The degree of lymphocytic mitochondrial transmembrane potential and blood magnesium concentrations during coronary artery bypass grafting

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Abstract. Magnesium (Mg) plays an important role in lymphocyte function. Low blood concentration of Mg may result in intralymphocyte imbalance and in turn may be associated with intensified apoptosis of peripheral blood lymphocytes. Due to its multistage character; extracorporeal circulation (ECC) may augment Mg disorders adding to the above mentioned pathology. The aim of this study was to assess the correlation between lymphocyte apoptosis and Mg concentration in the blood during the course of coronary artery bypass grafting (CABG) and in the early postoperative period. Method. Twenty male patients undergoing CABG with ECC under general anaesthesia were included in the study. For detection of apoptotic lymphocytes in the circulation, inner mitochondrial transmembrane potential (ΔΨm) was measured with the use of chloromethyl-X-rosamine (CMXRos) and flow cytometry. Spectrophotometry was used for Mg blood concentration measurements. Peripheral blood samples were obtained in seven stages: 1) just before anaesthesia, 2) 2 hours after the beginning of surgery, 3) immediately after surgery, 4) 12 hours after the beginning of surgery, 5) 24 hours after the beginning of surgery, 6) 36 hours after the beginning of surgery, 7) 54 hours after the beginning of surgery. Results. The statistically significant increases of lymphocyte apoptosis were noted in stages from 2 to 7. Blood Mg concentrations decreased in stages 2 and 3. There was negative correlation between Mg blood concentration in stages 2 and 3 and the intensity of lymphocyte apoptosis in the stage 5. Conclusions. 1) CABG with extracorporeal circulation was associated with a decrease of magnesium concentration in the blood and an increase of lymphocyte apoptosis intensity. 2) The decrease of magnesium blood concentration may increase the degree of lymphocyte apoptosis. 3) Lymphocyte apoptosis after extracorporeal circulation has a two-phase course. Keywords: CABG, apoptosis, lymphocyte, mitochondrial transmembrane potential, plasma magnesium concentration
It is well known, that magnesium (Mg) is a very important intracellular electrolyte for cell homeostasis [1]. Mg acts as a cofactor of many enzymes and is involved in a variety of biological functions, including structural and regulatory intracellular balance. Mg deficiency is connected with the development of heart diseases including hypertension and atherosclerosis. This electrolyte stabilizes mitochondrial linkage and regulates oxidative and ATP synthesis processes [2, 3]. Hypomagnesemia may cause changes in mitochondrial transmembrane potential and deregulate the mitochondrial electrolyte balance [4]. It may lead to accumulation of sodium and calcium ions in intracellular spaces which may be particularly important for the disturbance of mitochondrial respiratory activity (regulated by calcium ions) [1, 5]. These changes cause hyperproduction of reactive oxygen species (ROS) [6, 7] and many authors suggest that the ROS accumulation results in programmed cell death or apoptosis [8]. It has also been reported that an increase in intracellular calcium and magnesium concentration promotes apoptosis [9]. Apoptosis is an active and physiological mechanism allowing cells to die in a controlled and organized manner with characteristic biochemical and ultrastructural changes. Apoptosis of immunocompetent cells such as lymphocytes is involved in the regulation of the immune response, particularly under stress conditions. Extensive trauma, surgical stress and general anaesthesia cause neuroendocrine and inflammatory activation with hyperproduction of catecholamines, as well as proinflammatory cytokines such as IL-6, TNF-α and this may affect the immune system. Apoptosis of lymphocytes plays an important role in the resolution of these processes and is partly secondary to their activation and with reference to T lymphocytes, is described as activation-induced cell death (AICD) [10]. There are many morphological and biochemical hallmarks of apoptosis that can be detected and measured with cytometry and other techniques. Such cells may be characterized by the collapse of the mitochondrial transmembrane potential ($\Delta \Psi_m$), which is considered to be the first detectable sign of apoptosis. Mitochondria are organelles with two well-defined compartments: the matrix, surrounded by the inner membrane and the intermembrane space, surrounded by the outer membrane. The inner membrane contains various molecules such as ATP synthases, adenine nucleotide translocators and electron transport chains, each of them being dependent on magnesium. Permeabilization of inner membranes leads to changes in mitochondrial membrane potentials [10]. It has been found that alterations in mitochondrial function play a key role in the effector phase of apoptosis induced by different agents [2, 10, 11]. These alterations include the disruption of $\Delta \Psi_m$, the generation of ROS and the opening of permeabilization transition pores (PTP) in the outer mitochondrial membrane through which pro-apoptotic factors such as cytochrome c are released into the cytoplasm. $\Delta \Psi_m$ can be easily measured by the use of some cationic lipophilic dyes such as chloromethyl-X-Rosamine (CMXRos) [12].

Many authors underlined that surgery and anaesthesia cause depression of cell-mediated immunity in the postoperative period [6, 13]. Apoptosis of lymphocytes and the resulting susceptibility to infections from common pathogens seem to be a particularly important problem during and after cardiac surgery with extracorporeal circulation (ECC). Probably a multitarget stage of ECC procedures as well as intraoperative therapy and electrolyte disturbances may contribute to this process.

The aim of this study was to assess the correlation between apoptosis of lymphocytes and Mg blood concentrations during CABG procedure and in the early postoperative period.

### Material and methods

The study was approved by the Bioethical Committee of the Feliks Skubiszewski Medical University of Lublin; Poland (No KE-0254/28/2003) and included the patients treated for stable angina pectoris (I° and II° according to Canadian Cardiovascular Society, or CCS) and scheduled for elective aortocoronary bypass (CABG-coronary artery bypass graft) with ECC.

In the evening preceding the operation the patients were premedicated with lorazepam and promethazine. One hour before anaesthesia all the patients received lorazepam and morphine. The patients underwent general anaesthesia with fentanyl, midazolam, and etomidate. Muscle relaxation was obtained by injecting a single dose of pancuronium. The anaesthesia was maintained throughout the procedure using midazolam-fentanyl infusion and inhalatory fractionated doses of Isoflurane. During the implantation of aortocoronary by-passes, circulation and ventilation were maintained by a heart-lung machine SIII (Stockert). The following substances were used for priming: Ringer solution, HAES solution, 20 % mannitol, Natrium bicarbonatum 20 mL, and heparin 75 mg. The same composition of priming was used in all patients. Cardioplegia was prepared using 0.9 % salt solution supplemented with 3 g of
potassium chloride and 20 mL of sodium hydroxy-carbonate. The degree of normovolemic hemodilution induced by a constant volume of priming (1 800 mL) was determined on the basis of haematocrit measurements and body weight. During surgery the patients received supplementation of potassium chloride to the level of 4.94 mmol/L ± 0.5.

All the patients were transported to the Postoperative Intensive Care Unit immediately after the procedure, where they received a short-term infusion of 5% glucose solution with insulin and 3 or 6 g of potassium chloride. None of the patients received Mg infusion during surgery or the postoperative period.

Peripheral blood samples were taken at seven different stages: 1) just before anaesthesia, 2) 2 hours after the beginning of surgery, 3) immediately after surgery, 4) 12 hours after the beginning of surgery, 5) 24 hours after the beginning of surgery, 6) 36 hours after the beginning of surgery, 7) 54 hours after the beginning of surgery. The samples were immediately transferred to the laboratory for detection of apoptotic cells.

Mononuclear cells were isolated by density gradient centrifugation (Gradisol, Aqua-Medica, Poland). Peripheral blood serum samples were taken and stored at -20°C until use.

The dissipation of $\Delta V_m$ was measured by the use of CMXRos (Mito Tracker Red CMXRos, Molecular Probes). Cells were incubated with the dye alone for 15 minutes at 37°C, for the next 15 minutes monoclonal anti-glycophorin A FITC-conjugated antibody (DAKO, Denmark) was added to contaminating lymphocytic population. Just after the performance of these procedures, cells were acquired and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA) with Cellquest Software by the same company.

The blood magnesium concentrations were determined by spectrophotometric methods.

The Wilcoxon paired test was used to analyse differences between the percentages of apoptotic cells and Mg concentrations, in comparison with stage 1. The Spearman rank correlation was used for the estimation of correlation between percentages of apoptotic cells and Mg concentrations. The p-value < 0.05 was considered significant. Data were presented as median and ranges. Statistica 6 software was used for all statistical calculations.

Results

The examinations were conducted in 20 men aged 53-70 (61.1 ± 6.9). Sixteen patients had myocardial infarction during the past 3 years and 18 were treated due to concomitant arterial hypertension (I° or II° according to WHO classification). None of the patients was treated for endocrinological, neurological and other systemic diseases nor was resuscitated because of circulatory arrest.

The mean duration of the procedure was 205 min ± 35 and of anaesthesia 235 min ± 30. In all the patients the aorta was typically clamped and the mean closure time was 45.1 min ± 15.5. The aorto-coronary anastomosis was performed in shallow hypothermia at 34.5°C ± 0.4. In all the cases the heart-lung machine disconnection was uneventful and there was no need of intra-aortic contrapulsation. Four patients did not require pharmacological support after the end of ECC, seven were subjected to continuous dopamine infusions in doses adjusted to their clinical condition and nine had dobutamine infusion.

The significant increase of lymphocyte apoptosis (LA) was noted from stage 2 to stage 7. A small decrease of LA degree was noted in stage 5, but the value was significantly higher in comparison with stage 1.

The blood magnesium concentrations significantly decreased in stages 2 and 3. The level of magnesium decreased in stage 5 but this change did not reach statistical significance (figure 1).

There were significant negative correlations between Mg blood concentration in stages 2 and 3 and late apoptosis of lymphocytes in the stage 5 (lymphocyte apoptosis occurs dependently after Mg decrease) (figure 2).

Discussion

The concentration of magnesium in the cells depends on levels of binding substances including nucleic acids, ATP or phospholipids, although the main factor regulating intracellular concentration of Mg is its concentration in serum. On the other hand the changes in serum Mg concentrations during ECC are not explicitly defined and recent reports stress the importance of the maintenance of normomagnesemia, particularly in patients with stunned myocardium [14, 15]. Our studies showed that patients undergoing surgical myocardial revascularization with ECC have a high risk of decreased serum magnesium concentrations. Polderman and Girbes [16] suggest that the mechanism responsible for this may be a combination of increased urinary excretion and intracellular shift, induced by a multistage character of ECC procedures. A decrease in body
temperature during surgery and high urinary magnesium excretion play the main roles in this pathology. Probably a kidney tubular dysfunction results in urinary magnesium excretion [16]. Furthermore, hypomagnesemia during CABG is attributed to degree of hemodilution [17]. Examining the changes in blood magnesium concentrations in cardiosurgical patients, Satur et al. [17] observed that initiation of normovolemic hemodilution caused a 17.3% decrease in serum Mg levels, which persisted until the first postoperative day. They concluded that the main reasons for magnesium depletion are: the most

![Changes of blood magnesium concentrations](image1.png)

![Percentages of apoptotic lymphocytes](image2.png)

**Figure 1.** Blood magnesium concentrations (A) and lymphocytic apoptosis (B) in several stages of experiment (Wilcoxon test). *p < 0.05; **p < 0.01; ***p < 0.001 - comparison with stage 1.
important – normovolemic hemodilution and secondly – intraoperative and postoperative cellular depletions.

Magnesium deficiency in serum results in relatively low intracellular magnesium leading to cell dysfunction. Through its regulatory effects on sodium-potassium pump and ATPase, Mg directly affects intracellular concentration of potassium and calcium and its increased extracellular levels favourably influences the cell tolerance to ischaemia and reperfusion. It is worth stressing that decreased Mg level in blood leads to increased permeability of cell membranes and reduces $\Delta \Psi_{in}$. This effect seems to be relevant in cases of programmed cell death [18, 19]. Examining the pathophysiology of changes in $\Delta \Psi_{in}$ in rats, Marcocci et al. [18] observed a signifi-

**Figure 2.** Spearman’s correlations between percentages of apoptotic lymphocytes in stage 5 and blood Mg concentrations in stage 2 (A) and in stage 3 (B).
cant decrease in intracellular Mg concentration and simultaneous calcium ions (Ca) accumulation leading to cell oedema. According to Lang et al. [19] this mechanism is responsible for the initiation of cell apoptosis. A decrease in intracellular magnesium concentration results in a dysfunction of the sodium-potassium as well as magnesium-calcium pump [4, 20] leading to intracellular calcium accumulation and cell oedema [3, 21]. This leads to metabolism disorders and ROS accumulation, which also favours apoptosis [19, 21, 22]. On the other hand Chien et al. [4] suggest that achieving high levels of free intracellular magnesium is central to the apoptotic process. In their opinion magnesium mobilisation is an early event in cell suicides and disruption of ΔΨ\textsubscript{m} is dependent on this phenomenon. However the release of magnesium from mitochondria could merely be a consequence of the opening of mitochondrial pores as ΔΨ\textsubscript{m} is reduced. Bossy-Wetzel et al. [23] imply that this magnesium release is independent of the loss of ΔΨ\textsubscript{m}. According to them the release of mitochondrial cytochrome c is reported to occur prior to, and to be independent on, the disruption of ΔΨ\textsubscript{m} on the other hand the cytochrome c release is strictly dependent on magnesium ions. Thus it may be supposed that the acute disorders of magnesium play an important role for the apoptotic process. However, it is difficult to determine explicitly the precise cause of this cell pathology during CABG. According to Macno et al. [21] and Bortner and Cidlowski [22], the other main reason for such changes is the initial normotonic shrinkage of the cell caused by hyperosmotic stress. Considering the above, it can be supposed that normovolemic hemodilution used intraoperatively disturbs lymphocyte homeostasis stimulating apoptosis. The examinations performed seem to confirm this hypothesis as significantly increased apoptosis was already observed during extracorporeal circulation procedures. Korycinska et al. [24] demonstrate that the degree of lymphocyte apoptosis is dependent on normovolemic hemodilution. They report more marked cell suicides in patients with a high dilution of blood. On the other hand, it may seem that the process in question was not only initiated by normovolemic hemodilution. Many authors stress unfavourable, apoptosis-stimulating effects of commonly used anaesthetics [13, 25, 26]. According to Delogu et al. [8, 25, 26], both operative stress and the drugs used initiate lymphocyte apoptosis. Therefore, it may be supposed that the fentanyl and pancuronium used in our study intensify apoptosis. Examining the effects of opioids on LA, the authors mentioned above observed that the opioid already disturbed the lymphocyte ΔΨ\textsubscript{m} in the 90th minute of cell exposure to fentanyl, and the highest intensification was noted in 120th minute. It is also noting that the intracellular changes observed were accompanied by excessive ROS production, which may confirm the intracellular pathology described earlier [8, 26]. However, it is difficult to compare these results with our findings as all our patients received continuous fentanyl infusions throughout the operation and anaesthesia.

Analyzing LA intensification during extracorporeal circulation procedures, one should not neglect the effects of the procedure itself. One of the most harmful factors impairing cell homeostasis are filters and oxygenerators. It may seem that the extent of damage and cell apoptosis is likely to depend on the kind of the oxygenerator used. However, Leans et al. [27] who studied the effects of various oxygenerators on the extent of lymphocyte apoptosis, did not find relevant differences in ”lymphocyte response” to the procedure with cardiopulmonary bypass. According to them, LA intensification results from the procedure itself rather than from the equipment used.

The double phase of LA is interesting although difficult to explain. It may result from the simultaneous activation of caspase-dependent and caspase-independent pathways by different factors such as surgical stress or mechanical damage [28]. Possible mechanisms for these phenomenon are still discussed, including: cell – cell interactions, intracellular hydrodynamic disturbances and waste accumulation [29, 30]. Postoperative treatment – particularly dopamine or dobutamine infusion is not without importance too [31]. Ciocio et al. [31] in their analysis of the suicide of cells incubated in different dopamine or dobutamine solutions, presented dose-dependent increases of LA, with the highest intensity observed after 24 hours of incubation. Interestingly, the pathology was inhibited by a β-blocker – propranolol. Thus we believe that the second increase of LA observed by us may have resulted from dopamine or dobutamine infusion, although this correlation in patients after extracorporeal circulation is not explicitly documented and requires further studies.

The negative correlation between blood Mg level and LA intensity is also difficult to explain. It seems that high Mg blood concentrations may confirm the protective effects of Mg on the cell, particularly in stress situations. Studying the effects of high magnesium levels in blood on nerve cells, Park and Hyun [32] noted significantly weaker nerve cell apoptosis in patients treated with high doses of Mg. Likewise, Fernandez-Gomez et al. [33] stress the beneficial effects of high magnesium levels on ΔΨ\textsubscript{m}. According
to them, this element substantially inhibits mitochondrial membrane damage and ROS production, thus decreasing the extent of cell apoptosis. In our study the negative correlation between Mg blood concentration in stages 2 and 3 and apoptosis of lymphocytes in stage 5 confirm the opinion that hypomagnesemia may lead to increased apoptosis even several hours later. Thus it seems that LA observed in our study may result both from the operative procedure itself and from changes in blood magnesium levels.

Conclusions

1. The extracorporeal procedure caused a decrease of magnesium blood concentrations and an increase of lymphocyte apoptosis.
2. The decrease of magnesium concentration in the blood is one of the causes of increased lymphocyte apoptosis.
3. The lymphocyte apoptosis has a two-phase course after extracorporeal circulation.

References

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