect 0.001 nmol of hydrogen peroxide.

Activity of released myeloperoxidase

A modification of the method described by Schmitt *et al.*[10] was used to measure the activity of released myeloperoxidase: 1 mM 2,2'-azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS; Sigma, Deisenhofen) was dissolved in 0.1 M citrate buffer (Behring, Marburg). The PMNL were isolated as described before ($2 \times 10^6\ ml^{-1}$) and incubated with the respective anaesthetic for 15 min at 37°C. Then 0.5 µg cytochalasin B (Sigma, Deisenhofen) was added to 100 µl PMNL suspension. The preparation was incubated for five minutes at 37°C. After adding 100 µl opsonized zymosan further incubation was conducted for 10 min at 37°C. Then, 1 ml ABTS solution was added. After centrifugation at 700 × g for five minutes at 20°C, 1 ml supernatant was removed and mixed with 1 µl hydrogen peroxide solution (30%; Merck,

TABLE I the influence of various concentrations of methohexitone on PMNL superoxide anion production (nmol · 10^{-6} PMNL · min^{-1}), hydrogen peroxide generation (nmol · 10^{-6} PMNL · min^{-1}), and myeloperoxidase activity (units L^{-1} supernatant) *in vitro* ($n = 10$; means + SD)

	Methohexitone			
	0 μg · ml⁻¹	*1.7 μg · ml⁻¹*	*17 μg · ml⁻¹*	*170 μg · ml⁻¹*
Superoxide anion	3.003 ±0.579	2.465 ±0.703	2.562 ±0.727	2.245* ±0.764
Hydrogen peroxide	0.851 ±0.500	0.832 ±0.598	0.794 ±0.482	0.665† ±0.406
Myeloperoxidase	0.650 ±0.221	0.461 ±0.151	0.480 ±0.219	0.598 ±0.329

*$P \leq 0.001$;
†$P \leq 0.01$.

TABLE II The influence of various concentrations of flunitrazepam on PMNL superoxide anion production (nmol · 10^{-6} PMNL · min^{-1}), hydrogen peroxide generation (nmol · 10^{-6} PMNL · min^{-1}), and myeloperoxidase activity (units L^{-1} supernatant) *in vitro* ($n = 10$; means ± SD)

	Flunitrazepam			
	0 μg · ml⁻¹	*0.004 μg · ml⁻¹*	*0.04 μg · ml⁻¹*	*0.4 μg · ml⁻¹*
Superoxide anion	3.003 ±0.579	2.553 ±0.729	2.524 ±0.697	2.412* ±0.907
Hydrogen peroxide	0.851 ±0.500	0.845 ±0.524	0.829 ±0.509	0.823 ±0.523
Myeloperoxidase	0.650 ±0.221	0.513 ±0.188	0.441 ±0.169	0.498 ±0.204

*$P \leq 0.01$.

Darmstadt). Extinction was measured photometrically at 405 nm. Using a calibration curve previously established with horse radish peroxidase activity of released myeloperoxidase was determined. The assay was able to detect 0.001 mU of peroxidase activity.

The variability of the three assays was ≤5%.

Anaesthetics

The following concentrations were tested:

- Methohexitone (Brevimytal Natrium; Eli Lilly, Bad Homburg, Germany): 1.7, 17, and 170 μg · ml^{-1}.
- Flunitrazepam (Rohypnol; Hoffmann-La Roche, Grenzach-Wyhlen, Germany): 0.004, 0.04, and 0.4 μg · ml^{-1}.
- Droperidol (Dehydrobenzperidol; Janssen, Neuss, Germany): 0.1235, 1.235, and 12.35 μg · ml^{-1}.

The medium concentrations (17 μg methohexitone ml^{-1}, 0.04 μg flunitrazepam ml^{-1}, and 1.235 μg droperidol ml^{-1}) are of clinical relevance.[11-13]

Statistics

All tests were performed in duplicate and the results were mean values of two estimations. The Pearson Stephens test was used to check normal distribution. The Bartlett test examined homogeneity of variance ($P \leq 0.1$). If the requirements were met, analysis of variance for repeated-measures and Scheffé test were conducted. If the requirements were not fulfilled, Friedman analysis of variance and Miller test were performed. A probability of $P \leq 0.05$ was regarded as moderately significant, $P \leq 0.01$ as significant, and $P \leq 0.001$ as highly significant.

Results

Means and standard deviations are specified in Tables I–III.

The high concentration of methohexitone caused an inhibition of superoxide anion production ($P \leq 0.001$) and a reduction of hydrogen peroxide generation ($P \leq 0.01$). The alterations of myeloperoxidase activity were not statistically significant.

The high concentration of flunitrazepam inhibited superoxide anion generation ($P < 0.01$) but there was no effect on hydrogen peroxide production or myeloperoxidase activity.

Droperidol did not influence the PMNL functions tested.

Discussion

The human organism has a wide range of mechanisms which provide protection from infection. Immunity to

TABLE III The influence of various concentrations of droperidol on PMNL superoxide anion production (nmol · 10^{-6} PMNL · min^{-1}), hydrogen peroxide generation (nmol · 10^{-6} PMNL · min^{-1}), and myeloperoxidase activity (units L^{-1} supernatant) *in vitro* (n = 10; means ± SD)

	Droperidol			
	0 μg · ml⁻¹	*0.1235 μg · ml⁻¹*	*1.235 μg · ml⁻¹*	*12.35 μg · ml⁻¹*
Superoxide anion	3.459	3.231	3.387	3.138
	±0.517	±0.483	±0.710	±0.550
Hydrogen peroxide	1.122	1.092	1.105	0.970
	±0.267	±0.217	±0.290	±0.334
Myeloperoxidase	0.449	0.394	0.425	0.425
	±0.164	±0.138	±0.118	±0.177

bacteria depends on the activity of PMNL.[14-16] After being produced gradually in bone marrow PMNL are delivered to the blood. Attracted by chemotactic factors they permeate the walls of blood vessels and migrate in the direction of bacteria which are subsequently ingested forming phagosomes. These vacuoles are fused with cytoplasmatic granules thus creating phagolysosomes, in which bacteria are usually killed and degraded. A distinction can be made between oxygen-dependent and oxygen-independent bactericidal mechanisms. Oxygen-dependent killing is complex. The PMNL are equipped with an oxidase which probably consists of cytochrome b, flavoprotein, and ubiquinone. This enzyme is able to transport electrons from NADPH to oxygen to form superoxide anion which, presumably, is bactericidal. Beyond that it is the basic material of several other oxygen products being able to kill bacteria. For example, hydrogen peroxide is generated by a dismutase reaction. Compared with other aggressive oxygen metabolites (superoxide anion, singlet oxygen, hydroxyl radical) hydrogen peroxide is stable. Therefore, it can pass through cell membranes and biological fluids without degradation and develop a long-range bactericidal effect. Myeloperoxidase is an enzyme included in the azurophilic granules of PMNL and, among other actions, it catalyses the generation of bactericidal hypochlorous acid.

Methohexitone is a barbiturate frequently used for induction of anaesthesia. In our study high concentrations inhibited superoxide anion production and hydrogen peroxide generation but clinically relevant concentrations had no effect. Myeloperoxidase activity was not influenced by methohexitone. Previous studies with methohexitone on PMNL properties have shown no impairment of chemiluminescence,[17-19] but that it inhibited PMNL adherence.[20] Moudgil *et al.*[21] and Kress *et al.*[22] found an inhibition of migration.

Flunitrazepam is a benzodiazepine used as premedicant, sedative, and induction agent. At high concentrations it depressed superoxide anion generation, but at clinically relevant concentrations it did not. This is compatible with observations made by Goldfarb *et al.*[23] There were no effects of flunitrazepam on hydrogen peroxide production or myeloperoxidase activity. Kress and Weiler[24] investigated the influence of flunitrazepam on chemiluminescence and at very high concentrations this PMNL function was inhibited.

Droperidol is a substituted butyrophenone possessing neuroleptic, antiemetic, and hypotensive properties. Most often it is used with fentanyl to conduct neuroleptanaesthesia. In the course of our investigation we were unable to detect any disturbance of superoxide anion generation, hydrogen peroxide production, or activity of released myeloperoxidase caused by droperidol. Nakagawara *et al.*, using a similar method,[25] noticed a 50% reduction of superoxide anion generation caused by 32 μg · ml^{-1} of droperidol but this is a concentration far beyond clinical relevance.

In conclusion, our data suggest that the customary doses of methohexitone, flunitrazepam, or droperidol do not enhance bacterial infections by inhibiting superoxide anion production, hydrogen peroxide generation, or myeloperoxidase release. However, clinical recommendations based on *in vitro* results are problematic on principle. For example, the concentrations of anaesthetics in infected tissues are unknown. Therefore, additional clinical studies should be conducted to support our findings.

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