Laboratory Investigation

Spinal anaesthesia but not general anaesthesia enhances neutrophil biocidal activity in hip arthroplasty patients

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The purpose of this study was to compare neutrophil cidal activity during general or spinal anaesthesia. Assays were performed on neutrophils extracted from the blood of patients after surgery had been under way for one hour. First, the ability of the neutrophils to kill a standard laboratory strain of S. aureus was examined. Neutrophils extracted from the blood during surgery in the spinal anaesthetic group and incubated with the staphylococci for one hour killed twice as many bacteria than those from two groups of patients that received halothane or isoflurane general anaesthesia ($P < 0.05$). This effect persisted, to a lesser extent, in the spinal group neutrophils after two hours of incubation with the bacteria. Second, neutrophils from patients under the same conditions of surgery and anaesthesia were tested to examine the effect of the different anaesthetic techniques on neutrophil biocidal mechanisms. Neutrophils extracted during surgery in the spinal group released more superoxide in response to phorbol-12-myristate-13-acetate (PMA) than those from both groups of patients that received general anaesthesia ($P < 0.05$). It is concluded that there is an increased state of reactivity of the neutrophil cell membrane NADPH oxidase system in patients receiving spinal anaesthesia than in patients receiving general anaesthesia.

Cette étude vise à comparer l'activité bactéricide des neutrophiles pendant l'anesthésie générale ou rachidienne. Les analyses sont effectuées à partir de neutrophiles extraits du sang de patients une heure après le début de la chirurgie. D'abord, l'habileté des neutrophiles à tuer une colonie standard de S. aureus est examinée. Les neutrophiles extraits de patients sous rachianesthésie et incubés avec des neutrophiles pendant une heure tuent deux fois plus de bactéries que le sang se patients sous anesthésie générale à l'halothane ou à l'isoflurane ($P < 0,05$). Cet effet persiste, mais à un degré moindre, dans le sang du groupe rachianesthésie après un contact de deux heures avec les bactéries. Ensuite, on a examiné sous les mêmes conditions d'anesthésie et de chirurgie les neutrophiles de patients anesthésiés avec les différentes techniques pour en déterminer le mécanisme bactéricide. Les neutrophiles extraits pendant la rachianesthésie libèrent plus de superoxyde en réponse au phorbol-12-myristate-13-acétate (PMA) que ceux des deux groupes de patients sous générale ($P < 0,05$). En conclusion, on constate une augmentation de l'état réactif du système NADPH oxydase de la membrane cellulaire du neutrophile chez les patients sous rachianesthésie comparativement à ceux qui sont sous anesthésie générale.

Key words
ANAESTHETIC TECHNIQUES: general, spinal;
SURGERY: hip arthroplasty;
IMMUNE RESPONSE: bacterial, chemiluminescence.

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We demonstrated in patients undergoing hip arthroplasty that neutrophil movement is enhanced under spinal but not under general anaesthesia.[1] It is important to establish the extent to which anaesthetics and anaesthesia modify neutrophil function in the perioperative period. Innate mechanisms, essentially neutrophils primed by and acting in combination with complement, constitute the most im-

portant early defence strategy against bacterial infection during and for the first 48 hr after surgery.[2] But, in addition, neutrophils activated by inflammation result in host tissue damage and thus a balanced system for biological advantage is established. It has been suggested that epidural anaesthesia combined with general anaesthesia for surgery and continued into the postoperative period for pain relief may reduce morbidity, including perioperative infection.[3] In addition, Anand *et al.* have demonstrated, in neonates undergoing cardiac surgery, that using sufficient opioid to block the stress response to surgery also reduced perioperative infection and improved survival.[4] We decided to examine the possibility that neutrophil cidal function, modified by the anaesthetic technique during surgery, might in part explain these findings.

Several studies have examined the effect of anaesthesia and anaesthetics on the ability of neutrophils to phagocytose and kill bacteria. The majority of the studies have exposed neutrophils extracted from the blood of volunteers to anaesthetic agents and have produced inconsistent results. The volatile agents halothane and nitrous oxide and to a lesser extent isoflurane have been found to depress neutrophil biocidal activity.[5,6] In contrast, no effect of isoflurane at 0.5–3% could be demonstrated in another study.[7] Tsuchiya demonstrated a fMLP-induced stimulation of superoxide production by halothane.[8] Halothane 1% produced a 10% diminution in the killing of K. pneumoniae by neutrophils while isoflurane 0.5–3% had no effect on the killing of E. coli, S. aureus or K. pneumoniae.[7,9] Experiments in which neutrophils are removed from their natural environment and then exposed to anaesthetic agents are difficult to interpret in terms of their pathophysiological function in patients undergoing surgery and anaesthesia. It is more likely that experiments in which neutrophils removed from patients undergoing anaesthesia and surgery, whilst exposed to inflammatory mediators, will yield relevant information. Other investigators have adopted this approach. Several studies have shown a depression of neutrophil cidal activity in neutrophils extracted from patients undergoing general anaesthesia alone.[10–14] This depression was not seen when surgery was in progress at the time of neutrophil sampling in patients receiving general anaesthesia.[15] Neutrophils extracted from patients undergoing surgery under epidural anaesthesia also did not appear to have cidal function different from that prior to anaesthesia.[10]

Methods

This work was approved by the ethical and research committee of the University of Cape Town. Permission to obtain blood samples from each patient was obtained before inclusion in the study. Eighteen patients were randomised and divided into three groups. Data from these patients were used to compile the results for the S. aureus killing experiments. An additional seven patients were randomised and studied in the same three groups. Information from these 25 patients was used to present the chemiluminesce data. Two groups received general anaesthesia which included either halothane or isoflurane (GA-HALO, GA-ISO). The third group received SPINAL anaesthesia. All patients were of ASA status 1 or 2 and were suffering from osteoarthritis. All patients were receiving non-steroidal analgesics excluding aspirin up to but not including the day of surgery. Premedication of temazepam 20 mg two hours before induction of anaesthesia was administered to all patients. The GA-HALO group received morphine 0.15 $mg \cdot kg^{-1}$ before induction of anaesthesia with a sleep dose of thiopentone. Tracheal intubation was facilitated by alcuronium 0.2 $mg \cdot kg^{-1}$ and anaesthesia was maintained utilising 30% oxygen in nitrogen and halothane 1% from a previously calibrated vaporizer. The $PETCO_2$ was maintained at 40 mmHg. The GA-ISO group was managed in a similar fashion except that isoflurane 1.5% was administered instead of the halothane. After a preload of 10 $ml \cdot kg^{-1}$ Plasmalyte B (Ringer's lactate without calcium in which the lactate has been replaced by bicarbonate) anaesthesia was induced in the SPINAL group by the subarachnoid injection of 3.5 ml plain isobaric bupivacaine 0.5% into the L_{3-4} or L_{4-5} interspace. This produced a loss of sensation to a median level of T_8 (range T_4–T_{10}). Patients in this group were lightly sedated by an infusion of propofol of around 0.2 $mg \cdot kg^{-1} \cdot h^{-1}$, after a loading dose of 0.5 $mg \cdot kg^{-1}$. The rate was adjusted to maintain a sedation score of 3 on the Mackenzie scale.[16] Blood pressure was maintained above 100 mmHg systolic throughout the study period in all patients by infusion of Plasmalyte B. Patients received no other fluids or blood during the study. No bone cement was administered while the study was in progress.

Neutrophil function assays

CHEMILUMINESCENCE

Blood (5 ml) was sampled before induction of anaesthesia, from a large free-flowing vein and placed in a standard EDTA tube and kept in ice. Blood was sampled again 90 min later when surgery had been in progress for one hour. Blood was sampled twice from volunteers (control group) under identical conditions 90 min apart to ensure that no changes occurred due to keeping the first sample on ice for 90 min longer before all the assays were performed together for each patient. The peak light emission from neutrophils obtained intraoperatively from each pa-

TABLE I Final constituents of experimental combinations for the measurement of chemiluminescence response

	LUC-PMA	*LUC-PMA*	*LUC-OPZYM*	*LUM-OPZYM*
0.2 ml blood sample diluted				
1:1000 in PBS	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Veronal buffer	0.68 ml	0.68 ml	0.68 ml	0.68 ml
Lucigenin 1 uM	0.1 ml		0.1 ml	
Luminol 20 μM		0.1 ml		0.1 ml
PMA 12.5 μM	20 μl	20 μl		
Opsonized zymosan			20 μl	20 μl

tient was compared with the peak light emission from the same patient's leucocytes obtained before induction of anaesthesia. Additional experiments were performed on the blood from four volunteers in the presence and absence of propofol 10 $\mu g \cdot ml^{-1}$.

We tested light emission from leucocytes in diluted whole blood samples in the presence of the following combinations of stimulants, Opsonised zymosan-Luminol (OPZYM-LUM), phorbol-12-myristate-13-acetate-Lucigenin (PMA-LUC), OPZYM-LUC and PMA-LUM. The final concentrations of cells and reactants are shown in Table I. For each combination the peak height of light emission or maximal brightness from the cells for each patient intraoperatively was compared with the cells from the same patient before anaesthesia. The light units in each case was divided by the leucocyte count of each sample to correct for changes in leucocyte relative number that might have occurred intraoperatively. The 1 ml of reactant solution was counted in a Beckman liquid scintilation counter using single-photon monitor option for three hours. The solutions were prepared and kept in the dark until prior to starting the counts, the stimulants OP-ZYM or PMA were added. The stimulant solutions were prepared as follows: PMA (P8139) from Sigma Mo, USA). A stock solution of 5 $mmol \cdot L^{-1}$ was diluted with redistilled water to yield a working solution of 12.5 $\mu mol \cdot L^{-1}$ (0.25 $\mu mol \cdot L^{-1}$ final). Opsonised Zymosan (Z4250 from Sigma Mo, USA) was prepared by dissolving 25 mg in 5 ml 0.9% saline. After heating to 90°C for 30 min an equal volume of fresh pooled serum was added and kept at room temperature for 30 min and then centrifuged. The precipitate was taken up in a 5 × dilution of saline and frozen at −20°C in aliquots until use. Veronal buffered saline containing Mg^{++}, Ca^{++}, albumin and glucose was made up and adjusted to a PH of 7.3. Lucigenin (M8010 Sigma) and Luminol (A8511 Sigma) were dissolved in water to the concentrations shown in Table I.

S. aureus killing assay

We used the technique of Quie as modified by Haddad *et al.* to investigate the ability of neutrophils extracted from the blood of our patients to kill S. aureus, a common surgically associated pathogen.[17] Preinduction blood (30 ml) was kept in heparin tubes on ice until the intraoperative sample was obtained and then both samples were processed together. Once again neutrophils obtained from volunteers' blood samples and stored under the same conditions were always assayed together with the patient neutrophils to control for experimental aberration. Neutrophils from four volunteers were also tested for killing ability in the presence and absence of propofol as above.

Neutrophils were extracted into RPMI-HEPES buffered cell culture medium from the 30 ml whole blood sample by centrifugation on Ficol (Histopaque-1077, Sigma Diagnostics Mo, USA) cushions and 6% dextran sedimentation. This yielded a harvest of at least 97% neutrophils of which at least 94% were found to be viable on a tryphan blue dye exclusion test. The cells were adjusted to a final concentration of $2 \times 10^6 \cdot ml^{-1}$ in RPMI buffered with HEPES prior to incubation with the staphylococci. Staphylococci (Oxford NCTC 6571) were grown overnight in TSB broth. An aliquot of 1 ml was transferred the next day into fresh TSB and incubated for four hours to get the organisms into log phase growth. At the end of this time they were spun down and resuspended in RPMI at 1×10^7 colony forming units (CFU) determined spectrophotometrically in a PYE UNICAM PU8800 from a previously determined standard curve. Then 500 μl of cells were added to 100 μl of freshly thawed pooled serum and 100 μl of bacteria (20 bacteria:1 cell). An additional tube excluding cells was included as a bacterial control. The tubes were gently agitated in a water bath at 37°C. Aliquots of 100 μl were removed at 0, 1 and 2 hr. After ultrasonication (MSE at 6 amps for 15 sec) to disrupt the neutrophils, serial dilutions were made in sterile saline and plated out on agar plates and incubated overnight. Remaining viable organisms could be counted the next morning as CFU. Killing was assessed at one and two hours by the equation:

$$(\text{CFU bacteria without cells} - \text{CFU bacteria with cells}) / \text{CFU bacteria without cells} \times 100 = \% \text{ kill.}$$

TABLE II Demographic details of patients studied

	Control ($n = 6$)	*GA-halo* ($n = 9$)	*GA-iso* ($n = 7$)	*Spinal* ($n = 9$)
Age (yr)	36	55	53	60
(Range)	(24–48)	(50–70)	(36–73)	(48–73)
Weight (kg)	68	65	67	68
(Range)	(55–78)	(56–80)	(74–84)	(55–78)
Sex (M:F)	4:2	4:5	4:3	6:3

For each patient's cells, intraoperative killing was compared with the killing by the same patient's cells prior to induction. Killing by neutrophils from the blood of volunteers was always assayed in parallel as a biological control.

Statistical analysis was performed using ANOVA examination for intergroup differences followed by Students t test for intergroup comparisons. Significance was assumed when $P < 0.05$.

Results

The anaesthetic groups revealed no differences with regard to age, weight or sex (Table II). After one hour of surgery there were no intergroup differences in the leucocyte count nor were there any differences apparent when the intraoperative leucocyte count was compared with the preoperative leucocyte count within groups (Table III). There was enhanced light emission from the neutrophils obtained intraoperatively in the SPINAL anaesthetic group which were stimulated by PMA in the presence of lucigenin (LUC-PMA) compared with that in the neutrophils from the same patients obtained prior to anaesthesia and surgery ($P < 0.05$). In addition, the enhanced emission by neutrophils stimulated by LUC-PMA was greater during surgery under spinal anaesthesia than in both general and anaesthesia groups. The neutrophils obtained during surgery under general anaesthesia in both these groups did not show any enhanced light production compared with neutrophils obtained prior to induction (Figure 1). In addition a modest enhancement of light emission occurred in the spinal group with intraoperative neutrophils stimulated by OPZYM in the presence of luminol compared with the preanaesthetic response ($P < 0.05$, Figure 1). The light emission was also increased during surgery in the spinal group with the other two combinations of stimulants and probes (OPZYM-LUC, PMA-LUM) but the differences were not greater than in the general anaesthesia groups. Light emission from cells obtained from volunteers and stored for 1.5 hr on ice was no different from the light emission from cells from the same subjects in whom the experiments were commenced immediately the blood was obtained (control group Figure 1).

The S. aureus killing experiments revealed an enhancement of killing by neutrophils obtained during surgery in the spinal group ($P = 0.017$) compared with their killing ability prior to surgery. In addition this killing increased compared to the intraoperative killing by neutrophils obtained during surgery under general anaesthesia. Once again the control killing experiments conducted in parallel with the patient cells showed that the changes in neutrophil function were not methodological (Figure 2).

Propofol had no stimulant or depressant effect on the neutrophil killing of S. aureus. At the same concentration it produced a small but statistically insignificant ($P > 0.05$) decrease in the light producion in the chemiluminescence combinations (Figure 3).

Discussion

Previous studies comparing neutrophil function during surgery with different anaesthetic conditions are broadly in agreement with our findings. General anaesthesia alone depresses neutrophil biocidal activity.[13–15] This depression is less marked when neutrophils are obtained from patients undergoing surgery in addition to anaesthesia.[12] Surgery conducted under epidural anaesthesia resulted in less depression of neutrophil function than during general anaesthesia in two studies.[10,11] This study demonstrated that neutrophils from patients undergoing surgery under spinal anaesthesia have an increased ability to kill S. aureus than in the two general anaesthesia groups. The increased neutrophil response in the spinal group may reflect the increased magnitude of the surgical trauma, the timing of the neutrophil sampling, or the different methodology employed in this, compared with previous studies. The enhanced light emission in the spinal group neutrophils stimulated by PMA in the presence of lucigenin suggested that enhanced release of superoxide by these neutrophils is a likely mechanism for the increased biocidal activity.

To explain these findings, let us assume that neutrophils are activated by tissue damage associated with surgery.[18] This would confer biological advantage for host survival in the presence of wounds. However, the activated neutrophil has a considerable potential for host tissue damage. Thus balance must exist between host defence and host damage. Spinal anaesthesia has differing effects from general anaesthesia on this control system.

Are hormonal factors circulating in the blood of the general anaesthesia patients inhibiting the increased neutrophil activity seen in the spinal group? After one hour of major surgery, plasma epinephrine and cortisol concentrations would be two to three times higher in the general anaesthesia groups than in the spinal group.[1,19,20] An epinephrine-mediated inhibition is unlikely in the gen-

TABLE III Total leucocyte counts of patients in the different anaesthetic groups (Numbers $\times 10^9 \cdot L^{-1}$)

	GA-halo (n = 6)		*GA-iso (n = 6)*		*Spinal (n = 7)*	
	Pre	*Intra*	*Pre*	*Intra*	*Pre*	*Intra*
Mean	7.6	8.4	6.8	6.8	7.9	7.8
Range: Low	5.0	4.8	4.6	4.1	5.3	5.0
High	13.6	13.1	9.1	8.4	11.3	9.6

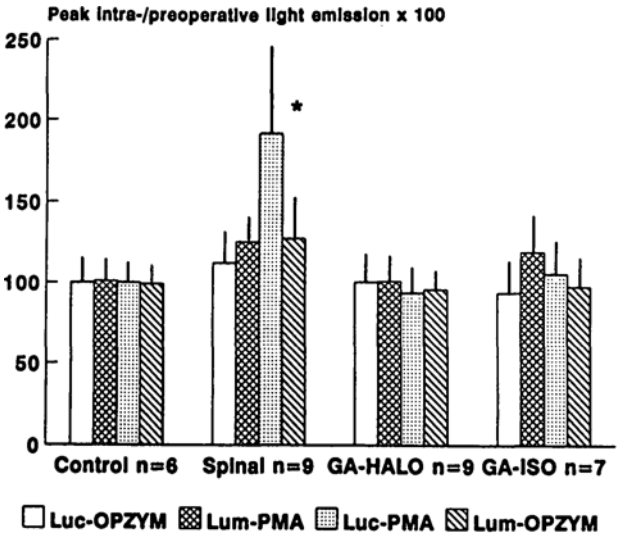

FIGURE 1 Changes in the chemiluminescence response to various stimulants (PMA vs OPZYM in the presence of lucigenin and luminol) between preoperative and intraoperative whole blood samples from patients in the different anaesthetic groups. The results are expressed as the mean of the relative and percentage changes in intrapreoperative light emission (columns) and the vertical bars indicate SEM. $*P < 0.05$ compared with the changes in the blood samples obtained from healthy volunteers (100% = no change).

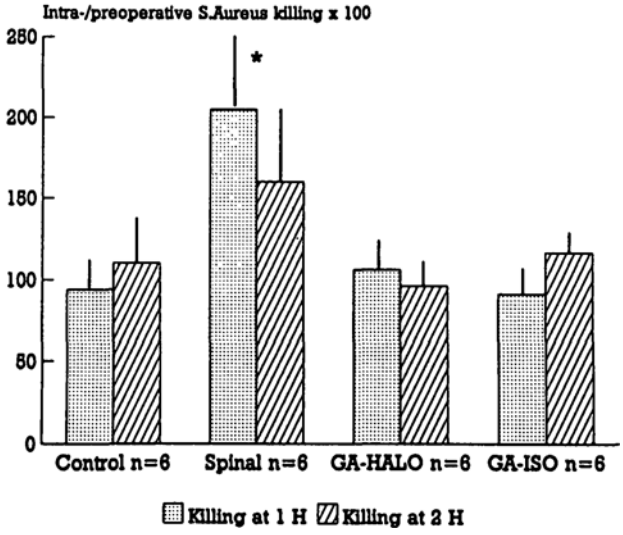

FIGURE 2 Differences in the killing ability of neutrophils extracted from the blood samples of patients from different anaesthetic groups pre- and intraoperatively and incubated *in vitro* for one and two hours in the presence of S. Aureus. The results are presented as the mean of the relative percentage changes in intrapreoperative killing ability of neutrophils and the vertical bars indicate SEM. $*P < 0.05$ compared with control samples.

eral anaesthesia groups as no epinephrine-mediated demargination, as would be represented by a neutrophilia, was apparent. Second, Moudgil was unable to demonstrate any effect of epinephrine at a concentration of 10^{-6}–10^{-3} M on neutrophil phagocytosis.[21] Cortisol-induced neutrophil inhibition would also have resulted in a demargination-induced neutrophilia. In addition macrocortin-mediated inhibition of function by neutrophils takes 1–2 hours to begin to develop.[22,23] Direct inhibition of the neutrophils by the anaesthetics administered to the general anaesthetic groups is unlikely in our experiments. Hallet showed that different mechanisms operate to release light when neutrophils phagocytose latex beads compared with light emission in the presence of NADPHase stimulants such as PMA or OPZYM.[24] Previous work which examined neutrophil biocidal activity in the presence of enflurane and isoflurane demonstrated an anaesthetic-induced inhibition of latex bead ingestion but inhibition in the presence of cell surface stimulants was unproven.[5–7,14–17] We employed the stimulants OPZYM and PMA in our methodology.

One of the most important innate neutrophil-mediated host defence mechanisms is the phagocytosis which is followed, by the intracellular killing and digestion of microorganisms by phagocytic cells. This process involves recognition and attachment, ingestion and killing of the ingested organisms. In the killing experiments organisms were coated in pooled human serum to enable recognition and attachment to the patient neutrophils. Ingestion is triggered by the attached, opsonised bacteria on the surface of the neutrophil. This oxygen-consuming metabolic burst by the neutrophil emits light which can be enhanced by the chemiluminescent probes lucigenin or luminol. Lucigenin amplifies light generated by superoxide radical released from the stimulated cell surface. Luminol am-

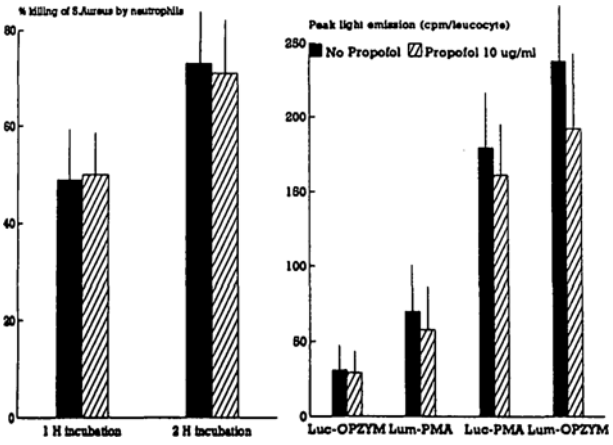

FIGURE 3 Comparison of the killing ability of neutrophils (left panel) and chemiluminescence response to various stimulants (right panel) in the blood samples taken from healthy volunteers and incubated without (open column) and with the presence of propofol at the concentration 10 $\mu g \cdot ml^{-1}$ (striped column). The results are expressed as the % of killing (left panel) or peak light emission in cpm white blood cell (right panel) and the vertical bars indicate SEM for four measured blood samples.

plifies light generated by the whole killing process right down to the myloperoxidase-dependent hypohalide acid bleaches.[25,26] The specificity of chemiluminescent methodology has been questioned but the evidence for this explanation presented by Allen is convincing.[26] The neutrophil-killing methodology consisted of incubating the cells with bacteria for one hour and then recording the kill rate by ultrasonic lysis of the neutrophil membrane to release the ingested bacteria and establish their viability. The results confirmed the findings of Haddad *et al.* and Quie that 85% of microbiocidal activity occurs within one hour of exposure of the bacteria to the neutrophils.

Our findings suggest that during surgery neutrophils are activated to kill S. aureus. This ability is inhibited by general anaesthesia but not by spinal anaesthesia. Mealhy showed that a factor present in the serum of patients during general anaesthesia inhibited neutrophils obtained from volunteers who had not been exposed to anaesthesia.[14] We are exploring the possibility that serum from patients undergoing surgery under spinal anaesthesia may enhance the killing of bacteria by neutrophils obtained from volunteers. In particular, complement activation may be different during surgery conducted by spinal anaesthesia compared with general anaesthesia. The evidence of Yeagher and Anand together with our findings suggest that a study to examine the incidence of perioperative sepsis in operations carried out during spinal and general anaesthesia is required. Stress response blockade provided by regional anaesthesia may be central to these findings.

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