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SCIENTIFIC ARTICLE

Total knee replacement induces peripheral blood lymphocytes apoptosis and it is not prevented by regional anesthesia – a randomized study

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KEYWORDS
Total knee replacement;
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Abstract
Background: Among the many changes caused by a surgical insult one of the least studied is postoperative immunosuppression. This phenomenon is an important cause of infectious complications of surgery such as surgical site infection or hospital acquired pneumonia. One of the mechanisms leading to postoperative immunosuppression is the apoptosis of immunological cells. Anesthesia during surgery is intended to minimize harmful changes and maintain perioperative homeostasis. The aim of the study was evaluation of the effect of the anesthetic technique used for total knee replacement on postoperative peripheral blood lymphocyte apoptosis.
Methods: 34 patients undergoing primary total knee replacement were randomly assigned to two regional anesthetic protocols: spinal anesthesia and combined spinal–epidural anesthesia. 11 patients undergoing total knee replacement under general anesthesia served as control group. Before surgery, immediately after surgery, during first postoperative day and seven days after the surgery venous blood samples were taken and the immunological status of the patient was assessed with the use of flow cytometry, along with lymphocyte apoptosis using fluorescent microscopy.
Results: Peripheral blood lymphocyte apoptosis was seen immediately in the postoperative period and was accompanied by a decrease of the number of T cells and B cells. There were no significant differences in the number of apoptotic lymphocytes according to the anesthetic protocol. Changes in the number of T CD3/8 cells and the number of apoptotic lymphocytes were seen on the seventh day after surgery.
Conclusion: Peripheral blood lymphocyte apoptosis is an early event in the postoperative period that lasts up to seven days and is not affected by the choice of the anesthetic technique.

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PALAVRAS-CHAVE
Artroplastia total do joelho;
Anestesia regional;
Anestesia geral;
Linfócitos;
Apoptose

Artroplastia total do joelho induz apoptose em linfócitos de sangue periférico e não é evitada por anestesia regional – estudo randômico

Resumo
Justificativa e objetivo: Dentre as muitas alterações causadas por uma ferida cirúrgica, uma das menos estudadas é a imunossupressão pós-operatória. Esse fenômeno é uma causa importante das complicações infecciosas relacionadas á cirurgia, como infecção do sítio cirúrgico ou pneumonia nosocomial. Um dos mecanismos que levam à imunossupressão pós-operatória é a apoptose de células imunológicas. Durante a cirurgia, a anestesia se destina a minimizar as alterações prejudiciais e manter a homeostase perioperatória. O objetivo deste estudo foi avaliar o efeito da técnica anestésica usada para artroplastia total de joelho sobre a apoptose em linfócitos de sangue periférico no pós-operatório.
Métodos: 34 pacientes submetidos à artroplastia total primária de joelho foram randomicamente designados para dois protocolos de anestesia regional: raquianestesia e bloqueio combinado raqui-peridural. Onze pacientes submetidos à artroplastia total do joelho sob anestesia geral formaram o grupo controle. Antes da cirurgia, logo após a cirurgia, durante o primeiro dia de pós-operatório e sete dias após a cirurgia, amostras de sangue venoso foram colhidas e o estado imunológico do paciente foi avaliado com o uso de citometria de fluxo, juntamente com apoptose de linfócitos usando microscopia de fluorescência.
Resultados: Apoptose em linfócitos de sangue periférico foi observada imediatamente no pós-operatório e acompanhada por uma redução do número de células T e B. Não houve diferença significativa no número de linfócitos apoptóticos de acordo com o protocolo anestésico. Alterações no número de células T CD3/8 e no número de linfócitos apoptóticos foram observadas no sétimo dia após a cirurgia.
Conclusão: Apoptose em linfócitos de sangue periférico é um evento precoce no período pós-operatório que dura até sete dias e não é afetado pela escolha da técnica anestésica.

Introduction

Surgical trauma leads to a complex systemic response including sympathetic nervous system activation, endocrine response, and inflammatory and immunological disturbances. Simultaneously with inflammatory response activation, which reduces the surgical stress damage area and facilitates repair processes, immunological system impairment occurs. This mechanism seems to have a defensive function – the organism defends itself from the autoimmunological response in a situation of its own antigens' excess and a stimulation of the processes of their recognition. Unfortunately, it also leads to adverse consequences – direct damage of natural defensive barriers such as skin and mucous membranes in association with impairment of defensive mechanisms that increases the possibility of infections. In oncologic surgery that also means metastatic progression and acceleration of neoplasmal disease.

Postoperative lymphopoenia is a phenomenon that has been known for a long time, and it applies to all lymphocyte populations and its intensification is directly proportional to the extent of the injury.[1] It is caused by a series of perioperative events and one of them has been intensively examined in recent years. Apoptosis is the process of programmed cell death, a term which was proposed in 1972 by Kerr et al. to describe morphologically different types of cell death.[2] Intensive research which has been continued in the years following allowed the specification of mechanisms leading to programmed cell death and precise control of the cell count. It is especially important in relation to the immunological system, since cell deficiency involves uncontrolled tumor cell growth and increases the risk of infection, whereas excess of immunological cells may lead to autoimmunological response. The main apoptosis pathways are: extrinsic, associated with particular ''death ligand'' (FasL, CD195), and intrinsic – mitochondrial, which depends on physical and chemical factors such as hypoxia or toxins that lead to changes in mitochondrial structure. The third path described in relation to cytotoxic lymphocyte T is perforin/granzyme-mediated apoptosis.[3]

Surgical trauma includes direct tissue damage as well as other factors including: administered anesthetics, blood loss followed by blood transfusions, hypothermia, immobility, in some procedures also general or local ischemia and reperfusion injury. All of the above can induce apoptosis of immune cells. Clinical research showed an influence of surgical procedures on circulating blood lymphocyte apoptosis.[4] This raises a question about an optimal anesthetic technique and anesthetics used in it. Research focused on the influence of anesthetics on lymphocyte apoptosis in *in vitro* conditions shows pro-apoptotic effect of almost all inhalational, intravenous and local anesthetics.[5,6] Unfortunately, data collected from clinical research are ambiguous. Comparative research from 2009 did not indicate regional anesthesia being superior to general anesthesia (GA).[7]

Over the last few years several interesting retrospective studies focused on estimating the long-term effects of regional anesthesia were presented. They proved that

cancer recurrence and metastasis is less frequent among patients who underwent radical prostatectomy procedure with epidural anesthesia compared to the same procedure with GA.[8] Two years later another study partially confirmed previous observations and showed the superiority of epidural anesthesia in patients undergoing a prostatectomy procedure.[9] Unfortunately, Canadian researchers did not confirm these findings, however, their retrospective research period was shorter – 3 years.[10] Similar long-term effects were observed in breast cancer patients who underwent a mastectomy. Rarer cancer recurrence and metastasis were observed in patients who had GA combined with paravertebral anesthesia.[11] These findings, despite the short period of observation and ambiguous results, suggest a need for intensive research on the influence of anesthesia on the systemic defense mechanisms.

Aim of the study

The aim of the study was the assessment of the effect of the anesthetic technique on lymphocyte counts in peripheral blood during perioperative period in patients undergoing primary total knee replacement (TKR) and the influence of lymphocyte apoptosis in these changes. The study was undertaken during an 18-month period between August 2009 and April 2011 at the Department of Orthopedic Surgery of the University Hospital in Bialystok. The study protocol was approved by the University Bioethics Committee of the Medical University of Bialystok, no R-I-002/268/2009. All patients were informed about the study protocol and gave written informed consent.

Patients and methods

45 consecutive patients scheduled for primary TKR were recruited for the study. The inclusion criterion was osteoarthrosis. Exclusion criteria were diseases such as RA (rheumatoid arthritis), systemic lupus erythrematosus (SLE), diabetes mellitus and treatment with glucocorticosteroids, methotrexate and other immunosuppressive and cytostatic drugs within 24 months preceding the surgery. Demographic characteristics of patients are presented in Table 1. Blood samples were taken at 4 time points: before the surgery (T1), directly after closing the surgical wound (T2), 24 h after the surgery (T3) and on the 7th day after the surgery (T4). The immunological status and microscopic study of apoptotic lymphocytes were assessed for all blood samples.

All patients qualified to regional anesthesia were randomly assigned to two anesthetic protocols: spinal anesthesia (SA) and combined spinal–epidural anesthesia (CSE). 11 patients undergoing surgery under GA served as control group.

GA: for induction of GA fentanyl was used (Fentanyl WZF, Polfa Warszawa, Poland) at a dose of 1 μg/kg bw, propofol (1% Propofol-Lipuro, B. Braun, Germany) at a dose of 2 mg/kg bw and suxamethonium chloride (Chlorsuccillin, Jelfa, Jelenia Gora, Poland) at a dose of 1 mg/kg bw. After orotracheal intubation the anesthesia was conducted with continuous infusion of propofol, remifentanil (Ultiva, GlaxoSmithKline, UK) and cisatracurium (Nimbex, GlaxoSmithKline, UK). Artificial ventilation was provided with the mixture of air and oxygen with FiO_2 0.4. Ten minutes before the end of the surgery the infusion of remifentanil was stopped and intravenous morphine (Morphini sulfas WZF, Polfa Warszawa, Poland) was administered at a dose of 0.1 mg/kg bw and 1.0 g of paracetamol (Perfalgan, Bristol-Myers Squibb Pharmaceuticals, USA). Postoperative pain management was provided with subcutaneous morphine per request and paracetamol 1.0 g every 6 h.

SA was provided with 0.5% hyperbaric bupivacaine (Marcaina Spinal Heavy®, Astra Zeneca Pharmaceuticals, UK) at a dose of 2.8–3.4 mL according to the height of the patient. Postoperative pain management was the same as in the group of GA.

CSE was done using the ''single space – double needle'' technique. In the lateral position on the operated side the spinal needle 27 G pencil-point shape (Balton, Poland) was introduced at the L3–L4 level. After a dose of 2.8–3.4 mL of hyperbaric bupivacaine (Marcaina Spinal Heavy®, Astra Zeneca Pharmaceuticals, UK) an epidural catheter (Perifix® B.Braun, Germany) was inserted. The epidural space was identified using the ''loss of resistance'' technique with a saline-filled low resistance syringe. Negative aspiration test was the confirmation of proper catheter position. After motor recovery and before first pain symptoms a bolus of 8–12 mL of 0.5% ropivacaine (Naropin®, Astra-Zeneca Pharmaceuticals, UK) was given. Subsequently continuous infusion of 0.2% ropivacaine with fentanyl 4 μg/mL at dose 6–10 mL/h was started. The infusion was stopped after 48 h and the epidural catheter was removed.

Surgery

Knee arthroplasty (TKR – total knee replacement) was performed using implant Triathlon® (Stryker Co., USA) or

Table 1 Demographic characteristics of patients undergoing TKA with respect to anesthesia protocol.

	Type of anesthesia			
	General	Spinal	CSE	Total
No. of patients	11	17	17	45
Age Median (min-max)	69 (59–84)	72 (59–78)	72 (59–77)	72 (59–84)
Sex (M:F)	2:9	2:13	3:16	7:38
Time of surgery in minutes Median (min–max)	95 (6–160)	100 (70–120)	100 (70–120)	105 (60–160)

Vanguard® (Biomet Inc., USA). Both systems require tibial and femoral component fixation with bone cement, and in both of them a plastic element for providing distance and friction reduction is inserted between the two metal components. All surgeries were done with the use of a pneumatic tourniquet at the femoral level after exsanguination of the extremity with elastic gum tape. The tourniquet was inflated to the pressure of 150 mmHg above systolic blood pressure and deflated after bone cement hardening. After obtaining surgical haemostasis the autotransfusion drain was left in. After stratified wound closure the autologous blood collection system for autotransfusion HandyVac™ ATS (Unomedical, A/S, Denmark) was initiated. The operation wound was covered with a sterile dressing. Time of surgery was assessed as starting from the time of leg exsanguination to skin closure.

Postoperative treatment

Autologous blood transfusion was provided according to the volume of collected blood, but not later than 6 h after the start of the ATS system. Surgical wound drainage was performed up to the second postoperative day. If necessary the leucoreduced Red Blood Cells Concentrate was given, but in no case was it on the day of the surgery. All patients had thromboprophylaxis with LMWH (low molecular weight heparins) according to the Polish Orthopedics Society Guidelines.[12] All patients were also given antibiotic prophylaxis with cefazoline (Biofazolin®, Polpharma, Poland) 1.0 g and amikacin (Biodacyna®, Polpharma, Poland) 0.5 g, 30 min before the start of the surgery. Postoperative rehabilitation was started with Continuous Passive Motion device 24 h after the surgery. Active rehabilitation was started on the third postoperative day.

Blood samples preparation

Blood samples for laboratory study were collected into 2 mL test-tubes with EDTA in 4 time points: before the surgery (T1), immediately after closing the surgical wound (T2), 24 h after the surgery (T3) and 7 days after the surgery (T4).

The immunological status of peripheral blood lymphocytes was assessed with flow cytometry using Simultest™ IMK-Lymphocyte Kit (BD Biosciences, San Jose, CA, USA) and a FASC Calibur BD cytometer. The kit allows for quantitative assessment of lymphocyte count according to the following surface antigens: T cells (CD3) with subpopulations CD3/4 (T helper), T CD3/8 (T suppressor), B cells (CD19), and NK cells (CD16/56). The assessment of apoptotic cells was performed after lymphocyte isolation with Histopaque-1077 and Histopaque-1119 (Sigma–Aldrich Co., USA), and centrifugation of peripheral blood samples at 3000/min and staining with ethidium bromide (10 μM) and acridine orange (10 μM). Acridine orange binds to DNA and stains its structure green. It also binds to cytoplasmic RNA staining it red-orange. Ethidium bromide does not cross the cytoplasmic membrane, so it only stains necrotic cells orange. The structure of lymphocytes was assessed with fluorescent microscopy at 1000× magnification. 100 consecutive cells were assessed as alive, apoptotic or necrotic.[13]

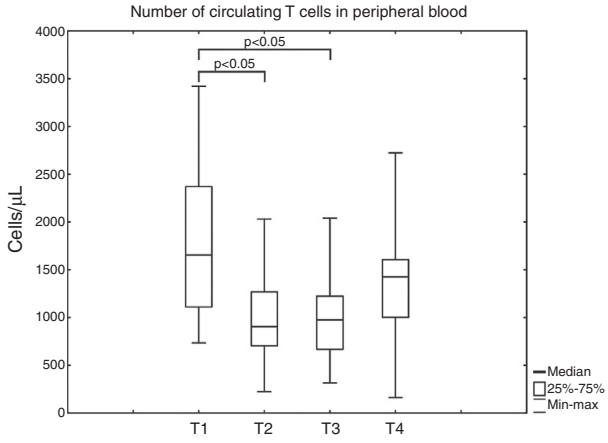

Figure 1 The number of circulating T cells (CD3) before the surgery (T1), just after surgery (T2), 24 h postoperatively (T3) and 7 days after surgery (T4). The box-and-whisker plots show minimal and maximal values, 25 and 75 percentiles and medians (horizontal bars).

Statistical methods

Statistical analysis was performed with ''Statistica 10.0'' software (Statsoft Inc., Tulsa, OK, USA). Data are presented as a median and as 25 and 75 percentile with minimal and maximal values. The *W* Schapiro-Wilk test was used to test for normality. Since the data did not follow normal distribution we used a non-parametrical test for analysis. We compared the data in consecutive time points of the study with the Wilcoxon test and patients' groups in single time points with Kruskal–Wallis test. A *p*-value less than 0.05 was considered statistically significant.

Results

Immunological status

Immediately after the surgery the number of circulating lymphocytes was decreased. The lowest cell count was seen in T cell population. At the T1 time point the number of T cells was reduced to 55% of baseline values ($p < 0.01$). A small increase of T cells was seen 24 h after surgery (up to 58% of pre-operative values). 7 days after the surgery (T4) the number of circulating T cells was still 14% lower than preoperatively (Fig. 1). The pattern of particular subtypes of T cell changes was different. The greatest decrease in the number of circulating T CD3/4 cells was seen immediately after the surgery – and was 45% of preoperative values. 24 h after the surgery we observed an increase in the number of circulating T CD3/4 cells to 56% of preoperative values. On the seventh day of the study the number of circulating T CD3/4 cells reached 90% of preoperative values (Fig. 2). A different pattern of change was observed in T CD3/8 cells. The greatest decrease in circulating T CD3/8 cells was seen 24 h after surgery – it decreased to 55% of preoperative values. The number of CD3/8 cells on the 7th day was the same as before the surgery (Fig. 3).

Immediately after surgery the number of circulating B cells (CD19) was significantly lower than before the surgery.

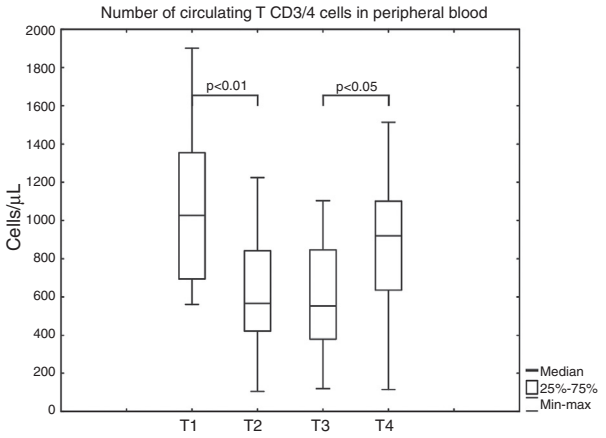

Figure 2 The number of circulating Th cells (CD3/4) before the surgery (T1), just after surgery (T2), 24 h postoperatively (T3) and 7 days after surgery (T4). The box-and-whisker plots show minimal and maximal values, 25 and 75 percentiles and medians (horizontal bars).

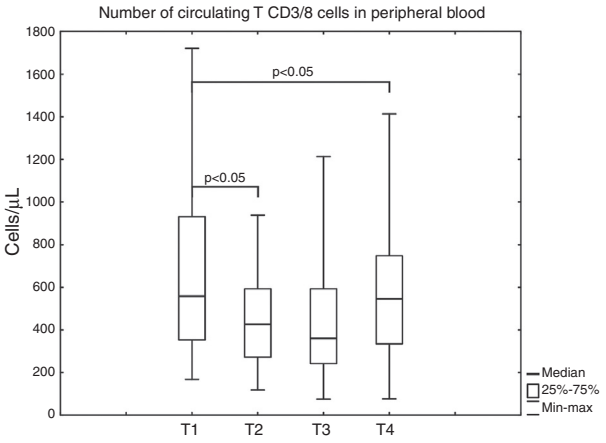

Figure 3 The number of circulating Ts cells (CD3/8) before the surgery (T1), just after surgery (T2), 24 h postoperatively (T3) and 7 days after surgery (T4). The box-and-whisker plots show minimal and maximal values, 25 and 75 percentiles and medians (horizontal bars).

24 h after the surgery an increase in the number of circulating CD19 cells was observed, and on the 7th day after the surgery the number of B cells was higher than before the surgery, although the difference did not achieve statistical significance (Fig. 4).

The smallest differences were observed in the number of circulating NK (CD16/56) cells. Differences in the number of circulating NK cells did not achieve the level of statistical significance during the time of the study (Fig. 5). Changes in the immunological status in the 3 groups of patients according to the anesthetic protocol were studied, but we did not observe any differences at all time points.

Peripheral blood lymphocyte apoptosis

An all time points we assessed the number of circulating lymphocytes for macroscopic signs of apoptosis using fluoroscopic microscopy. Before the surgery the number of

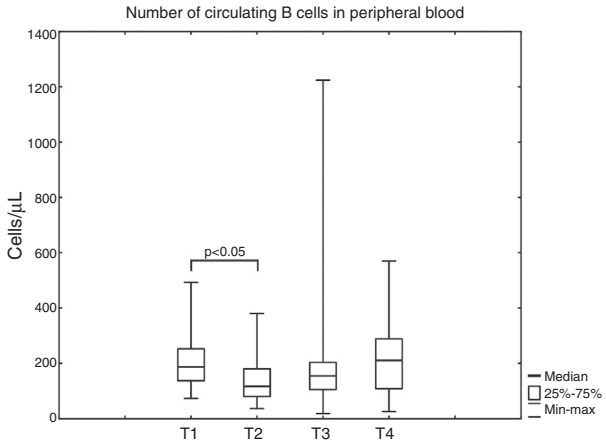

Figure 4 The number of circulating B cells (CD19) before the surgery (T1), just after surgery (T2), 24 h postoperatively (T3) and 7 days after surgery (T4). The box-and-whisker plots show minimal and maximal values, 25 and 75 percentiles and medians (horizontal bars).

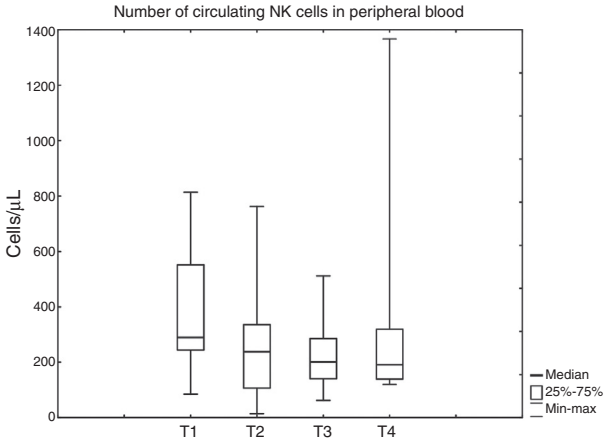

Figure 5 The number of circulating NK cells (CD16/56) before the surgery (T1), just after surgery (T2), 24 h postoperatively (T3) and 7 days after surgery (T4). The box-and-whisker plots show minimal and maximal values, 25 and 75 percentiles and medians (horizontal bars).

apoptotic lymphocytes varied from 0 to 8%. A statistically significant increase in the number of apoptotic lymphocytes was observed immediately after the surgery. The difference between the number of apoptotic lymphocytes before the surgery and after 24 h did not achieve statistical significance, but on the seventh day of the study it was higher than before the surgery. After comparing this data for particular anesthetic protocols no statistically significant differences between the groups were observed (Fig. 6).

Discussion

Postoperative lymphopenia is a well-established and precisely described phenomenon.[14] It affects all lymphocyte populations: T cytotoxic and T helper cells, B cells and NK cells, and its extent depends on the magnitude of surgical trauma. In our study a significant decrease of number of

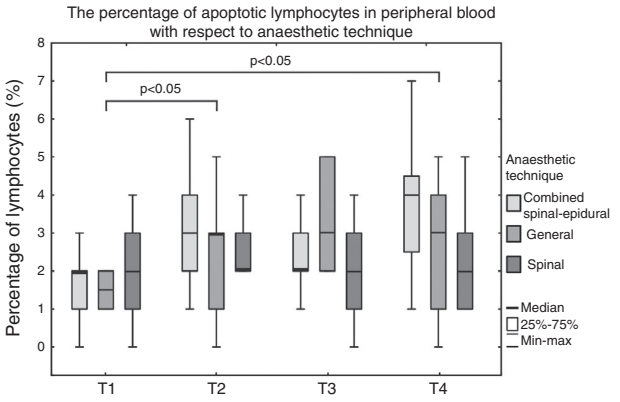

Figure 6 The number of circulating apoptotic peripheral blood lymphocytes with respect to anesthetic protocol (CSE group, GA group, spinal group) before the surgery (T1), just after surgery (T2), 24 h postoperatively (T3) and 7 days after surgery (T4). The box-and-whisker plots show minimal and maximal values, 25 and 75 percentiles and medians (horizontal bars). Statistically significant differences were seen between particular time points, but not between groups.

circulating T CD3/4 cells and T CD3/8 cells was observed very early, just after the end of surgery. Similar findings were seen in previous studies and this refers to postoperative and posttraumatic patients.[15,16] A decreased number of circulating T cells was seen 24 h after the surgery, but the number of Th cells (CD3/4) increased 24 h after the surgery achieving values similar to values seen preoperatively. The number of cytotoxic T CD3/8 cells was significantly lower than before the surgery and a statistically significant difference was observed until the seventh day after surgery. In contrast the number of circulating B cells (CD19) only immediately after the surgery was significantly lower than before the surgery. 24 h after the surgery we observed an increase in the number of circulating B cells, and 7 days after the surgery the number of cells was higher than before the surgery, although the difference did not achieve the level of statistical significance. The cause of such a different pattern of changes may be explained by a presence of foreign substances such as joint implants or bone cement.[17]

No difference was seen in the number of circulating NK cells. The number of circulating NK cells did not differ during the entire observational period. It is in contrast to most previous studies, where a significant decrease in the number of circulating NK cells was observed.[18,19] The explanation for this difference may be the difference in the patient population – in our study general patients without malignancy were studied whereas in the studies mentioned above the patients were oncologic. The number and function of NK cells is of particular interest to clinicians because these cells are the first line of defence against neoplasmal cells. The possibility of the influence of the anesthetic technique – general *vs.* regional anesthesia – on the number and function of NK cells was even studied in meta-analysis by Conrick-Martin and co-workers.[20] The results of this study do not confirm the superiority of regional techniques over GA but may be an important contribution to further studies. The slight influence of anesthesia and surgery on the number of circulating NK cells observed in our study may be explained by the exclusion of patients with neoplasmal disease and taking immunosuppressive agents from the research.

The apoptosis of lymphocytes in response to surgical trauma is an occurrence which was well confirmed in *in vitro* and *in vivo* settings.[4,21] It may be one of the causes of posttraumatic and postoperative lymphopenia. This is the reason the question of the effect of the anesthetic technique on peripheral blood lymphocytes may be clinically important. The optimal choice of an anesthetic procedure and agents should minimize the immunosuppressive effect of surgical insult. We may point out such apoptosis-inducing factors as: preoperative – psychological stress and fasting, and intraoperative – pneumatic tourniquet, tissue damage, bone cement and anesthetic agents used during the operation.[22–24] Some of the factors initiated earlier are maintained in the postoperative period, but some new ones, including long-term immobilization, presence of an implant and complex postoperative pain management with use of strong and long-acting opioids, also occur. In our study, immediately after the end of the surgery and the closing of the surgical wound, the percentage of lymphocytes with macroscopic features of apoptosis was significantly higher than preoperatively. On the first postoperative day, 24 h after surgery, the number of apoptotic lymphocytes was also higher than preoperatively but the difference did not achieve a level of statistical significance. This may be explained by the manner of presenting the results – the number of apoptotic lymphocytes was counted in 100 consecutive cells. A relatively lower number of apoptotic lymphocytes may be the result of the activation of lymphopoiesis and the appearance of new cells in peripheral blood. Confirmation of this explanation is an increase of the number of all lymphocyte populations in peripheral blood on the first postoperative day. An increased percentage of apoptotic lymphocytes were also seen 7 days after the surgery compared to preoperatively. It means that not only intraoperative but also postoperative factors may affect the processes of apoptosis.

The effect of the anesthetic technique on peripheral blood lymphocyte apoptosis was the topic of few clinical trials. Pro-apoptotic effect of volatile and intravenous anesthetics, local anesthetics and opioids was established *in vitro*.[5,6] In one of the studies comparing the effect of general and epidural anesthesia on peripheral blood lymphocytes apoptosis no difference was found in patients undergoing abdominal surgery.[7] The difference was the patients' population – in the previous study patients were undergoing abdominal surgery for nonmetastatic colon cancer. TKA is a strong proapoptotic stimulus because of major tissue damage, use of pneumatic tourniquet, blood loss and postoperative pain. The choice of drugs used in the study, propofol and ropivacaine, had discrete or no influence on the peripheral blood lymphocyte apoptosis.

Conclusion

During the postoperative period there is significant lymphopoenia which lasts up to seven days. The magnitude of this phenomenon depends on the lymphocyte population and to a greater degree refers to CD3/8 T cells and to a lesser degree to CD3/4 cells and CD19 cells (B cells). It is

not seen in respect to NK cells (CD16/56). The concomitant change is the increase of the percentage of lymphocytes with macroscopically seen apoptotic changes. The process of lymphocyte apoptosis may be partially responsible for postoperative lymphopoenia. The choice of the anesthetic technique: GA, SA or CSE, in this particular type of surgery, does not affect the number of apoptotic lymphocytes.

Conflicts of interest

The authors declare no conflicts of interest.

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