# Influence of $O_3/O_2$–pneumoperitoneum as an oxidative stressor on duration of anaesthesia, loss of different reflexes and cytokine mRNA expression

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## Summary

We analysed the effect of intraperitoneal insufflated ozonized oxygen on the anaesthetic strength generated by tribromoethanol, ketamine/xylazine, chloral hydrate, pentobarbital, and urethane in male Wistar rats. High dosages of anaesthetic drugs normally used for deep surgical anaesthesia were injected. The ozonized oxygen gas mixture was given five times daily on five consecutive days at 0.8 mg ozone/kg body weight before anaesthesia. The reflexes were measured 15, 30, 60, 90, 120, 180, and 240 min after injection of the anaesthetic drug. The sleeping time and the loss and regain of six different reflexes on noxious and non-aversive stimuli were recorded during the 4 h of observation. $O_3/O_2$–pneumoperitoneum ($O_3/O_2$–PP) reduced the sleeping time induced by tribromoethanol and ketamine/xylazine and increased it for chloral hydrate and pentobarbital. In accordance to the changes in the duration of anaesthesia, the $O_3/O_2$–PP induced significant changes in the loss of different reflexes. Additionally, the modulatory effect of the anaesthetic drugs on splenic cytokine mRNA expression was further influenced by $O_3/O_2$–PP. Thus, the influence of an oxidative stressor on anaesthetic potency and on the resting immune system has to be taken into account for experimental designs in which surgical anaesthesia is necessary for small laboratory animals.

**Keywords** Oxidative stress; ozone–pneumoperitoneum; injectable anaesthetic drugs; anaesthetic strength; cytokine

In many animal experiments the use of a non-inhaled anaesthetic drug is required. In non-recovery experiments urethane and chloral hydrate are commonly used anaesthetics, whereas in recovery experiments ketamine/xylazine, pentobarbital, and tribromoethanol are preferred. The duration and the depth of anaesthesia induced by these injectable anaesthetic drugs in small laboratory animals are known (Wixson *et al*. 1987, Field *et al*. 1993). But in most experiments additional substances given to the animal might influence the time and/or depth of anaesthesia. For example,

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chloramphenicol, cimetidine, ketoconazole or columbin when given peri-anaesthetically to small laboratory animals increased (Roder *et al*. 1993) or decreased (Wada *et al*. 1995) the duration of sleeping time of different anaesthetics. Most inhaled or injected xenobiotic anaesthetic drugs and the majority of clinically used drugs become metabolized by cytochrome P-450 enzymes. It has been discussed that these drugs and/or its metabolites are involved in the pathophysiology of some anaesthetics (Morgan 1997).

Changes in the duration of anaesthesia have been described when gaseous substances were given by inhalation. The inhalation of a gas, such as ozone ($O_3$) can influence the anaesthetic effects of barbiturates such as thiopental (McCracken *et al*. 1979) and pentobarbital (Kramer *et al*. 1983). For example, ozone was able to increase significantly the pentobarbital-induced sleeping time of aged mice, rats and hamsters (Graham *et al*. 1981, Canada *et al*. 1986), which depends on the modulation of the enzymatic activity of cytochrome P-450 (Graham *et al*. 1985). Furthermore, the inhalation of a helium–oxygen gas mixture reduced the lethality of ethanol plus pentobarbital (Malcolm *et al*. 1985). This lets us assume that ozone, which has recently been described as an endogenously-produced powerful oxidant gas (Babior *et al*. 2003), could have some influence on other anaesthetic drugs, especially when given by i.p. insufflation. This has, to our knowledge, not yet been investigated. The inhalation of gas mixtures such as ozonized oxygen ($O_3/O_2$) is toxic to the lungs of all animal species, dependent on doses and time (Menzel 1984). To avoid known pathophysiological effects we applied the $O_3/O_2$ gas mixture by a repetitive insufflation directly into the abdomen ($O_3/O_2$–pneumoperitoneum, $O_3/O_2$–PP). Up to now, no reports about influences of insufflated oxidative gases into the peritoneum on the duration and depth of anaesthesia have been available.

The aim of this study was to evaluate whether the anaesthetic potency of the five most frequently anaesthetics (tribromoethanol, ketamine/xylazine, urethane, chloral hydrate, and pentobarbital) used at high dosages can be effected by pre-treatment with $O_3/O_2$ gas mixture as an oxidative stressor. Therefore we measured the sleeping time, the loss of reflexes and depth of surgical anaesthesia. Because an early influence of some anaesthetic drugs on the immune status has already been found (Bette *et al*. 2004), we further evaluated if the effect of an oxidative stressor on depth and duration of anaesthesia is associated with changes in the anaesthesia-induced expression of cytokine mRNAs. Pure effects of different gases such as $CO_2$, $N_2O$, helium or air insufflated into the abdomen have been described to influence the intraperitonal immunity (Kamei *et al*. 2001, Matsumoto *et al*. 2001). Therefore, interactions between $O_3/O_2$ gas mixture and anaesthetic agents on the local immune system in the abdomen are possible and are additionally evaluated in this study by characterization of splenic cytokine mRNA expression.

## Material and methods

### *Animals*

Adult male HsdBrl:WH Wistar rats weighing 209–215 g were purchased from Harlan-Winkelmann GmbH, Borchen, Germany. The animals were kept in rooms with standardized airconditioning 20–22°C, 50–57% humidity and a 12 h artificial day/night rhythm. The rats had free access to food (1320 Rats/Mice Standard Maintenance Diet; Altromin, Lage, Germany) and water was available *ad libitum*. All animal experiments were approved by the RP Giessen (Az: 17a-19c20-15(1)) according to the German Animal Protection Law and were performed according to the guidelines of FELASA.

### *Experimental design*

The experimental design and the measuring parameters are summarized in Fig 1. For the induction of $O_3/O_2$–PP, rats were pre-treated with a gas mixture consisting of 5% volume ozone and 95% volume medical oxygen, which passed through a sterile filter and was insufflated through a 50 ml Perfusor

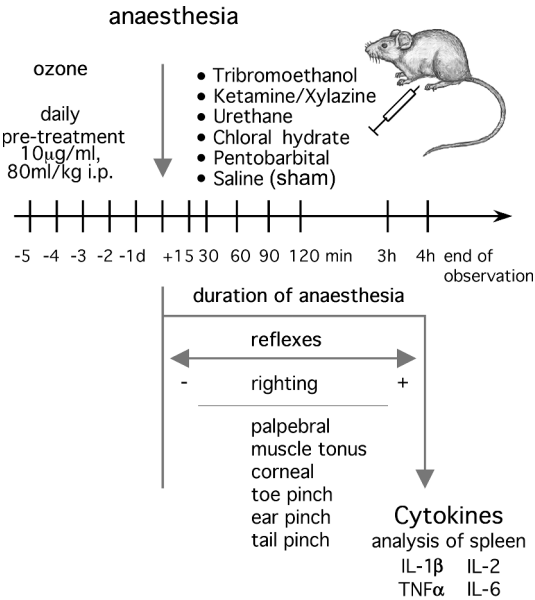


**Fig 1 Experimental design of the study**

R-Syringe (Braun Melsungen AG, Melsungen, Germany) with a standardized gas volume of 80 ml/kg body weight (b/w) by rapid (approximately 15 s) insufflation (needle size: 21 g) into the right lower abdomen by daily injections for 5 consecutive days (Schulz *et al*. 2003). The intra-abdominal pressure was less than 3 mbar after each insufflation. The concentration of ozonized oxygen we used was 10 µg ozone per ml of the gas mixture. It was generated from medical oxygen by the Ozonosan PTN 60 ozone gas processor, and concentrations of ozone were monitored by using an Ozonosan photometer (both from Dr Hänsler GmbH, Germany). The mean body weight development of $O_3/O_2$ pre-treated rats was increased over 5 days by $12.1 \pm 1\%$ thus showing a normal weight gain for growing male Wistar rats. As control, rats received no gas insufflations. One day after the last ozone insufflation the different anaesthetic drugs were applied intraperitoneally (i.p.) as described (Bette *et al*. 2004). In short: 2,2,2-tribromoethanol (formerly Avertin®, Fluka, Seelze, Germany) 400 mg per kg b/w at a volume of 7.9 ml/kg dissolved in tertiary amyl-alcohol as recommended by Hogan *et al*. (1986); ketamine/xylazine (Ketavet®, Pharmacia and Upjohn, Erlangen Germany/Rompun®, Bayer GmbH, Leverkusen Germany) 100 mg ketamine and 16 mg xylazine per kg b/w in a volume of 2.7 ml/kg; urethane (Fluka, Seelze, Germany) 1500 mg per kg b/w in a volume of 7.5 ml/kg; chloral hydrate (Atarost®, Atarost, Germany) 400 mg per kg b/w in a volume of 4.0 ml/kg; pentobarbital (Narcoren®, Merial GmbH, Hallbergmoos, Germany) 50 mg per kg b/w in a volume of 1.5 ml/kg. 2,2,2-tribromoethanol, urethane, and chloral hydrate as crystalline substance were dissolved in physiological saline and passed through a sterile filter before i.p. injection. For sham injection saline at 0.9% solution, 1.7 ml/kg b/w was injected i.p. Normal untreated rats were used as additional controls. The total number of animals used in this study was $n = 72$. The number of animals in each group was $n = 6$.

Four hours after application of the anaesthetic drug, animals were killed by the use of inhaled isoflurane (Forene®, Abbott GmbH, Wiesbaden, Germany) followed by an intracardial injection of embutramid/mebezonium/tretracain hydrochlorid (T61) at 0.5 ml/kg b/w (Intervet International GmbH, Wiesbaden, Germany ad us.vet). The use of inhaled isoflurane followed by T61 for euthanasia lasted less than 2 min, which makes it highly unlikely that these substances may have interfered with the experimental outcome. The spleen was removed immediately, embedded in Tissue Tek® (Miles, Elkhart, USA) and frozen in −50°C cold methylbutane (Fluka, Seelze, Germany). There were three animals in each group from which the spleen was removed.

## *Determination of the anaesthetic period*

*Duration of anaesthesia (sleeping time)*
The sleeping time was determined as the time between loss (−) and regain (+) of the righting reflex defined by the ability of the animal to perform three consecutive righting responses when it was placed in dorsal recumbence and ambulation.

*Quantification in loss of different reflexes*
The loss of a reflex was defined as the loss of a response to a specific stimulus. A non-response associated with or directed at the site of stimulation was recorded as negative for nociception. The scoring of the reflexes was performed 15, 30, 60, 90, 120, 180 and 240 min (end of observation) after the i.p. injection of the anaesthetic. For each animal the numbers of all negative reflexes—loss of response after stimulation—at all time points were added to a total amount ($s = 42$ per animal was the maximum). The righting reflex was not included to measure the depth of anaesthesia but was used to monitor the duration of anaesthesia. The principal investigator collected all the data. The statistical significance between $O_3/O_2$ pre-treated and non pre-treated animals was evaluated by one-way analysis of variance (ANOVA).

*Sleeping time*
The sleeping time was defined as the time between loss and regain of the righting reflex. The loss of the righting reflex occurred when the animal made no attempt to perform it when placed in dorsal recumbency. The regain of the righting reflex was defined as the point at which the animal performed three consecutive righting responses when placed in dorsal recumbency.

*Noxious stimuli*

- *Toe pinch reflex* A pair of forceps was attached to the lateral digit of the right hind paw for approximately 2–3 s at moderate pressure by the experienced investigator. The loss of the leg withdrawal or loss of all other movements during the time of pinching was recorded for nociception. The toe pinch reflex was used to define the level of anaesthesia.
- *Tail pinch reflex* A pair of forceps was attached to the distal 2 cm of the tail for approximately 2 s at moderate pressure. The loss of tail movement or other body movements during pinching were recorded for nociception.
- *Ear pinch reflex* The forceps was attached to both ears for 2 s at moderate pressure and a loss of ear/head movement and all other movements during the time of pinching were recorded for nociception.

*Non-aversive stimuli*

- *Muscle tonus* The losses of muscle rigidity/or loss of resistance to deflection or extension of the limbs were recorded.
- *Palpebral reflex* The losses of blinking and/or head movement in response to a light touch to the medial and lateral canthus of the eye were recorded.
- *Corneal reflex* The loss of blinking, globe retraction and/or loss of head movement in response to a touch to the centre of the cornea were recorded.

In situ *hybridization*
*In situ* hybridization was performed for IL-1β, IL-2, IL-6 and TNFα as described (Bette *et al*. 2004). In short: rat specific cDNA fragments for IL-1β, IL-2, IL-6 and TNFα were generated by reverse transcription. *In situ* hybridization was performed by usage of $^{35}$S-radioactive-labelled sense and antisense ribonucleotide probes. Autoradiograms were taken by exposing the sections to an autoradiography film (Hyperfilm-βmax, Amersham Biosciences, Freiburg, Germany) for 1–3 days. The *in situ* hybridization signals were quantified by densitometry measurements using the MCID image analysis system (Image Research, Ontario, Canada). The relative optical density of four spleen tissue slices ($s = 4$) per animal with three animals per group ($n = 3$) was measured for each cytokine mRNA. The optical density of the X-ray film was defined as physical background and was subtracted from each sample before the mean relative optical density was calculated for each group and cytokine mRNA. For statistical differences the one-way analysis of variance (ANOVA) was used.

## Results

*Duration of anaesthesia*

The urethane and tribromoethanol produced sleeping times which lasted longer than the observation period of 240 min (Table 1 and for experimental design see Fig 1). All other anaesthetic drugs generated a sleeping time shorter than 240 min, with sleeping times of chloral hydrate > ketamine/xylazine > pentobarbital (Table 1). The end of the sleeping time was determined by the regain of the righting reflex. The i.p. pre-treatment with $O_3/O_2$ gas mixture showed a minor, but non-significant, influence on the sleeping time, except for tribromoethanol. Here $O_3/O_2$–PP dramatically shortened the sleeping time from > 240 min to 175 min (Table 1). In contrast, a clear significant influence of $O_3/O_2$–PP was seen when the loss of all recorded reflexes was calculated (Table 1). A highest significant reduction in the loss of all reflexes was observed for tribromoethanol (Table 1). The significant changes induced by $O_3/O_2$–PP in the loss of reflexes for ketamine/xylazine, urethane and pentobarbital accompanied the changes seen in the duration of the sleeping time. No statistically significant changes could be calculated for chloral hydrate. However, the tendency for an enhanced duration of sleeping time was paralleled by an enhancement in the mean loss of reflexes.

For the representative analysis of the influence of $O_3/O_2$–PP on the loss of reflexes we analysed five different noxious and non-noxious reflexes which disappeared at various times after application of the anaesthetic drug. Figure 2 shows the time-dependent loss of the palpebral reflex, a reflex which disappeared shortly after the application of the different anaesthetics. Figure 3 summarizes the kinetics in the loss of the later disappearing toe pinch reflex.

*Disappearance and reappearance of reflexes in rats anaesthetized with the different anaesthetic drugs*

Tribromoethanol anaesthesia caused a rapid loss of the palpebral reflex within 15 min (Fig 2A), and the toe pinch reflex within 30 min, after application (Fig 3A). The loss of the palpebral reflex was observed with some minor deviation throughout the whole observation period. In contrast, the toe pinch reflex was transiently regained from 60 to 240 min. The $O_3/O_2$–PP clearly reduced the loss of both reflexes. Both reflexes completely reappeared in all animals at later time points of anaesthesia (Figs 2 and 3A). The kinetics of the disappearance and reappearance of all other reflexes and the effect of the $O_3/O_2$–PP were similar to the reflexes described (data not shown).

Ketamine/xylazine completely depressed the palpebral reflex (Fig 2B) and the toe pinch reflex (Fig 3B) within 15 to 30 min, and both reappeared within 90 to 120 min. The $O_3/O_2$–PP reduced the mean probability in the loss of these reflexes, which was most prominent for the toe pinch reflex at 15 to 30 min (Fig 3B). The kinetic of all other reflexes and the influence of $O_3/O_2$–P were comparable.

**Table 1 Anaesthetic effectiveness of different anaesthetic drugs: modulation by $O_3/O_2$– pneumoperitoneum**

| | Sleeping time (min) | | | Loss of six reflexes (%) | | |
|---|---|---|---|---|---|---|
| Anaesthetic drug | No gas | $O_3/O_2$-PP | | No gas | $O_3/O_2$-PP | |
| Tribromoethanol | >240 | 175±6.7 | ↓*** | 76.6±9.7 | 40.5±15.8 | ↓*** |
| Ketamine/xylazine | 126±39.4 | 97±29.2 | ↓ | 55.6±10.0 | 36.1±5.3 | ↓** |
| Chloral hydrate | 143±26.6 | 161±10.4 | ↑ | 30.6±9.0 | 40.0±14.6 | ↑ |
| Pentobarbital | 86±23.0 | 115±32.3 | ↑ | 30.6±3.3 | 57.4±23.0 | ↑* |
| Urethane | >240 | >240 | ? | 65.9±9.1 | 44.0±11.3 | ↓** |

Summary of the mean differences in duration time of anaesthesia (sleeping time), defined by the loss of the righting reflex and the mean frequencies in loss of six different reflexes in normal or ozone-pre-treated ($O_3/O_2$-PP) male Wistar rats during 4 h of observation. Number of animals per anaesthetic drug $n = 6$; number of test samples for the reflexes $s = 42$ per animal. Statistically significant differences in an increase (↑) or decrease (↓) in the number of lost reflexes between non-pre-treated (no gas) and $O_3/O_2$ gas mixture pre-treated ($O_3/O_2$–PP) animals were marked (*$P < 0.05$), (**$P < 0.01$) and (***$P < 0.001$)

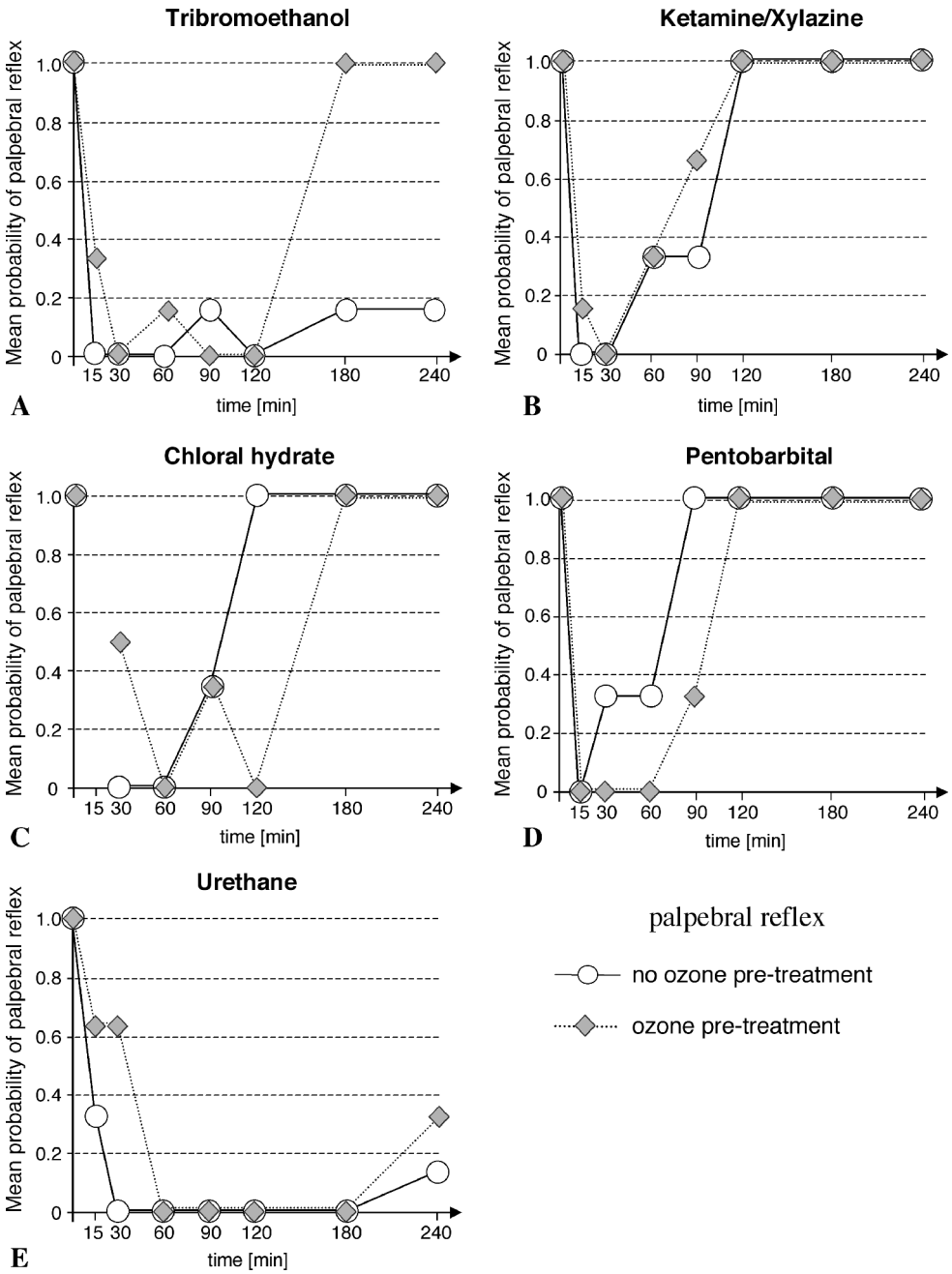


**Fig 2 The mean probability of the appearance of the palpebral reflex after anaesthesia induced by tribromoethanol (A), ketamine/xylazine (B), chloral hydrate (C), pentobarbital (D), and urethane (E) at several time points after injection is shown**. Zero means that the palpebral reflex was lost and 1.0 means that the reflex was present in all animals per group at the given time point after application of the anaesthetic drug. The animals were either sham-treated (no ozone pre-treatment, open circles) or received five repetitive $O_3/O_2$ gas mixture insufflations at daily intervals (ozone-pre-treatment, grey rhombus). Number of animals at each time point and group $n = 6$, sum of probes per group $s = 42$

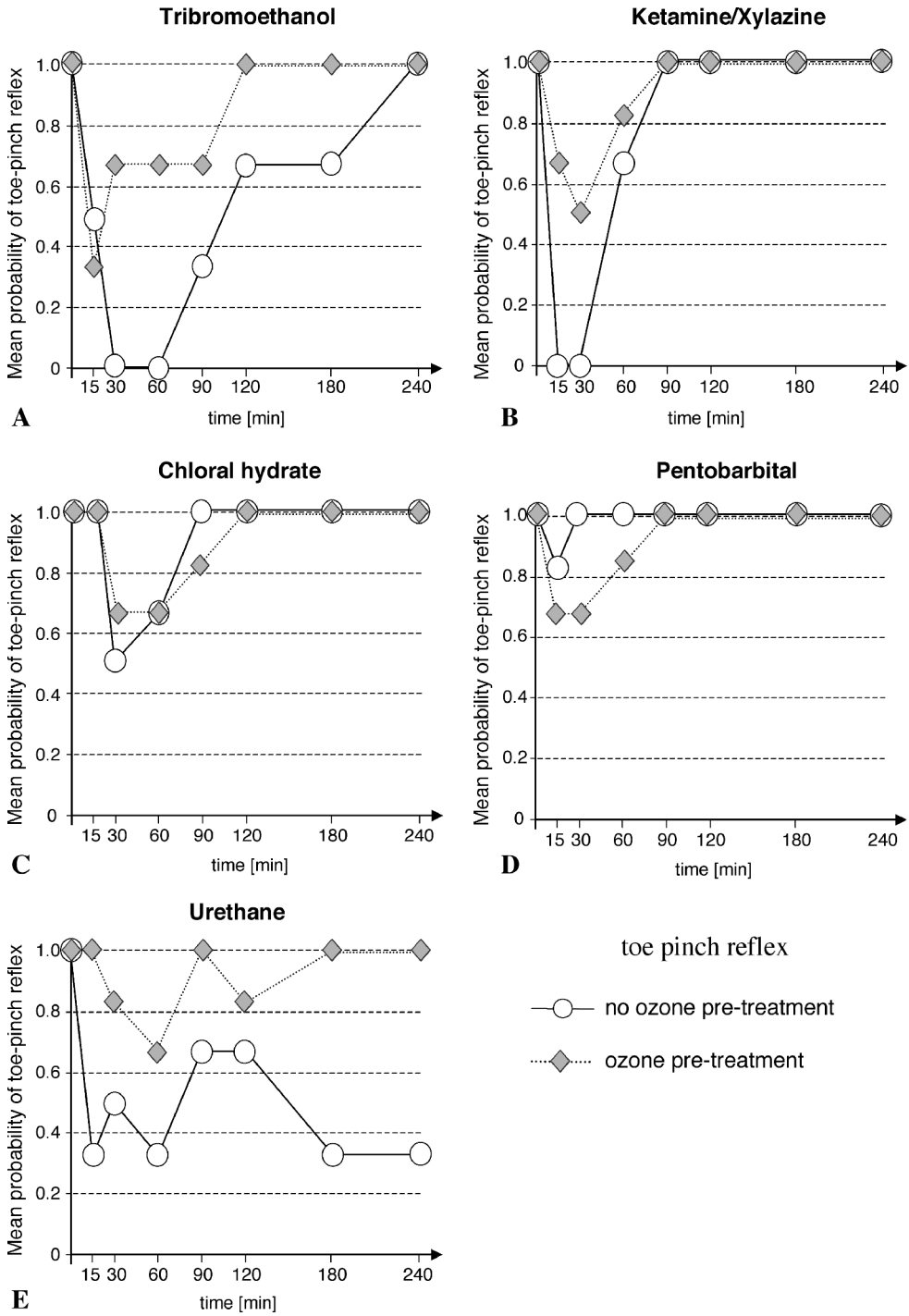


**Fig 3 The mean probability of the appearance of the toe pinch reflex after anaesthesia induced by tribromoethanol (A), ketamine/xylazine (B), chloral hydrate (C), pentobarbital (D), and urethane (E) at several time points is depicted**. The experimental groups and the number of animals were similar to those described in Fig 2

Chloral hydrate caused a complete loss of the palpebral reflex (Fig 2C) and the muscle tonus, and induced a moderate loss of the tail pinch reflex, toe pinch reflex (shown in Fig 3C), corneal reflex, and ear pinch reflex. In contrast to tribromoethanol, ketamine/xylazine and urethane, $O_3/O_2$–PP exhibited an enhancing and delaying effect on the loss of all reflexes.

Pentobarbital induced, in a manner similar to chloral hydrate, a complete loss of the palpebral reflex (Fig 2D) and the muscle tonus but only a moderate loss of the tail pinch reflex, toe pinch reflex (shown in Fig 3D), corneal reflex, and ear pinch reflex. The effect of $O_3/O_2$–PP was seen as an enhancement of the loss of reflexes, but less strong than seen for chloral hydrate.

Urethane induced a prominent loss of the palpebral reflex (Fig 2E), tail pinch reflex and muscle tonus, and a moderate loss of the toe pinch reflex (Fig 3E), corneal reflex, and ear pinch reflex. The $O_3/O_2$–PP significantly inhibited the loss of all reflexes (Figs 2 and 3E; for tail pinch reflex, muscle tonus, corneal reflex, and ear pinch reflex, data not shown).

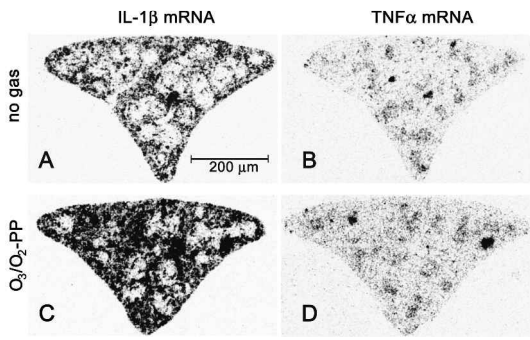


**Fig 4 Autoradiographic detection of *in situ* hybridization signals for IL-1β(A, C) and TNFα (B, D) mRNA in the spleens of pentobarbital narcotized rats which were either non pre-treated animals (no gas) or ozone pre-treated ($O_3/O_2$-PP) are shown.** Note a clear visible change in the number of hybridization signals (black dots) or signal intensity for IL-1β between the animals which received no gas and those with the $O_3/O_2$-PP. For TNFα mRNA no difference in the hybridization signals between no gas and $O_3/O_2$-PP can be seen

*$O_3/O_2$–PP modulated cytokine gene expression in the spleen of anaesthetized rats*

In order to test whether the depth and/or duration of an anaesthetic drug reflects the anaesthetic drug-induced changes in the amount of cytokine mRNAs, as described (Bette *et al*. 2004), the effect of an oxidative stressor able to reduce the anaesthetic effect, was evaluated. Semiquantitative *in situ* hybridization analysis of IL-1β, IL-2, IL-6 and TNFα mRNA in rat spleen revealed that the changes already found in the gene expression induced by a single anaesthetic drug were further influenced by the $O_3/O_2$–PP. Semiquantitative image analysis of autoradiograms derived from *in situ* hybridization experiments was performed to measure splenic cytokine mRNA levels. Autoradiograms of *in situ* hybridization

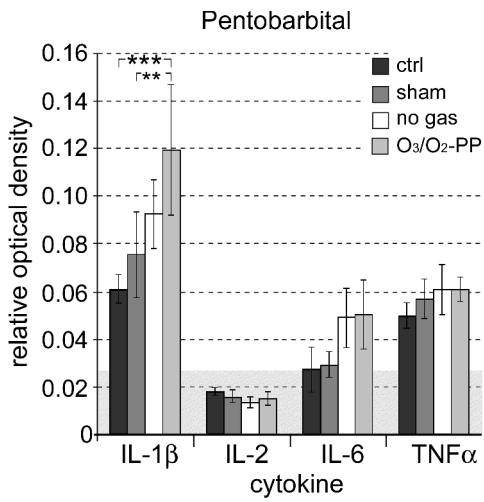


**Fig 5 Semiquantitative image analysis of autoradiograms derived from *in situ* hybridization experiments with $^{35}$S-labelled cRNA templates for IL-1β, IL-2, IL-6, and TNFα in the spleen of normal untreated rats (ctrl), saline injected animals (sham), rats which received no gas or which were pre-conditioned by $O_3/O_2$-PP prior to the i.p. injection of pentobarbital.** Data are expressed as the mean relative optical density ± standard deviation of four spleen tissue slices ($s = 4$) per animal with three animals per group ($n = 3$). The light grey zone between zero and 0.025 relative optical density marked in each graph reflects the relative optical density of the tissue alone estimated as no basal expression. Asterisks indicate statistically significant differences with $^{**}P < 0.01$ and $^{***}P < 0.001$

**Table 2 Alterations in splenic cytokine mRNA expression induced by different anaesthetic drugs**

| Anaesthetic drug | IL-1β No gas | IL-1β $O_3/O_2$–PP | IL-2 No gas | IL-2 $O_3/O_2$–PP | IL-6 No gas | IL-6 $O_3/O_2$–PP | TNFα No gas | TNFα $O_3/O_2$–PP |
|---|---|---|---|---|---|---|---|---|
| Saline (sham) | Basal expressed | | Not expressed | | Not expressed | | Basal expressed | |
| Tribromoethanol | ↔ | −* | ↔ | = | ↔ | = | ↓ | +** |
| Ketamine/xylazine | ↑ | −*** | ↔ | = | ↑ | = | ↓ | +*** |
| Chloral hydrate | ↑ | −*** | ↔ | = | ↑ | = | ↔ | = |
| Pentobarbital | ↑ | +** | ↔ | = | ↑ | = | ↔ | = |
| Urethane | ↓ | −* | ↔ | = | ↑ | = | ↓ | −* |

Analysis of *in situ* hybridization signals was performed by the use of a subjective rating by which only clear visible changes in the signal intensity observed on the X-ray film between two groups were taken into the account. The splenic cytokine mRNA levels of non pre-treated animals (no gas) were compared to that seen in sham injected control animals. Changes in cytokine levels induced by an anaesthetic drug were signed as (↓) reduction; (↔) no changes; (↑) enhancement. Alterations in the cytokine mRNA expression in ozone-pre-treated ($O_3/O_2$–PP) narcotized rats were evaluated with reference to that seen in non pre-treated narcotised animals and signified by (−) reduction; (=) no changes; (+) enhancement. Number of animals $n = 3$, number of test samples $s = 9$. Statistically significant differences between non-pre treated (no gas) and $O_3/O_2$ gas mixture pre-treated ($O_3/O_2$–PP) animals were marked (*$P < 0.05$), (**$P < 0.01$) and (***$P < 0.001$)

experiments of IL-1β and TNFα mRNA (Fig 4) and a graphical view of the calculated mean cytokine mRNA levels given as the mean relative optical density of the autoradiograms (Fig 5) are shown representatively for pentobarbital. For all anaesthetic substances the pre-conditioning with insufflated $O_3/O_2$ gas mixture led to significant changes in the levels of IL-1β mRNA as compared to those in non-pre-treated anaesthetized rats (summarized in Table 2).

Interleukin-2 and IL-6 mRNA were not induced or enhanced by any anaesthetic substance alone or in combination with $O_3/O_2$–PP (Table 2). The tribromoethanol- and ketamine/xylazine-induced reduction in splenic TNFα mRNA expression was abolished by $O_3/O_2$–PP, whereas the reduction in rats anaesthetized with urethane was significantly enhanced by this intervention (Table 2). Tumour necrosis factor alpha mRNA was not influenced by $O_3/O_2$–PP when chloral hydrate or pentobarbital was used as an anaesthetic substance (Table 2 and Fig 4).

## Discussion

Many drugs can influence the outcome of anaesthesia when applied perianaesthetically. The inhalation of gaseous substances such as oxygen (McCracken *et al*. 1979) or ozone (Graham *et al*. 1981, 1985, Canada *et al*. 1986) has been described to be a modulator of the sleeping time of some anaesthetic drugs. But hitherto, except for influences of inhaled ozone on the duration of anaesthesia, no data have been available from small laboratory animals, when an oxidant gas such as ozone is given extrapulmonary, which is characterized by insufflation into the peritoneum and known as pneumoperitoneum.

We found that the sleeping time and the mean loss or regain of different reflexes (strength of reflexes) of the five different anaesthetic drugs were influenced by extrapulmonary applied ozonized oxygen. The $O_3/O_2$–PP decreased tribromoethanol- and ketamine/xylazine-induced sleeping time, and probably that of urethane, but increased it for pentobarbital. The effect of an abdominal insufflation of ozone for pentobarbital is therefore similar to that described for inhaled ozone (Graham *et al*. 1981).

The $O_3/O_2$–PP reduced significantly the loss of reflexes on the response to noxious and non-aversive stimulants, when tribromoethanol, ketamine/xylazine and urethane were given, but increased it for pentobarbital. Thus, the effect of $O_3/O_2$–PP on the kinetics in the regain of reflexes is correspondent to the regain of recovery. An anaesthetic drug, which also exhibited a reduced sleeping time after $O_3/O_2$–PP, showed an earlier regain of all reflexes.

A likely mechanism, which could explain the difference in the strength of anaesthesia between non pre-treated normal rats and $O_3/O_2$ gas mixture pre-treated ones, could be based on the regulation of

xenobiotic enzymatic activity. For example Morgan (1997, 2001) has shown that the pro-inflammatory cytokines IL-1β, IL-6 and TNFα can decrease most of the cytochrome P-450 enzymes. A potentially less metabolized drug, such as an anaesthetic drug, may prolong sleeping time and enhance the depth of anaesthesia. We have previously described ketamine/xylazine, chloral hydrate, pentobarbital, and urethane as modulators of pro-inflammatory cytokine gene expression (Bette *et al*. 2004). In this study we show that $O_3/O_2$–PP also influences the anaesthetic-induced modulation of cytokine mRNAs. It is therefore feasible to consider that ozone pre-treatment influences the early anaesthetic-specific effects on the abdominal immune system. Furthermore, cytokine-dependent mechanisms regulated by ozone may be involved in the effects on anaesthesia which we observed.

The $O_3/O_2$–PP showed a further increase in IL-1β mRNA expression for pentobarbital alone. However, we speculate that this could be due to an $O_3/O_2$–PP-dependent enhanced accumulation of metabolites able to trigger pathological side effects of pentobarbital (Lumb & Jones 1984). These side effects by themselves might be the reason for the further increase in IL-1β mRNA observed in the spleen due to an activation of the immune system beyond that recorded for pentobarbital alone. In this context, Thompson *et al*. (2002) found an early increase in apoptosis in splenic follicles and in hepatic Kupffer and endothelial cells caused by pentobarbital, tribromoethanol, and ketamine/xylazine. It seems unlikely that the stimulatory effect of cytokines on P-450 enzyme (Morgan 1997) is the reason for $O_3/O_2$–PP-enhanced IL-1β mRNA because this effect is absent for ketamine/xylazine and chloral hydrate. Both anaesthetic drugs were also found to enhance IL-1β mRNA.

In conclusion, we found that insufflation of $O_3/O_2$ gas mixture into the abdomen of rats leads to an altered sleeping time and to changes in the loss of reflexes. The $O_3/O_2$-dependent alterations in the strength of anaesthesia are paralleled by a modulation of pro-inflammatory cytokine levels beyond those seen for a given anaesthetic drug alone.

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