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Influence of Antithymoglobulins on Ischemia/Reperfusion Injury in perfused non-human primate tissues: Histological and Cytological Investigation

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"….¡Moito sabés, miña vella,
moito de sabiduría!
¡Quen poidera correr mundo
por ser como vós sabida!
Que anque traballos se pasen
aló polas lonxes vilas,
tamén ¡que cousas se saben!,
tamén ¡que cousas se miran!…

…Amén, miña vella, amén;
mais, polas almas benditas,
hoxe dormirés nun leito
feito de palliña triga,
xunta do lar que vos quente
ca borralliña encendida,
e comerés un caldiño
con patacas e nabiza. …."

Rosalia de Castro, 1863

A mi abuela Estrella

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Abbreviations:

- IRI: Ischemia-Reperfusion Injury
- OFR: Oxygen-free Radicals
- ATG: Antithymocyte Globulin
- ALS: Antileukocyte sera
- MHC: Major Histocompatibility Complex
- HLA: Human Leukocyte Antigen
- ADCC: Antibody-dependant Cellular Cytotoxicity
- ISR: Institute for Surgical Research
- WBC: White Blood Cells
- RBC: Red Blood Cells
- Plat: Platelets
- Hct: Haematocrit
- Hb: Haemoglobin
- EC: Endothelial Cells
- TNF-α : Tumor Necrosis Factor-alpha
- ICAM-1: Intercellular Adhesion Molecule-1
- VCAM: Vascular Adhesion Molecule
- CD: Cluster of Differentiation
- LCA: Leukocyte Common Antigen
- IL: Interleukin
- CIM: Cyto-Immunological Monitoring
- PMN: Polymorphonuclear
- PECAM: Platelet Endothelial Cell Adhesion Molecule
- IVM: Intravital Microscopy
- H/E: Haematoxylin-Eosin
- ANOVA: Analysis of Variance
- PBS: Phosphate-buffered Saline
- SI: Short Ischemia
- LI: Long Ischemia
- PB: Peripheral Blood
- LGL: Large Granular Lymphocyte
- GE: Granulocyte Esterase

1. INTRODUCTION

Depriving a tissue of its blood supply leads to a severe cellular dysfunction and ultimately cell death, resulting in serious consequences for the tissues and the whole organism (58). Ischemia-reperfusion injury (IRI) is a vital problem in organ transplantation as no organ can be transplanted without suffering from ischemia and posterior reperfusion injury (72, 100). IRI constitutes an acute inflammatory process by which cells or organs are damaged first by temporary ischemia, hypoxia and accumulation of toxic metabolites and later by reperfusion due to cell activation (118). This process involves cell surface adhesion molecule expression (99), which is crucial for the recruitment and infiltration of effector cells in the reperfused tissue (14, 10, 69, 146). These mechanisms are also involved in the rejection process of transplanted solid organs (5, 66, 71). Activation of endothelial cells (EC) and white blood cells (WBC), in particular neutrophils, is one of the main underlying mechanisms in IRI (185, 195).

IRI is strongly associated with the localisation of neutrophils in the ischemic regions, which occurs within the initial hours of reperfusion. After implantation of an allograft, host blood perfuses the donor organ, triggering a cascade of receptor ligand interactions (51, 73) responsible for endothelial damage and cell activation (151). Emigration of neutrophils is evident both by histological (4, 133) and cytological methods (200). Presence of neutrophils in the tissue due to reperfusion causes injury beyond the ischemic aggression, as interventions against adhesion properties of neutrophils and leukotactic activity have significantly reduced the amount of damaged tissue (83, 103, 104, 113-115, 203).

IRI is also associated with the production of oxygen-derived free radicals (OFR), that cause cell membrane damage as well as oedema and have a very important role in activating the complement cascade (109), resulting in the rapid expression of the adhesion molecules on the endothelial cell surface, thus enhancing the reperfusion injury (86, 122). OFR include the

superoxide anion and the hydroxyl ion. OFR can be cytotoxic to cellular components as a result of degradation of proteins and nucleic acids and lipid peroxidation of membranes and may not be controlled by endogenous anti-oxidative mechanisms such as superoxide dismutase (135).

At the time of reperfusion, the EC surface becomes a site of intense interaction between different types of leukocytes and their adhesion molecules, leading to intermediate adhesion (rolling) through the action of selectins and to permanent adhesion of the leukocytes to the EC (sticking) triggered by integrins. Different molecules including tumor necrosis factor-alpha (TNF-α), intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule (VCAM) rapidly undergo conformational changes that increase their avidity for endothelial ligands for both lymphocytes (ß2 integrin leukocyte function associated antigen) and neutrophils (ß1 integrin very late antigen-4) (27, 65, 116). These interactions between oxygen radicals, adhesion molecules, activated WBC and EC may represent the key step in the process of intragraft antigen recognition by allowing the lymphocytes and the neutrophils to migrate into the graft, initiating or developing processes which lead to allograft destruction (101, 168). Transendothelial migration of WBC, especially lymphocytes and neutrophils, are also stimulated by means of local endothelial chemokine activation (112).

Long-term outcome of the graft may also be influenced by IRI and subsequent acute rejection episodes related to endothelial and vascular damage (169). Organs that are used for transplantation undergo varying periods of cold ischemia. Long periods of cold storage result in an increased susceptibility to damage upon reperfusion (95). For this reason organ preservation is fundamental for the outcome of transplanted organs.

To summarise, it is evident that immunological interactions leading to activation of different WBC subsets play an essential role in IRI and later outcome of graft survival, so that immunosuppression becomes a vital tool to prevent and treat these phenomena.

During vertebrate evolution the immune system has evolved in such a way that it is able to distinguish self from nonself. The necessity for such an advanced antigen recognition system lies on the fact that an infected host first needs to identify pathogens before it can initiate their elimination. During ontogenesis the immune system is educated to avoid reacting against self structures (autoantigens). This process of eliminating or controlling self reactivity does not always reach the level of perfection as is reflected by the existence of a large number of autoimmune disorders. In these types of diseases, immunocompetent cells or their products mount pathological reactions towards autoantigens. Susceptibility or resistance develop many types of autoimmune diseases controlled by multiple genetic components. Candidates are the polymorphic gene products of the major histocompatibility complex (MHC) and those of the T-cell-receptor system which are involved in the initial steps of immune recognition (163). In addition, activated T-cells and many other immunocompetent cells have the capacity to secrete a large variety of cytokines. These so-called biological response modifiers play a key role in the immune system since they control the potential to augment or diminish the strength of immunological responses. At the other end of the spectrum of immune regulation, one finds the problems that clinicians face in bone marrow and solid organ transplantation. The recipient's immune system may recognize structures on foreign tissues (alloantigens) resulting in the rejection of the graft. Especially the gene products of the MHC are known to act as major barriers for successful transplantation. Besides immunosuppressive protocols, matching of MHC alleles between donor and recipient is known to have beneficial effects on graft survival.

The crucial role of immunological mechanisms in the rejection of grafts was first demonstrated by Medawar (120) in 1944, who discovered important characteristics of this process, such as latency or memory induction. The involvement of cellular and humoral mechanisms in the immune response was described by Mitchinson in 1954 (128). Essential investigations in the field, e.g., the HLA-system or the differentiation of T- and B- cells were carried out in the 60´s

(37, 56, 126, 163). At the same time the first immunosuppressive drugs, corticosteroids, were developed.

The initial experiments performed with cytotoxic agents such as benzene, toluene or irradiation were interrupted due to the undesirable side-effects and high rates of morbidity and mortality (181). Non-selective antiproliferative agents such as Cyclophosphamide, Azathioprine and Micophenolic acid analogues were used, alone or in combination with steroid therapy (15, 62, 87, 153, 173). This resulted in an improvement of the transplant outcome while it maintained considerable harm to the patients due to their toxicity (150). Drugs that inhibit cytokine synthesis such as Cyclosporine A, Tacrolimus or cytokine receptor binding monoclonal antibodies such as anti-CD25 as well as inhibitors of signal transduction like Syrolimus improved the immunosuppressive therapy by prolonging the graft survival (141), diminishing the acute rejection episodes and permitting the reduction of steroids (150) in the therapy with the subsequent diminution of the side-effects. However, they show a high nephrotoxicity in combination therapies and other undesirable adverse events such as hypertension, neurotoxicity and hyperlipidaemia (141).

Specific molecules against EC ligands, T-cell receptors or G-protein-ligand are used still experimentally and although the results are very promising they are not used in clinical protocols at the moment (2, 127).

Despite these latest improvements of immunosuppressive agents the IRI paradigm is still a main obstacle in organ transplantation, vascular damage and graft rejection as most of the immunosuppressive agents are specific for one action yet not covering the whole spectrum of possible endothelial-molecular-cellular interactions.

There is, however, a group of drugs named generally polyclonal antilymphocyte preparations, which may alleviate the ischemia/reperfusion paradigm, as they show a modulatory effect in the immune system not only against the cellular mechanisms but also against the humoral

mechanisms, including adhesion molecules (33, 187). This thesis will focus on the action of antithymocyte globulins (ATG) upon ischemia-reperfusion injury.

Antileukocyte sera (ALS) were first described in 1899 by Metchnikoff. He proposed that xenoantibodies coat foreign cells, leading to their destruction (124, 125), a proposal that led to the application of animal antihuman polyclonal antibodies to reduce rejection reactions in clinical practice decades after. Several species were investigated to see which one would be the best source of ALS for its application on human beings. Horse would be "a priori" the best source due to the large quantity of product available in an individual animal. However, the efficacy of horse antibodies varies considerably. Sheep and goats were also dismissed due to their unreliability. Rabbits were identified as the best antibody producers despite the small quantity of serum available per animal. The antibodies present in these sera were generally named antilymphocyte, i.e., thymocyte globulins.

These antibodies bind to cell surface receptors, thereby opsonizing lymphocytes for complement-mediated lysis or reticuloendothelial cell-dependant phagocytosis. ATGs recognise most of the molecules involved in the T-cell activation cascade such as CD2, CD3, CD4, CD8, CD11a, CD18, CD25, HLA DR and HLA class I (24). Although lymphocyte depletion constitutes the primary mechanism of the immunosuppressive effects of ATG, other mechanisms such as blocking of adhesion molecules and apoptosis induction are involved.

As explained above, depletion and modulation of peripheral blood B- and T-cells is the main effect of ATG (129, 166, 183, 211). T-cell depletion involves active cell death, demonstrated by annexin V binding (41) and TUNEL analysis (157). Maximal depletion occurred in blood by means of a complement-dependent lysis. A second mechanism of T-cell depletion would be an activation-associated apoptosis, Fas- and TNF-independent (52, 53). Profound immunosuppression is evidenced by a peripheral blood T-cell lymphocyte count of less than 150 T-cells/ml (88). Monitoring of the lymphocyte subsets (CD2, CD3, CD4, CD5, CD7, CD8,

CD14, CD19 and CD25) confirmed the broad range of T-cell specifities of ATGs (1, 55, 160, 190). Over 85% depletion was shown after the first two weeks of treatment for CD2, CD3, CD4, CD8, CD25, CD56 and CD57 lymphocytes while monocytes underwent less marked depletion and B-cells were almost unaffected (19). ATGs present also functional effects on preactivated T-cells, which may be relevant in their activity on acute cellular rejection. ATG induces Fas-Ligand (Fas-L) (CD95L) and TNFα expression on resting T cells (22). OKT3 shares this property although it is more active on preactivated than on resting T cells (7). Induction of Fas-L expression on resting cells is completely inhibited by Cyclosporine, decreased by corticosteroids but only marginally affected by Rapamycin. Preactivated T-cell blasts as well as NK-cells may also be attacked by ATGs by a mechanism of Antibody-Dependant Cellular Cytotoxicity (ADCC) (134).

In addition to depletion and apoptosis induction, some major functional effects are also achieved by ATGs such as modulation of leukocyte surface antigens (23) and blocking of adhesion molecules (20), both being relevant to transplantation.

Modulation by ATG applies to molecules that control T-cell activation (T-cell receptors CD2, CD3, CD4, CD5, CD6, CD8) and also to molecules involved in leukocyte endothelium interaction such as the ß2 integrins, especially LFA1 (CD11a). Even low concentrations of ATG induce a nearly complete disappearance of LFA1 on monocytes, granulocytes and lymphocytes (91).

Blocking of adhesion molecules at high dosage is another property of ATGs. Important lymphocyte activation molecules such as ß1 and ß2 integrins and even endothelial inflammatory molecules such as ICAM1 are efficiently blocked by ATG (23). This property may reduce one of the most important features of IRI, the deleterious effects of reperfusion in the microvasculature of tissues and solid organs.

These properties have also an outstanding clinical relevance. ATGs are included in many clinical protocols as pre-induction therapy and post-transplantation therapy (12, 31, 78, 102) in spite of their side effects (85, 111, 131, 171). These unique properties of ATG preparations, not achieved by other immunosuppressive agents, make ATG an interesting subject of study as lymphocyte antigens and adhesion molecules play a crucial role in IRI.

AIM OF THE STUDY

Among others, IRI is a major problem in organ transplantation. Polyclonal ATGs are able to block the main cellular features of IRI, including cell activation and release of inflammatory and adhesion molecules. At the time, three different polyclonal ATGs from three companies are available. These drugs do not have a common origin and therefore may possess different molecular and clinical properties. All of them have been raised in rabbits although from different antigens. ATG-Fresenius (S) ® (Fresenius GmbH, Bad Homburg, Germany) is serum from rabbits which have been immunized with the Jurkat cell line while Thymoglobuline® (Imtix-SangStat, California, USA) and Tecelac® (Biotest GmbH, Dreieich, Germany) are developed from rabbit immunization with human thymocytes. Our objective was to test the influence of these three different pATGs on ischemia/reperfusion injury in a non-human primate model as well as to compare their activity. To study the influence of ATGs on IRI, the following parameters were evaluated:

- WBC subpopulations
- Influence on blood parameters
- WBC activation and co-activation
- Tissue infiltration and damage

2. MATERIAL AND METHODS

2.1. Animals

This study was approved by the Bavarian government (N° AZ-211-2531-33/2000). The animals in which our experiments were performed were monkeys, from two different species, cynomolgus monkeys (*Macacca fascicularis*) and baboons (*Papio hamadryas*). The distribution in gender and age between the different monkeys as well as the body weights are given in the table 1.

14 cynomolgus monkeys (*Macacca fascicularis*) and 5 baboons (*Papio hamadryas*) were purchased from two different centres (DPZ [1], ZVM[2]) and housed in the husbandry of the Institute for Surgical Research (ISR) for two days prior to the experiments. Animals were feed with fresh fruit, vegetables and pellets[3]. The animals were handled according to the directives from ISR and the local Bavarian government.

The animals were assigned to four groups according to the ATG used for the perfusion experiments. The blood of all monkeys was of blood group 0.

Species	Number	Male	Female	Age (months)	Weight (Kg)
Cynomolgus	14	8	6	52,57 ± 35,84	4,02 ± 0,85
Baboon	5	3	2	47 ± 27,29	5,38 ± 3,58

Table 1: Distribution of the animals according to species, sex, age and weight.

[1] Deutsches Primatenzentrum , 37077 Göttingen, Germany
[2] Zentrale Versuchstierhaltung der Innenstadt. Klinikum LMU, München, Germany
[3] ssniff Mü Z©; ssniff Versuchstier-Diäten GmbH, Soest, Germany

Species	Biotest	Fresenius	Merieux	Control
Cynomolgus	4	3	4	3
Baboon	2	1	1	1

Table 2: Distribution of the animals according to ATG

2.2. Donors

300 ml of blood were taken from 19 different volunteers 1 hour prior to the begin of the reperfusion. Both male and female donors were accepted. In some cases a second donation was needed and obtained from a different donor. All donors were free from chronic or acute diseases. The blood was collected with a butterfly catheter[4] and stored in 50 ml syringes[5] previously rinsed with heparin[6] in an incubator maintaining a constant temperature of 37°C. The blood of all donors was of blood group 0.

2.3. Design of the Groups

Isolated limbs of the animals were considered as single experiments except those in which perfusion was not possible due to technical reasons. The limbs were classified into eight different groups according to the ATG present in the perfusing blood and the length of the ischemia time.

The polyclonal ATGs employed were:

- Tecelac ®, ATG-Biotest, Biotest GmbH, Germany
- ATG-Fresenius (S)®, Fresenius kabi GmbH, Bad Homburg, Germany
- Thymoglobuline®, ATG-Merieux, Imtix-SangStat, California USA

[4] LEM 21 G-0,80© pfm Produkte für die Medizin; 50996 Köln, Germany
[5] Braun Injekt 50ml B.Braun Melsungen AG, 34209 Melsungen, Germany
[6] Heparin-Natrium Braun 25000 i.e. B.Braun Melsungen AG , Melsungen, Germany

The ischemia times ranged from:

- Short Ischemia (SI): 60 ± 10 minutes
- Long Ischemia (LI): 120 ± 10 minutes

A total of 60 single experiments was performed and divided into groups named according to the ATG used and the ischemia time as follows:

Group	Abbreviation	Number of experiments
Biotest Short Ischemia	BSI	11
Fresenius Short Ischemia	FSI	7
Merieux Short Ischemia	MSI	7
Control Short Ischemia	CSI	9
Biotest Long Ischemia	BLI	9
Fresenius Long Ischemia	FLI	5
Merieux Long Ischemia	MLI	4
Control Long Ischemia	CLI	8

Table 3: Design of the experimental groups.

2.4. Anaesthesia and surgical procedure

The animals were pre-medicated by an intramuscular (i.m.) injection of 15 mg/kg of Ketamine[7], 2 mg/kg of Xylazine[8] and 0,1 mg/kg Atropine[9]. After 10-20 minutes the animals were intubated

[7] Ketavet© Pharmacia & Upjohn GmbH 91058 Erlangen, Germany
[8] Rompun© BayerVital GmbH 51368 Leverkusen, Germany
[9] Atropinsulfat Braun 0,5mg. B.Braun Melsungen AG ,34209 Melsungen, Germany

with the help of an animal laryngoscope[10] using an endotracheal guide[11] and ventilated by means of a ventilator[12]. Ventilator settings were adjusted to obtain normocapnia.

Catheterisation of the internal jugular vein was performed in order to have an access for the intravenous administration of narcotics, analgesics, muscle relaxants and electrolytes. Anaesthesia was maintained by continuous intravenous injection of 0,08 mg/kg/h Fentanyl[13] and 9, 6 mg/kg/h of Midazolam[14]. The internal carotid artery was also catheterised and the catheter connected to a computer[15] by means of a signal transducer[16], controlling heart rate and systolic as well as diastolic pressure. The limbs of the animal were carefully shaved and disinfected with an external antiseptic[17]. The animals were then fixed in supine position on a special vacuum pouch.

The surgical procedure consisted of isolating the main arterial and venous vessels of both arms and legs so that we could perfuse each of them apart from the main body circulation. This operation was performed first in the right inferior extremity and then in the left one. A longitudinal incision in the inguinal flexure along the sartorious muscle after trespassing the muscle fascia was performed, allowing us to dissect the adjacent tissues to the common femoral artery before the division of the lateral femoral artery. Catheterisation of both arterial and venous vessels with plastic catheters as well as flushing of the limb with Ringer's Lactate of a temperature of 4°C were performed, draining the monkey's blood out of the limb and inducing cold ischemia.

To reach the arm vessels we started with a transversal incision in the axilar region, following the opposite order as in the lower extremities (first left arm, then right arm). Once located, the brachial artery was fixed with two threads and the axillary vein was isolated. After having

[10] Intubationssysteme Draeger, Germany
[11] Safety-Flex© Mallinckrodt Medical; Athione, Ireland.
[12] 100-IVB Infant Ventilator; Sechrist Industries.Inc, Anaheim, USA
[13] Fentanyl© Janssen GmbH, Neuss, Germany
[14] Dormicum© H. La Roche AG, Grenzach Whylen, Germany
[15] AMD 486. Digital GmbH, Germany
[16] Plugsys©, HugoSachs Elektronik. March-Hugstetten, 79232 Germany
[17] Kodan©, Shülke&Mair GmbH, Norderstedt, Germany

dissected both vessels we ligated them and catheterised them with plastic catheters of 0,9 mm to 1,1 mm diameter. The arm was then flushed with Ringer's Lactate[18] of 4°C temperature via the artery. Our purpose was not only to drain the monkey arm's blood but also to minimise the reperfusion injury by means of reducing the time of warm ischemia and to study the effect of different ischemia times in the tissues .

A plastic string[19] placed close to the hip respectively shoulder joint was used for compression in order to prevent reflux of the perfusing human blood into the systemic circulation. Blood gases as well as cell counts were evaluated in arterial blood samples from the animal during the surgical preparation.

2.5. Preparation of human blood

The haematocrit of the blood used to perfuse the monkey limbs was adjusted to a value of 30% ($\pm$ 2%) by adding Krebs-Henseleit-buffer[20] (144). This facilitates the perfusion by decreasing the viscosity of the blood without jeopardising tissue oxygenation (194). For intravital microscopy (IVM), plasma was stained with sodium fluoresceine[21] and the leucocytes with Rhodamine 6-G (0,096 mg/ml)[22] (97), added to the blood prior to the reperfusion.

The three different ATGs[23] were added to the human blood 30 minutes before the perfusion with a standard dosage of 1ml/kg. The human blood was perfused at a constant temperature of 37 C° into the animal limbs through the arterial vessel with the help of a perfusion system that will be explained in further chapters.

[18] Ringer's-Lactat-Infusionslösung; B.Braun Melsungen AG, 34209 Melsungen, Germany
[19] ISR LMU. München, Germany
[20] ISR-LMU; München, Germany
[21] Fluoresceinisothiocyanate Isomer I, Sigma Chemical Co., St. Louis, USA
[22] Rhodamin© Merck, Darmstadt, Germany
[23] ATG-Biotest, Biotest GmbH, Dreieich, Germany
ATG-Fresenius, Fresenius kabi GmbH, Bad Homburg, Germany
ATG-Merieux, Imtix Sangstat, California, USA

2.6. Perfusion system

The perfusion system (Fig. 1) (194) consisted of a custom made steel blood reservoir[24] with a volume of 500 ml. The blood was pumped from the reservoir into a custom-made aluminium oxygenator[25] by means of a roller pump[26]. Both reservoir and oxygenator were equipped with a heating system[27], allowing maintenance of a constant blood temperature of 37 °C (194). A computer[28] was linked to the system via either a pressure transducer[29] or a flow probe[30], allowing to adjust both parameters. The human blood flowed through a hemo-filter (pore size: 60µm.)[31] and a bubble trap [32] and was finally directed to the isolated arterial vessel of the limb. After circulation through the limb, the blood was drained from the vein and re-directed to the reservoir.

2.7. Intravital Microscopy

Intravital microscopy (IVM) was performed to investigate the microcirculatory parameters and endothelial-leukocyte interaction. For this purpose we stained the plasma with sodium fluoresceine and the leukocytes with Rhodamine 6-G before placing the human blood into the system. Heparin served as anticoagulant.

A capillary window was opened in the surface of the elbow for the upper limbs and below the knee for the lower. During perfusion, microcirculation of these capillary windows was directly investigated using a fluorescence intravital microscope[33] with epi-illumination and different filter blocks[34]. The IVM investigation is the main subject of the doctoral thesis of D. Chappell. The

[24] ISR-LMU, München, Germany
[25] ISR-LMU, München, Germany
[26] IPS,Ismatec SA, Zürich, Switzerland
[27] ISR-LMU, München, Germany
[28] AMD-486DX,Digital GmbH, München, Germany
[29] COMP DT-Xx, Ohmeda, Murray-Hill, NJ, USA.
[30] Transonic Flow Probe, Transonic Systems Inc. Ithaca, NY, USA
[31] Swinnex 25,Milipore,Eschborn, Germany
[32] Becton Dickinson, Heidelberg, Germany
[33] Orthoplan©; Leitz GmbH; Wetzlar, Germany
[34] Fa. Leitz, München, Germany

microscopic pictures were transferred via a CCD-camera[35] to a video monitor[36] and recorded with a SVHS video recording unit[37]. The system settings are illustrated in figure number 1.

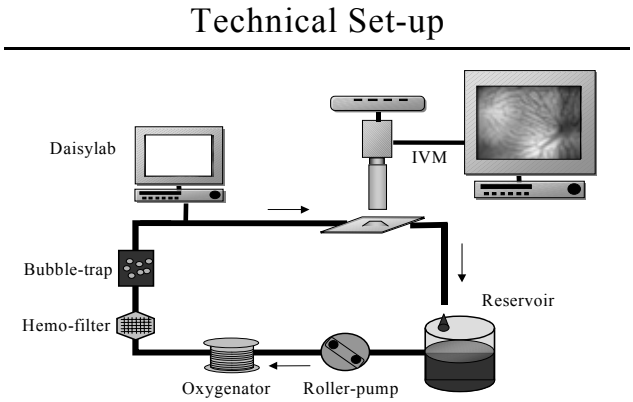


Figure 1: Perfusion system (with permission, Xenotransplantation 2001 8:95)

2.8. Blood samples. Blood parameters

2.8.1. Monitoring of the animal

Blood samples were collected during the two different phases of our experiments. During the surgical procedure, 2 ml blood samples were taken with the help of a 2 ml syringe[38] from the carotid artery at the time points 0, 15, 30, 45, 60, 90, 120 minutes. These samples were transported in Eppendorf cups[39] and analysed with a Cell Counter[40] to measure the following blood parameters during the surgical procedure: leukocytes, erythrocytes, platelets, haematocrit and haemoglobin. The same blood samples were used at the time points 0, 30 and 60 minutes to measure the PO_2, PCO_2, pH and HCO_3 levels with the help of a gas analyser. Additional samples were collected in order to measure gas levels when needed to control the state of the animal.

[35] C2400-08,Fa.Hamamatsu Photonics,Herrsching, Germany
[36]Wv-5470, Panasonic, München, Germany
[37] AG-7350, Panasonic, München, Germany
[38] Braun Injekt 2ml B.Braun Melsungen AG, 34209 Melsungen, Germany
[39] Sarstedt, 51588 Nümbrecht, Germany
[40] Coulter© Counter Ac T8, Coulter Electronics. Ltd , Luton, UK

Blood samples were collected from the donated blood before and after the blood was diluted to a haematocrit of 30% and the parameters mentioned above were also measured. These blood samples were named –1 for the donor blood and 0 for the diluted blood.

2.8.2. Blood samples during the perfusion

During the perfusion of the limbs, blood samples were collected from the perfusion system. 2 ml arterial blood samples were taken at the time points 1, 5, 10, 15, 30, 45 and 60 minutes after onset of reperfusion to measure blood parameters of the perfusate, storing these 14 ml (2 ml x 7 time points) of blood to perform cytological and immunocytochemical studies. At the time points 1, 15, 30, 45 and 60 minutes 2 ml of blood were collected to measure the oxygenation level of the perfusate.

Blood parameters studied in our experiments were:

- Erythrocytes (RBC) : Expressed in $x10^6/\mu L$
- Leukocytes (WBC) : Expressed in $x10^3/\mu L$
- Platelets (Plat) : Expressed in $x10^3/\mu L$
- Haematocrit (Hct) : Expressed in %
- Haemoglobin (Hb) : Expressed in mg/dl

2.9. Smears

Blood smears were performed to evaluate and quantify the presence and morphology of myeloid and erythroid cells in whole blood. Blood was anticoagulated with heparin. One drop of well mixed blood was spread over a glass slide[41], dried and stained by hand or automatically with:

- May-Grünwald solution[42] for 3-4 minutes
- Aqua dest. rinsing[43] 2 minutes

[41] Menzel-Glaser GmbH, München, Germany
[42] ISR-LMU, München, Germany

- Giemsa solution[44] (1/20) for 15-20 minutes
- Aqua dest. rinsing 2 minutes

The dyes were prepared freshly and filtered daily. After drying, the slides were covered with a plastic slide[45]. A general view for rough information was performed using a x100 magnification, while x400-x600 magnifications were used to evaluate preparations with a light microscope[46].

2.10. Cyto-immunological Monitoring (CIM)

CIM is a non invasive and non traumatic method for diagnosing inflammatory events in the postoperative phase of transplantation. CIM is based on the observation that lymphocytes as well as their activated forms disseminate from the rejecting graft modulating the recipient's lymphoid organs (64).

CIM consists of two tests. The first of them is a fast cytological differentiation of white blood cells separated from the peripheral blood that inform roughly about inflammatory events. This test should be used when there is no clinical sign of rejection or inflammation. The second test is called extended test and consists of the differentiation of mononuclear cell subpopulations by immunological methods and should be applied immediately in case of clinical signs of acute rejection or infection. We performed CIM as a reliable method to establish the diminution and activation of these cell populations.

After the smear was prepared, the rest of the blood was separated over a Ficoll-Hypaque gradient of a density of d=1,077 according to the method of Böyum (64). 0,5 ml of blood mixed with the

[43] B.Braun Melsungen AG 34209 Melsungen, Germany
[44] ISR-LMU, München, Germany
[45] Dako GmbH, Hamburg, Germany
[46] Carl Zeiss,37030 Göttingen, Germany

same amount of PBS[47] were carefully layered over 0,5 ml of Ficoll. Every micro tube[48] (1,5 ml) was spun at 3200 rpm for 2 minutes in a centrifuge[49] and the interphase ring consisting of mononuclear cells was transferred to a new microtube and washed two times with PBS. The mononuclear concentrate was divided into the machine cuvettes[50] and centrifuged for 5 minutes at 500 rpm. After this procedure the cytopreps were dried and stained following the May-Grünwald-Giemsa technique or frozen for immunoperoxidase staining and further immunohistochemical studies.

Cytological slides of the mononuclear concentrate were studied according to a fixed schedule of microscope magnification. A general view for rough information was performed using x100 magnification, while x400-x600 were used to evaluate preparations with no signs of activation. In case of cell activation x1000 magnification was employed. Evaluation was performed with the help of a light microscope. All immature and polymorphonuclear cells were taken into consideration.

2.11. Biopsies

Biopsy material was obtained from muscle and connective tissue from the limbs of the monkeys at the end of the experiment. The biopsies were snap-frozen in liquid nitrogen[51] at a temperature of –192 C° and stored at –80 C° in an industrial fridge[52] for later immunohistological investigation. Other specimens were stored in 8% formaline[53] for 48 to 72 hours and fixed in

[47] Biochrom KG, D-1000 Berlin, Germany
[48] Nunc Cryo Tube© vials, Nalge nunc International, Denmark
[49] Centrifuge 5415 C©, Eppendorf GmbH, 23331 Hamburg, Germany
[50] Eppendorf GmbH, 23331 Hamburg, Germany
[51] Messer Griesheim, 57555 Euteneuen, Germany
[52] Colora UF85-4605©, Colora MessTechnik, Germany
[53] Apotheke Klinikum Großhadern-LMU, München, Germany

paraffin[54] for later immunohistochemical and histological studies. The biopsies were assigned to eight groups according to the ATG employed and the ischemia duration.

Histological sections of 6 µm were cut from the paraffin-embedded tissue, dehydrated and fixed in xylol[55] for later immunohistochemical studies.

The frozen biopsies were cut in 4-µm to 7-µm cryostat[56] sections at a temperature of –15 C°, air-dried and fixed in acetone[57] for 10 minutes. H/E was performed in one of every ten slices to check the adequate orientation of the preparation.

Group	Frozen Biopsies	Paraffin blocks
BSI	20	20
FSI	14	14
MSI	12	12
CSI	10	10
BLI	16	16
FLI	10	10
MLI	8	8
CLI	8	8

Table 4: Distribution of the biopsies within study groups according to the technique employed.

2.12. Histological and immunohistochemical techniques

Histological and immunohistochemical techniques were performed to evaluate the state of the WBC subpopulations, the infiltrate associated to IRI and the cytotoxic effect of the drugs employed. Several techniques were used in order to localise the different types of cells, vessels

[54] Dako GmbH,Hamburg, Deutschland
[55] Apotheke Klinikum Großhadern-LMU, München, Germany
[56] Microm HM 560, Microm GmbH, 69190 Waldorf, Germany
[57] Apotheke Klinikum Großhadern-LMU, München, Germany

and muscle fibres. It was also important to determine the localisation of the cell subsets related to the duration of ischemia. These different histological techniques are described as follows:

- Haematoxylin / Eosin (H/E):

The H/E staining is one of the most often performed histological techniques due to the great cellular details obtained by means of a simple procedure. Haematoxylin stains the nuclei of the cells with a dark blue colour. Eosin stains the muscle fibres and fibrin with red and the cytoplasm of cells with a pale pink colour. Haematoxylin is a cationic colorant while Eosin is an anionic colorant belonging to the xanthenes family.

The preparations were introduced for four minutes in Haematoxylin[58], washed with PBS and introduced in Eosin[59] (solution 1%) during 1-2 minutes. After washing with ethanol[60] 70% to eliminate the rests of Eosin, the slices were covered with a plastic or glass slide, fixed with balsam or synthetic glue[61].

- Masson's Thrichrome:

This technique allows us to differentiate muscle fibres, cell nuclei, fibrin and collagen. Nuclei, centrosomes and secreting granules are dyed black, muscle fibres red and collagen blue. The preparations are immersed in Eosin solution 30 minutes after deparaffination, washed with aqua dest.[62], immersed for 30 minutes in a Haematoxylin solution and again washed with aqua dest. After that, differentiation in alcohol is performed during 10-30 minutes and the preparations are

[58] Apotheke Klinikum Großhadern-LMU, München, Germany
[59] Apotheke Klinikum Großhadern-LMU, München, Germany
[60] Apotheke Klinikum Großhadern-LMU, München, Germany
[61] Dako Diagnostika GmbH,22047 Hamburg, Germany
[62] Apotheke Klinikum Großhadern-LMU, München, Germany

stained with fuchsine[63] for 5 minutes, washed with aqua dest. and covered with a plastic or glass slide. The histological slides are then mounted and fixed with balsam or synthetic glue.

- Granulocyte Esterase (GE):

This technique allows differentiation of granulocytes from other WBC. Granulocytes are stained in a dark violet colour with black nuclei in contrast to no positive staining of lymphocytes and monocytes.

The preparations are immersed for 30 seconds in formaldehyde solution[64], afterwards washed with aqua dest., then immersed shielded from light during 30 minutes in ASDCL solution[65] (Fast-Red violet[66] + Sodium Nitrite + Buffer + Chloroacetate) , washed with aqua dest., stained for two minutes with simple Mayer´s Haematoxylin solution, washed with normal water, then washed with aqua dest. and finally air dried and covered with a plastic slide.

- CD45 (LCA) :

The primary antibody anti-CD45 is also called Leukocyte Common Antigen (LCA) due to its capacity to stain all cell subsets of WBC by staining the tyrosin-phosphatase present in signal transduction of these cells. We were interested in demonstrating the presence or absence of WBC after the perfusion with or without ATG. We performed this immunohistochemical staining according to standard rules, described in continuation, using an anti-human monoclonal antibody from mouse[67] .

[63] Apotheke Klinikum Großhadern-LMU, München, Germany
[64] Apotheke Klinikum Großhadern-LMU, München, Germany
[65] Apotheke Klinikum Großhadern-LMU, München, Germany
[66] Dako Diagnostika GmbH,22047 Hamburg, Germany
[67] Dako Diagnostika GmbH,22047 Hamburg, Germany

The immunohistochemical procedure was performed in 4 nm cryostat sections, air dried and fixed in acetone[68] for 10 minutes. After incubation with human serum in phosphate-buffered saline (PBS)[69] for 10 minutes, the sections were incubated with 100 ml of 1:100 and 1:250 dilutions, in PBS, of the primary antibody overnight at 4 C°. The secondary reaction was performed according to Hsu *et al.*(77) with an ABC complex[70] for indirect immunostaining. The immunoreaction was developed with 3; 3′-diaminobenzidine tetrachloride[71] (DAB) and H_2O_2[72] to prevent cross reactions with endogenous peroxidase, dehydrated and mounted in a glass mountant[73].

- Interleukin 4 (IL-4)

Interleukin 4 (IL-4, B-cell growth factor-1, BSF-1) is a T-cell derived cytokine that plays an important role in the activation of resting B-cells, being irreplaceable in the process of activation of Th2 lymphocytes.The same procedure as explained for CD45 was applied in this case, using an anti-human monoclonal antibody[74] against interleukin 4. Immunostaining of IL-4 was performed to determine whether ATG had an influence on the cellular expression and release of this cytokine and subsequently on the activation of helper lymphocytes.

- Thrombocytes and Endothelial Cells (CD31) :

Thrombocytes were stained with indirect immunoperoxidase to localise their presence, aggregation and participation in thrombus formation. Immunostaining was done with an anti-human monoclonal antibody for CD31[75].

[68] Apotheke Klinikum Großhadern-LMU, München, Germany
[69] Apotheke Klinikum Großhadern-LMU, München, Germany
[70] Sigma GmbH, Hamburg, Germany
[71] Dako Diagnostika GmbH,22047 Hamburg, Germany
[72] Apotheke Klinikum Großhadern-LMU, München, Germany
[73] Dako Diagnostika GmbH,22047 Hamburg, Germany
[74] R&D Systems, Waldorf, Germany
[75] Dako Diagnostika GmbH,22047 Hamburg, Germany

The immunohistochemical procedure was performed in 4-7 µm paraffin sections, air dried, and fixed in acetone for 10 minutes. After incubation with human serum in phosphate-buffered saline (PBS) for 10 minutes, the sections were incubated with 100 ml of a 1:10 dilution, in PBS, of the primary antibody at 4°C overnight. The secondary reaction was performed with the streptavidin-biotin LSAB©[76] complex for indirect immunostaining. The immunoreaction was developed with 3; 3´-diaminobenzidine tetrachloride and H_2O_2 to prevent cross reactions with endogenous peroxidase, dehydrated and mounted in a glass mountant.

- Fibrin:

Fibrin was stained according to Weiger's technique. This method allows to differentiate fibrin (homogeneous, brilliant pink) from muscle fibres (red). After deparaffination the slices were fixed in Chrome[77] and treated with permanganate[78] . The nuclei were stained with Lithium Carmine[79], washed in aqua dest., drained in filter paper[80], stained with Gentian violet[81] for 15-20 seconds, drained in filter paper, stained with Lugol solution[82] for 15-20 seconds, drained in filter paper, differentiated with Aniline Oil[83] and covered with a plastic or glass slide.

2.13. Histological evaluation

Semiquantitative evaluation of the histological section was done in seven tissue fields per biopsy, chosen by coincidence. Different criteria of evaluation were considered according to the staining performed. This method is extensively described in the literature (17, 38, 74, 149). We have

[76] Dako Diagnostika GmbH,22047 Hamburg, Germany
[77] Apotheke Klinikum Großhadern-LMU, München, Germany
[78] Apotheke Klinikum Großhadern-LMU, München, Germany
[79] Apotheke Klinikum Großhadern-LMU, München, Germany
[80] Dako Diagnostika GmbH,22047 Hamburg, Germany
[81] Apotheke Klinikum Großhadern-LMU, München, Germany
[82] Apotheke Klinikum Großhadern-LMU, München, Germany
[83] Apotheke Klinikum Großhadern-LMU, München, Germany

modified the one described by Koo *et a*. (95). The following criteria were established for the histological evaluation:

- Muscle damage: loss of architecture, necrosis or signs of ischemia were considered:
    - normal architecture: 0
    - light damage: 1
    - moderate damage: 2
    - severe damage: 3
- Soft tissue infiltration: Connective tissues present in the biopsy as well as connective perimisial structures were considered.
    - any WBC in these structures: 1
    - more than 10 cells per field: 2
    - more than 25 cells per field: 3
- Vascular infiltration: Cells present in the vascular spaces were considered, free or attached to the endothelial walls.
    - free cells in the vessels: 1
    - free and attached to the endothelial cells: 2
    - free cells, attached cells, granulocytes, occluding or almost occluding the vessel lumen: 3
- Perivascular infiltration: Presence of WBC in any tissue adjacent to the vessels:
    - presence of cells attached to endothelial walls: 1
    - presence of cells in perivascular connective tissue: 2
    - presence of cells in perivascular connective tissue and muscular tissue:3

- Muscular infiltration:
    - no cells in muscle fibres: 0
    - 1-5 cells pro muscular fibre: 1
    - >10 cells pro muscular fibre: 2
    - >25 cells pro muscular fibre: 3

Results are expressed as the mean values obtained from the different fields studied. The preparations were labelled with a code and evaluated blindly.

Fibrin, Masson's T and CD31 immunostaining were performed to localise fibrin and thrombus formation and the criteria were established as follows:

- Presence of fibrin or thrombocytes in the vascular spaces:
    - presence of fibrin or thrombocytes without occlusion: 1
    - occlusion of the vessel: 2
    - occlusion of the vessel and destruction of the vascular or endothelial integrity: 3

A fixed number of vessels, n=7, were studied blindly per section. The results are expressed as the mean of the sections studied.

The immunoreaction with IL4 was classified as positive or negative depending on the presence or absence of this molecule in the sections studied.

2.13. Statistical analysis

Statistical descriptive analysis of the results was performed with Excel[84]. Data are presented as median $\pm$ standard deviation. The analytical studies were performed with Excel and S-plus

[84] Office XP, Microsoft©, NJ, USA

statistical programmes[85]. The method employed was Analysis of Variance (ANOVA) for different variables. The variable "time of perfusion" was studied with Excel. After ANOVA, Tukey's test for normally distributed values and Dunnet's or Dunn's tests for non normally distributed values were used for posterior multiple comparisons. The variables studied with S-plus were "drug" and "time of ischemia". Scheffe´s test was employed "a posteriori" when statistical significances were detected between the groups for the variables "time of ischemia" and "drug". We used Scheffe´s test as we needed tests appropriate for multiple comparisons. Multiple comparison tests are characterised by considering the number of tests that could be made. Scheffe´s test is a valid, fairly conservative test, sufficiently generalised to be applicable to unequal designs. Applying this test, all possible contrasts can be tested for significance, or confidence intervals constructed for corresponding linear functions of parameters (184).

The analytical graphics were designed applying the smooth/splining technique to the previously mentioned statistical tests. Nonparametric function estimation with stochastic data, otherwise known as smoothing, has been largely studied (32). Smoothing spline ANOVA models are a versatile family of smoothing methods that are suitable for both univariate and multivariate problems. This method allows constructing multivariate models with ANOVA. Statistical significance was accepted when p<0, 05.

[85] S-plus ©, Microsoft©, NJ, USA

3. RESULTS

3.1 Blood Parameters

Median and standard deviation values of the following parameters: WBC, RBC, platelets, haematocrit and haemoglobin for every time point are presented in the following pages. Statistical analysis to investigate the differences between groups was performed. Time point 0 (blood diluted to a haematocrit of 30%) was taken as control for the study of "time of perfusion" as variable.

3.1.1. WBC

- Short Ischemia: BSI-FSI-MSI-CSI

The number of circulating WBC in PB of the CSI showed no decrease. In contrast to this, the number of WBC was significantly decreased during reperfusion in the FSI group ($p<0.05$; Tukey test). Decreases of the number of WBC of BSI and MSI showed no statistical significance.

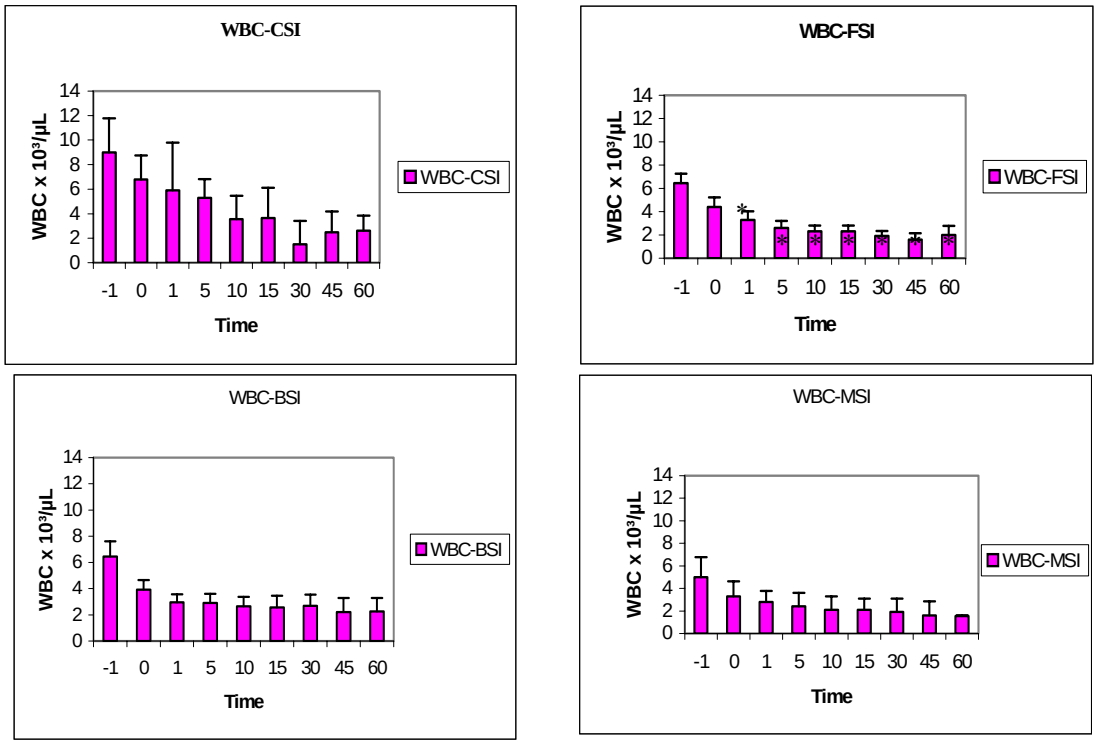


*Fig. 2: WBC-Short Ischemia: Number of WBC in peripheral blood during the reperfusion in SI time. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

- Long Ischemia: CLI, FLI, BLI, MLI

The number of circulating WBC in PB of the CLI showed no significant decrease. In contrast to this, the number of WBC was significantly decreased during reperfusion in the FLI group ($p<0,05$; Tukey test). Decreases of the number of WBC of MLI showed no statistical significance (figure 3). However, reperfusion in the BLI group showed a significant decrease in the number of WBC after the 15 minute ($p<0,05$, Tukey test).

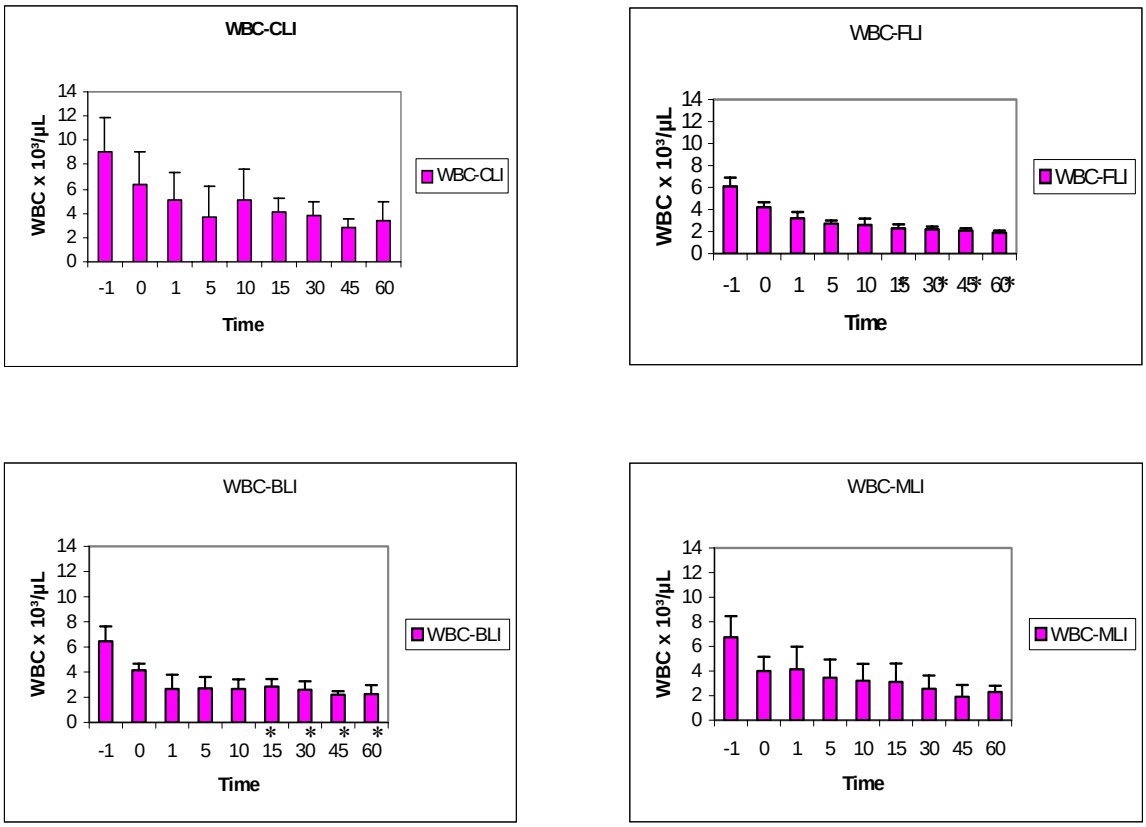


*Fig. 3: WBC-Long Ischemia: Number of WBC in peripheral blood during the reperfusion in LI time. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

Further statistical tests with ANOVA were performed between groups according to the length of the ischemia time and the drug employed. Differences between the groups were studied. Statistically significant differences were found:

- Drug: $p < 0.001$

- Ischemia Time: p = 0.056

Scheffe´s test ($p < 0.05$) was applied "a posteriori" to the drug and the ischemia time variables to determine which groups presented differences between them according to the following factors:

- The number of circulating WBC in the three ATG groups was statistically lower than the number of WBC in the control group both for SI and LI times ($p<0,05$).
- Applying the test to the ischemia time, only the group Merieux presented significant differences between short time and long time of ischemia, showing a higher number of WBC in MLI than in MSI ($p<0,05$).

These significances can be expressed graphically through smoothing-spline of ANOVA and Scheffe´s test, allowing one to create a theoretical model of the variable in relation to the groups studied.

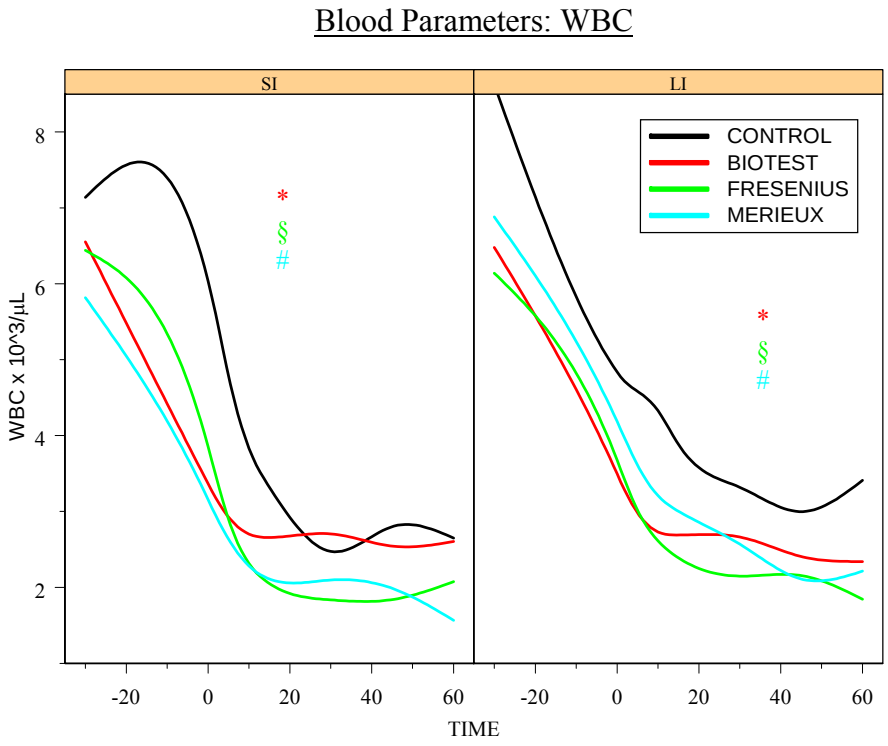


*Fig. 4: Smoothing-Spline of WBC: The number of circulating WBC in the three ATG groups was statistically lower than the number of WBC in the control group both in SI and LI times (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0,05).*

Fig. 5: Smoothing-Spline of WBC: Only Merieux-ATG groups experienced statistically significant differences between long and short ischemia in the number of WBC, these being significantly higher in LI times (# Merieux LI vs. Merieux SI: p< 0,05). The other groups, including control, showed no statistical significance.

3.1.2 RBC

- Short Ischemia: BSI-FSI-MSI-CSI

Analysis of the circulating number of RBC during perfusion with human blood treated with a standard dosage of ATG (1mg/kg) after a short ischemia period showed the following results. CSI, MSI and FSI groups presented no significant variation of the number of RBC during the reperfusion. BSI group, however, showed a significant increase of the number of circulating RBC at the end of the reperfusion (p< 0,05, Dunn's test).

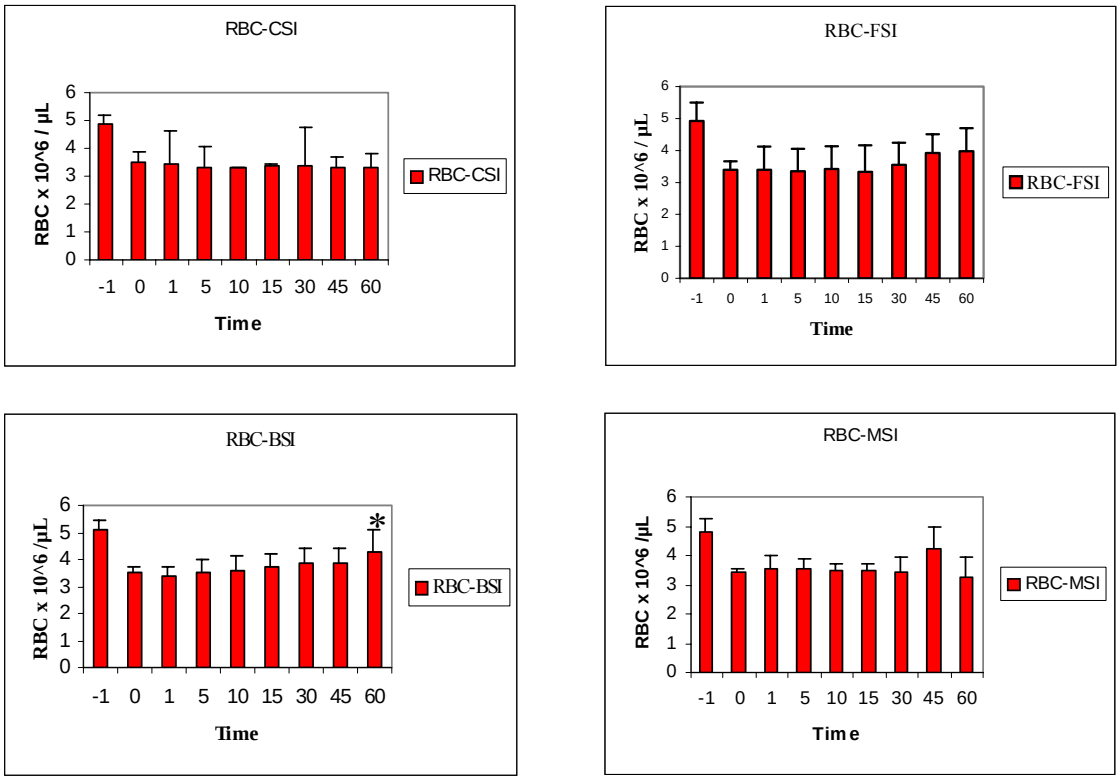
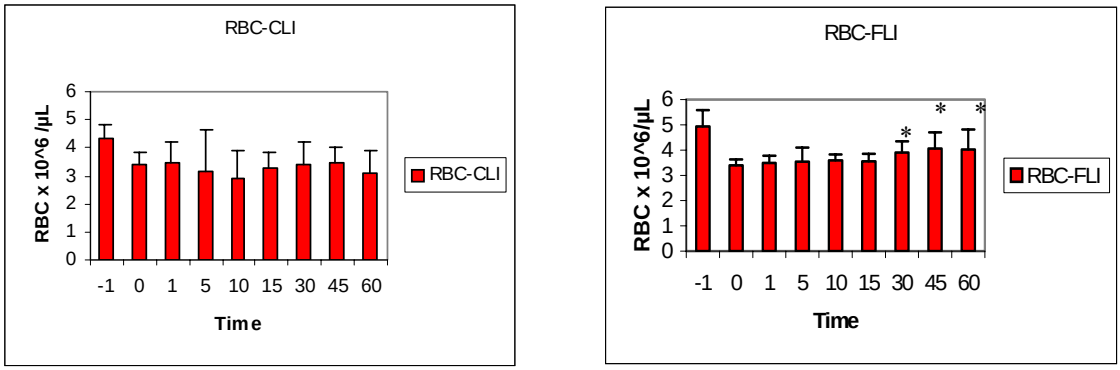


*Fig. 6: RBC-Short Ischemia: Number of RBC in peripheral blood during the reperfusion in SI time. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

- Long Ischemia: CLI, FLI, BLI, MLI

The number of RBC after long ischemia showed the following results. BLI and FLI groups show an increasing tendency (p< 0,05, Tukey's test) while the values of erythrocytes in the control and MLI group present no statistically significant difference between the time points of the reperfusion.



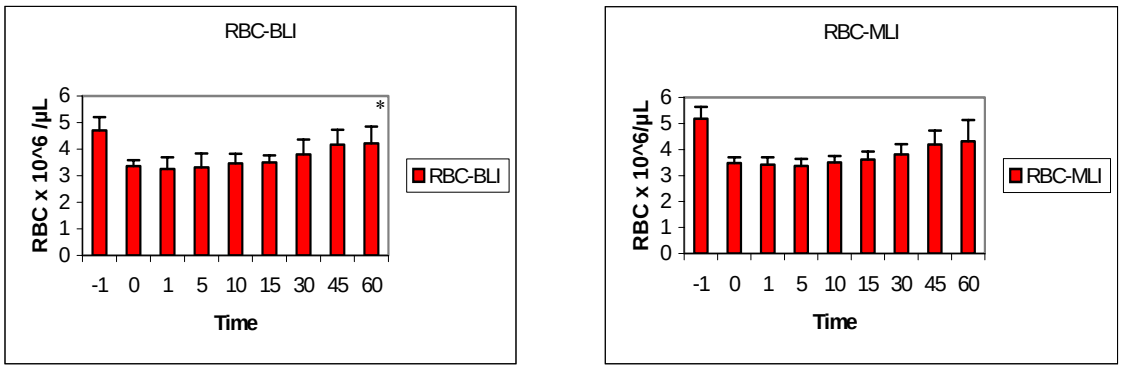


*Fig. 7: RBC Long Ischemia: Number of RBC in peripheral blood during the reperfusion in LI time. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

Comparisons between ATG groups and controls were performed as well as comparison between each ATG group. The circulating number of RBC showed statistical differences for the next variables:

- Drug: $p < 0.001$
- Ischemia Time: No significance was demonstrated

We applied the Scheffe´s test to the drug variable to determine differences between ATG groups and control in relation to RBC. All three ATG groups presented statistically significant higher values of RBC during the reperfusion time with a $p < 0.05$. No further studies were performed taking "ischemia time" as variable, because no significant differences were found in the general ANOVA test.

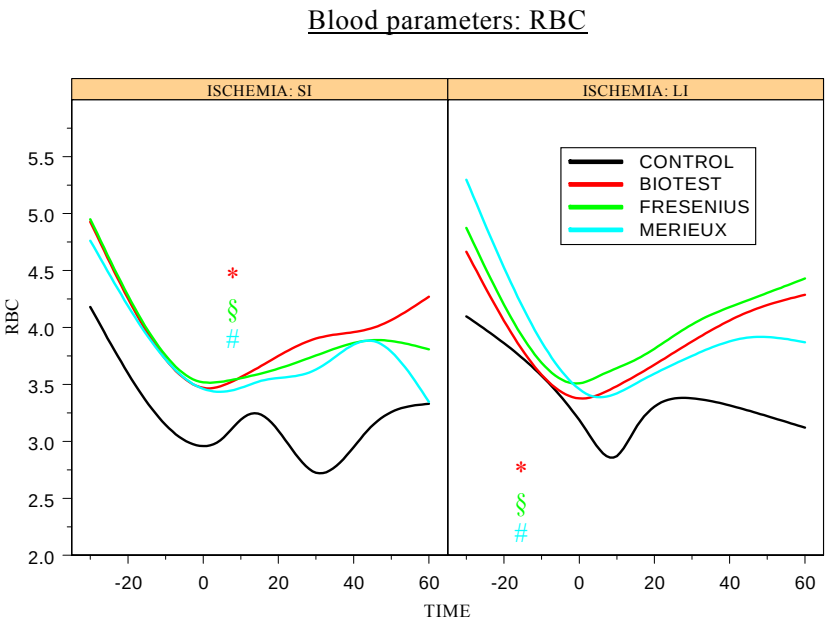


*Fig. 8: Smoothing-Spline of RBC for SI and LI: The number of RBC in the three ATG groups was significantly higher than the number of RBC in the control group both in SI and LI ischemia times (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0,05).*

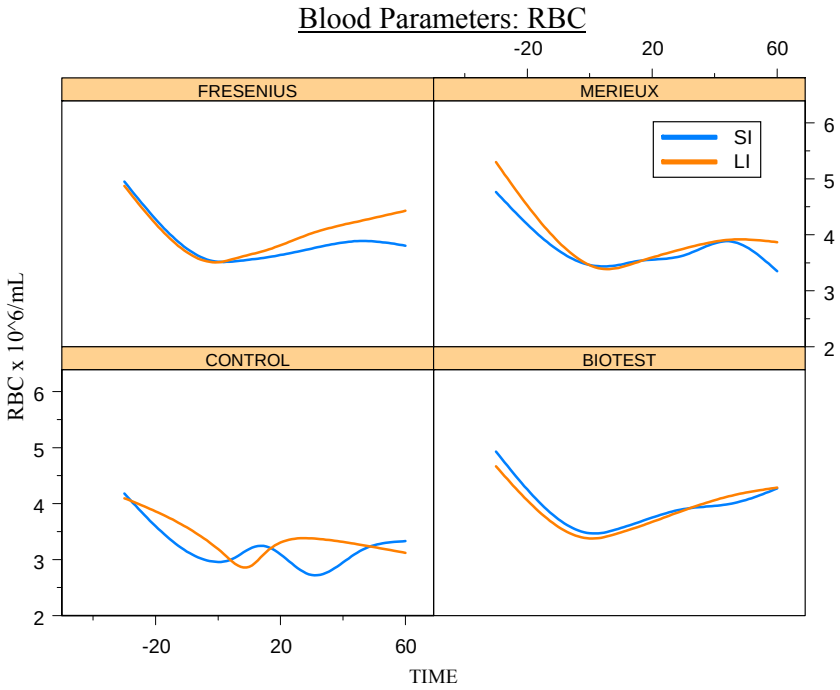


Fig. 9: Smoothing-Spline of RBC for SI and LI: No differences according the ischemia time were found within the groups.

3.1.3. Platelets

- Short Ischemia: BSI-FSI-MSI-CSI

Analysis of the number of circulating platelets in peripheral blood throughout reperfusion with human blood after short ischemia showed the following results. In the short ischemia groups only the BSI group showed a statistically significant decrease of the number of platelets during the reperfusion ($p < 0,05$, Tukey's test).

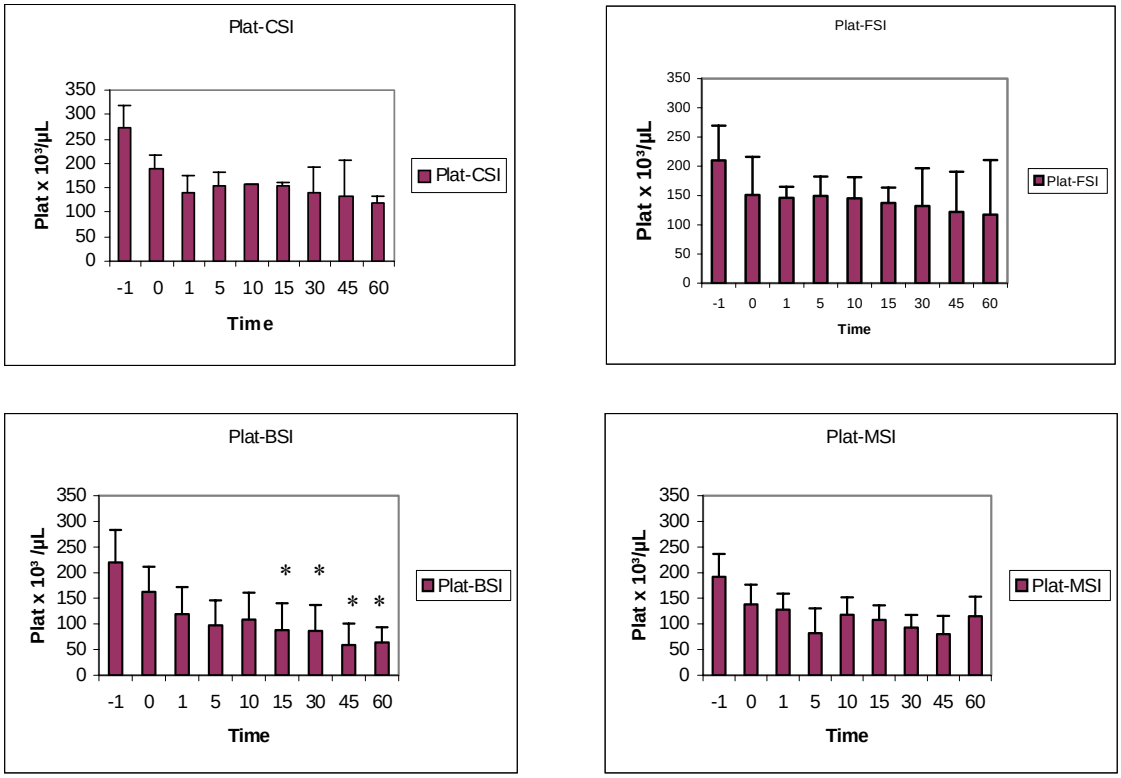


*Fig. 10: Thrombocytes-Short Ischemia: Absolute numbers of platelets in PB during reperfusion after SI time. Observe the decrease of the number of thrombocytes in the Biotest-ATG group (BSI). Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

- Long Ischemia: CLI, FLI, BLI, MLI

Analysis of the number of circulating thrombocytes after long ischemia showed the following results. BLI and MLI presented a significant decrease of the number of platelets during the

reperfusion (p< 0,05, Tukey's test). In contrast to this, FLI and CLI showed no significant differences.

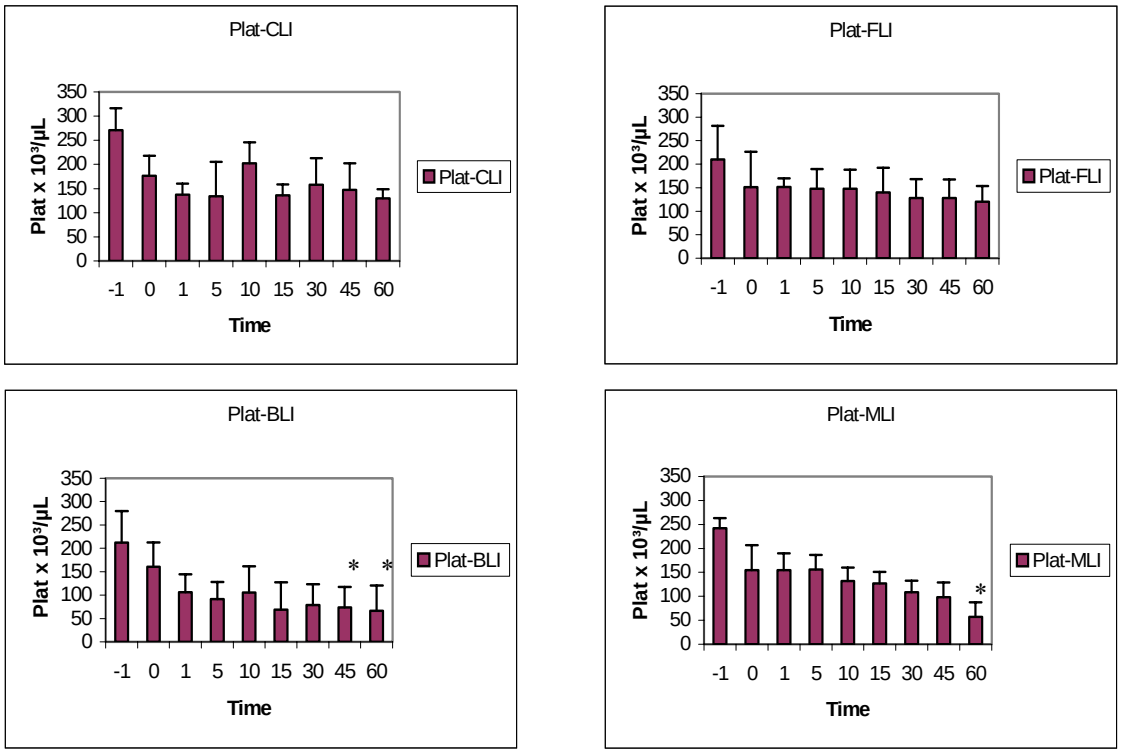


*Fig. 11: Thrombocytes-Long Ischemia: Absolute numbers of platelets in PB during the reperfusion after LI time. Observe the decrease of the number of thrombocytes in the Biotest-ATG group (BSI). Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

The number of peripheral thrombocytes showed differences between the ATG groups and control as well as differences within the ATG groups. Variable "drug" showed a statistical significance of p< 0,001, while "ischemia time" showed no general significance. Scheffe´s test was applied to "drug" and "ischemia time" variables to assess further differences within the ATG and the control groups. According to the ATG added, the results were as follows: The numbers of platelets remained at values between 100 and 250 x 10^3/µL for both the control groups and the Fresenius groups. The numbers of platelets for the Merieux and the Biotest groups decreased. The statistical significance accepted was p< 0, 05. Control showed a statistically significant

higher number of platelets when compared with Biotest and Merieux groups for both long and short ischemia times.

Applying the Scheffe´s test to the ischemia time, only the Merieux ATG group showed a significant difference on the number of peripheral thrombocytes with different ischemia times. The number of thrombocytes was higher in the MLI group. Control and the other two ATG groups showed no significant difference. However, the ischemia time as an independent variable has an influence when comparing the different drugs in short other long time of ischemia. ATG-Fresenius presents no statistical difference in number of platelets when compared to the Merieux group in LI Time, although this difference exists in the SI time. By means of smoothing-spline a multivariate model of the number of platelets under these conditions was designed (Figs. 12, 13).

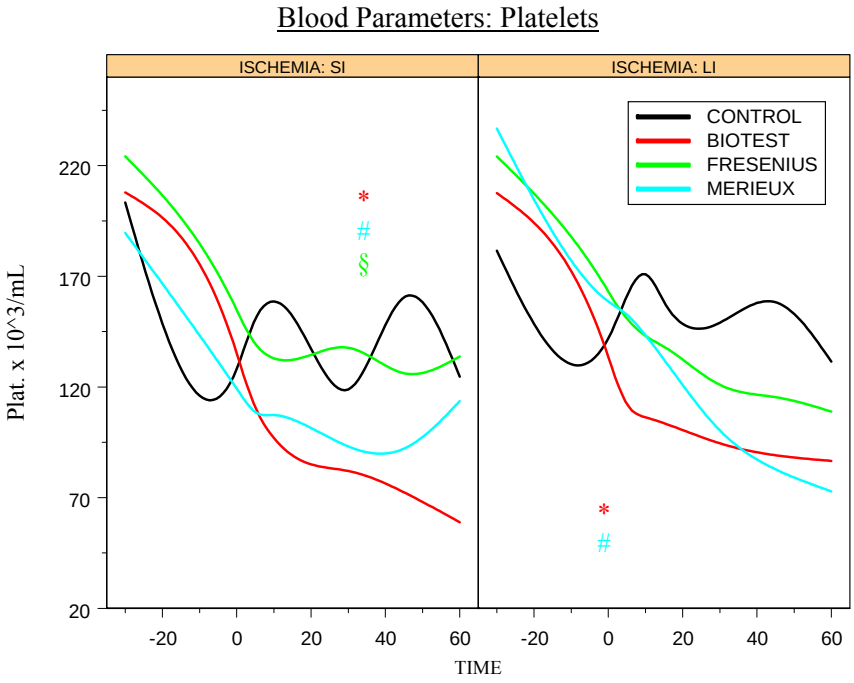


*Fig. 12: Smoothing-Spline of Thrombocytes for SI and LI : The number of platelets in the control group was significantly higher than the number of platelets in the Biotest-ATG and Merieux-ATG groups both for short and long ischemia times. Number of platelets of the Fresenius-ATG group was significantly higher than the number of thrombocytes of the Merieux-ATG group (*Control vs. Biotest-ATG; # Control vs. Merieux-ATG; § Fresenius-ATG vs. Merieux-ATG: p<0, 05).*

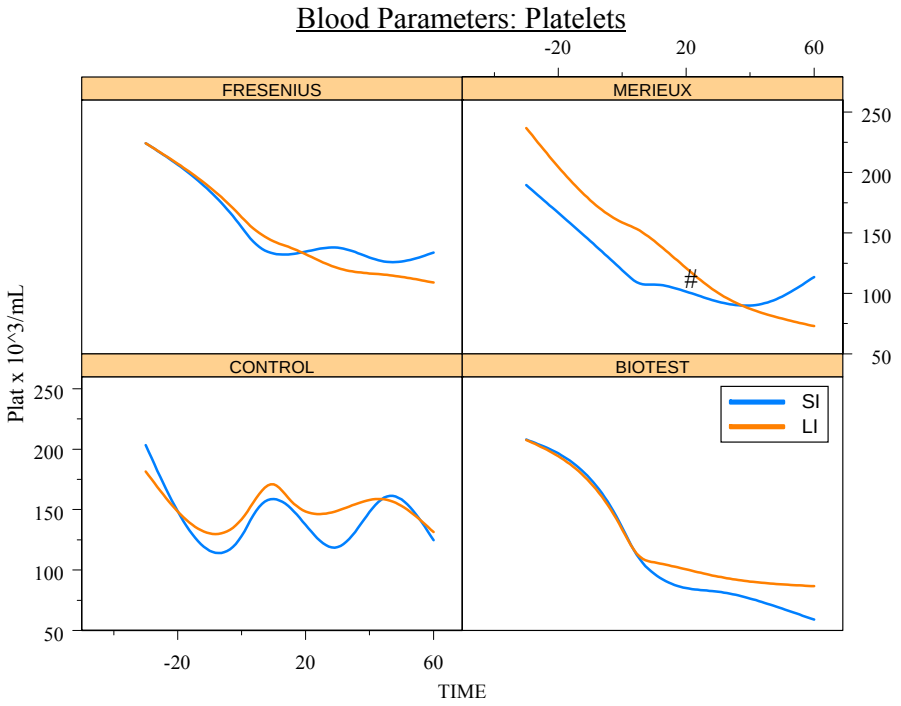
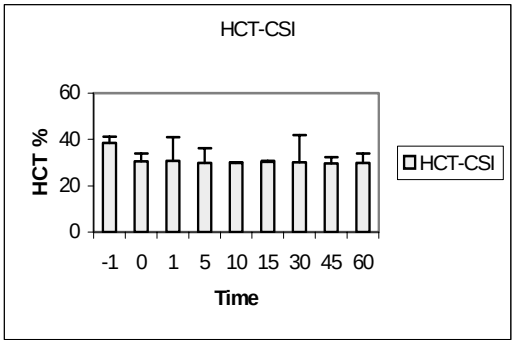


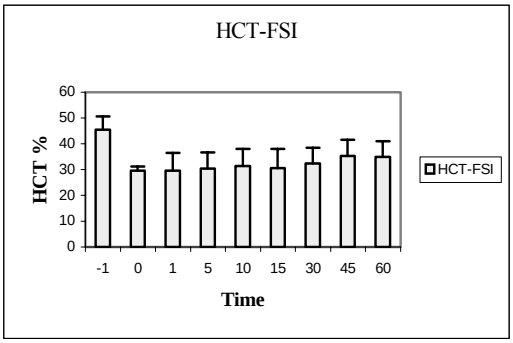
Fig. 13: Smoothing-Spline of Thrombocytes for SI and LI : After statistical analysis, only the Merieux ATG group showed a significant difference in the number of peripheral thrombocytes with different ischemia times, the amount of thrombocytes being higher in the LI group. (# Merieux LI vs. Merieux SI: p<0, 05)

3.1.4 Haematocrit

- Short Ischemia: BSI-FSI-MSI-CSI

Analysis of the human blood haematocrit during the whole process of reperfusion with human blood showed the following results. Analysis of the perfusion time is made after time point 0 (blood dilution), which serves as control. BSI group showed a statistically significant increase of Hct ($p<0,05$, Dunn's test) while FSI, MSI and CSI presented no significant differences.





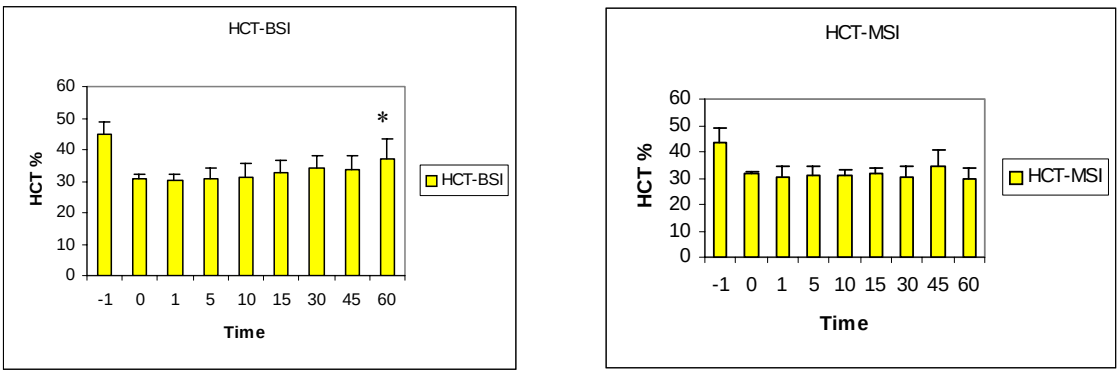


*Fig. 14: HCT in %- Short Ischemia: haematocrit in % during the reperfusion after SI time. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

- Long Ischemia: BLI, FLI, MLI, CLI

Analysis of the haematocrit during reperfusion with human blood after a LI period expressed the following variations. BLI and FLI presented a statistically significant increase of the Hct at the end of the reperfusion (p< 0,05, Dunn's test). In contrast to this, CLI and MLI experienced no significant variation.

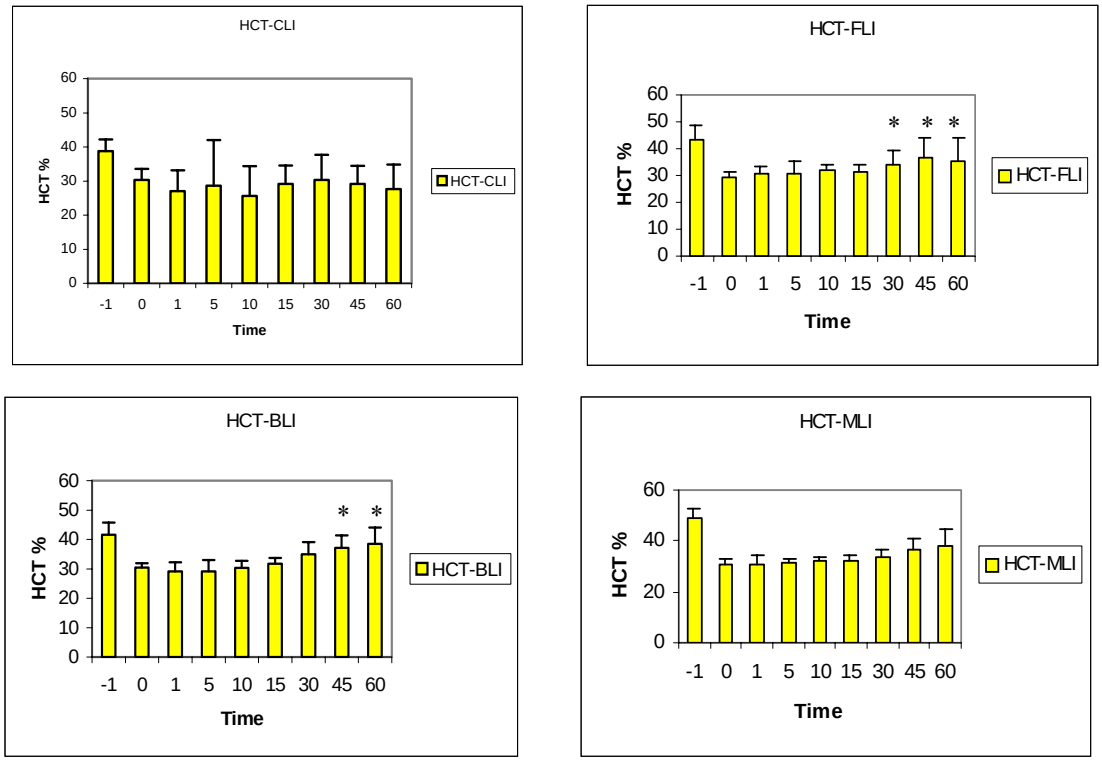


*Fig. 15: HCT in %- Long Ischemia: haematocrit in % during the reperfusion in LI time. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

As explained in material and methods the blood was diluted with Krebs-Henseleit-Buffer solution to a haematocrit of 30%. The variations of this value were studied with statistical methods and the results compared within the different groups. Ischemia time as a variable showed no statistical signification. However, “drug”, taken as variable, showed statistical signification with p<0,001. After applying the Scheffe´s test to the “drug” variable, we observed that the decrease of haematocrit of the control groups was statistically significant with a p<0.05. This difference exists also when the analysis is performed for both times of ischemia.

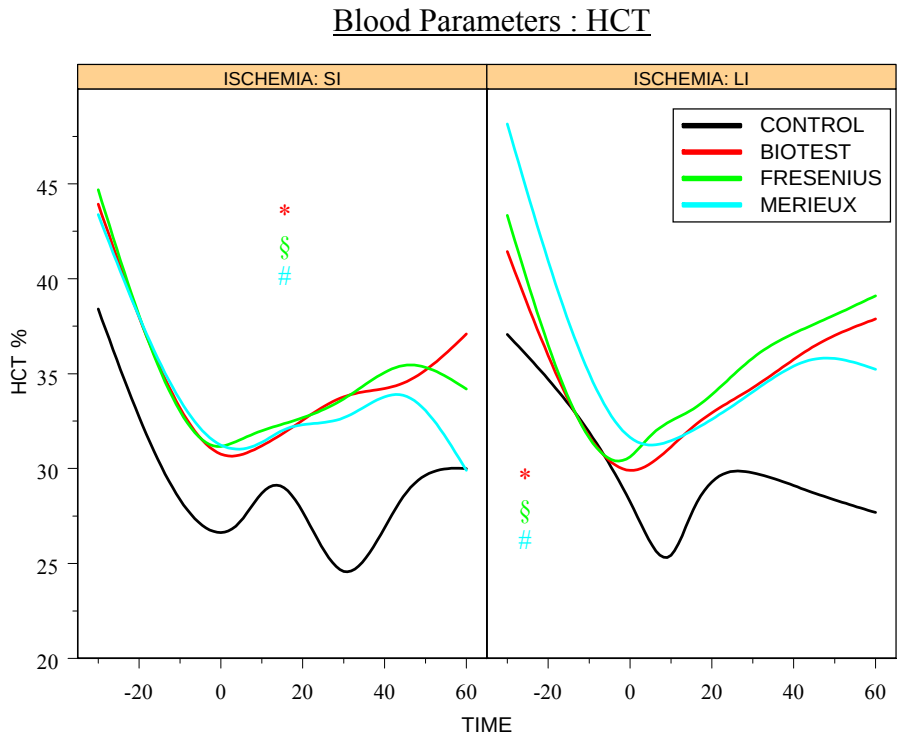


*Fig. 16: Smoothing-Spline of haematocrit in %: The Hct in the three ATG groups was significantly higher than in the control group both in short and long ischemia times (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0,05).*

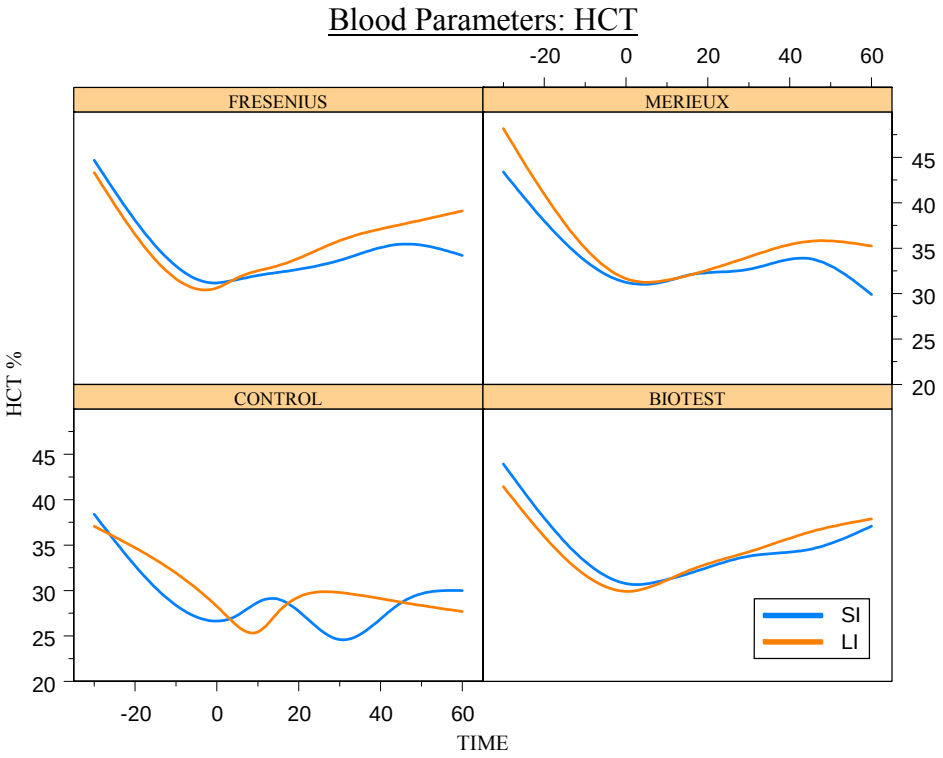
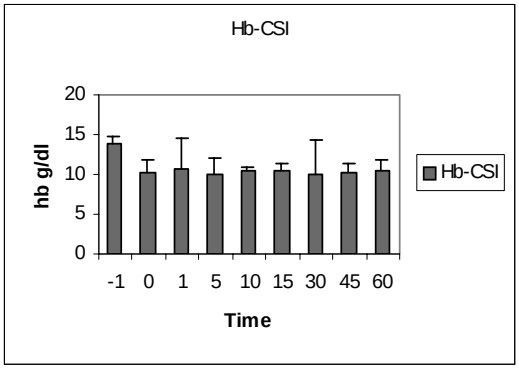


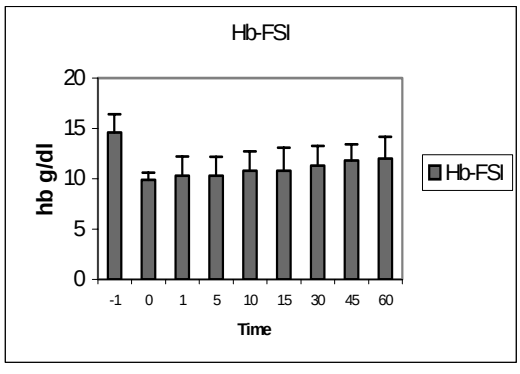
Fig. 17: Smoothing-Spline of haematocrit in %: No statistically significant differences were found according to the length of the ischemia times within the study groups.

3.1.5 Haemoglobin (Hb)

- Short Ischemia: BSI-FSI-MSI-CSI

Amount of haemoglobin in the different groups during the whole reperfusion was investigated. BSI group showed a statistically significant increase of Hb ($p < 0,05$, Tukey's test) while FSI, MSI and CSI presented no significant differences.





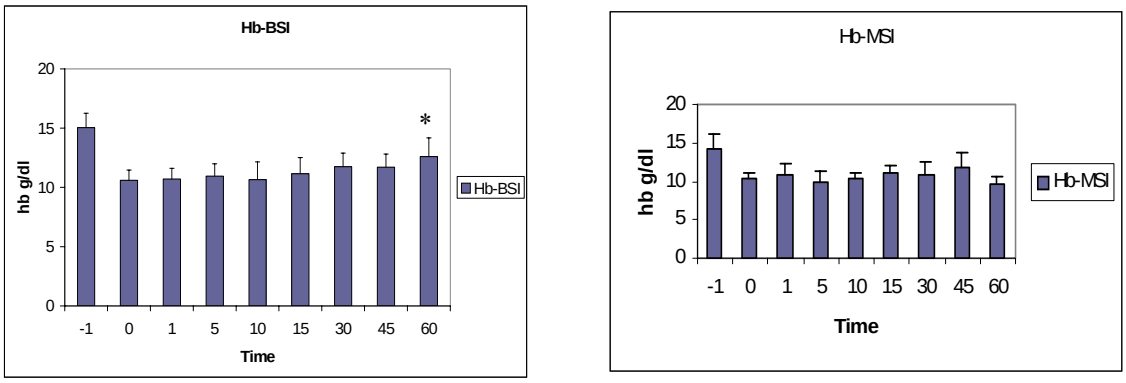


*Fig. 18: Hb (g/dl)-Short Ischemia: Amount of Hb of the study groups after a SI period. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

- Long Ischemia:

Haemoglobin values in the different study groups after a LI period showed the following results. BLI and FLI presented a statistically significant increase of the Hct at the end of the reperfusion (p< 0,05, BLI: Dunn's test; FLI: Dunnet's test). In contrast to this, CLI and MLI experienced no significant variation.

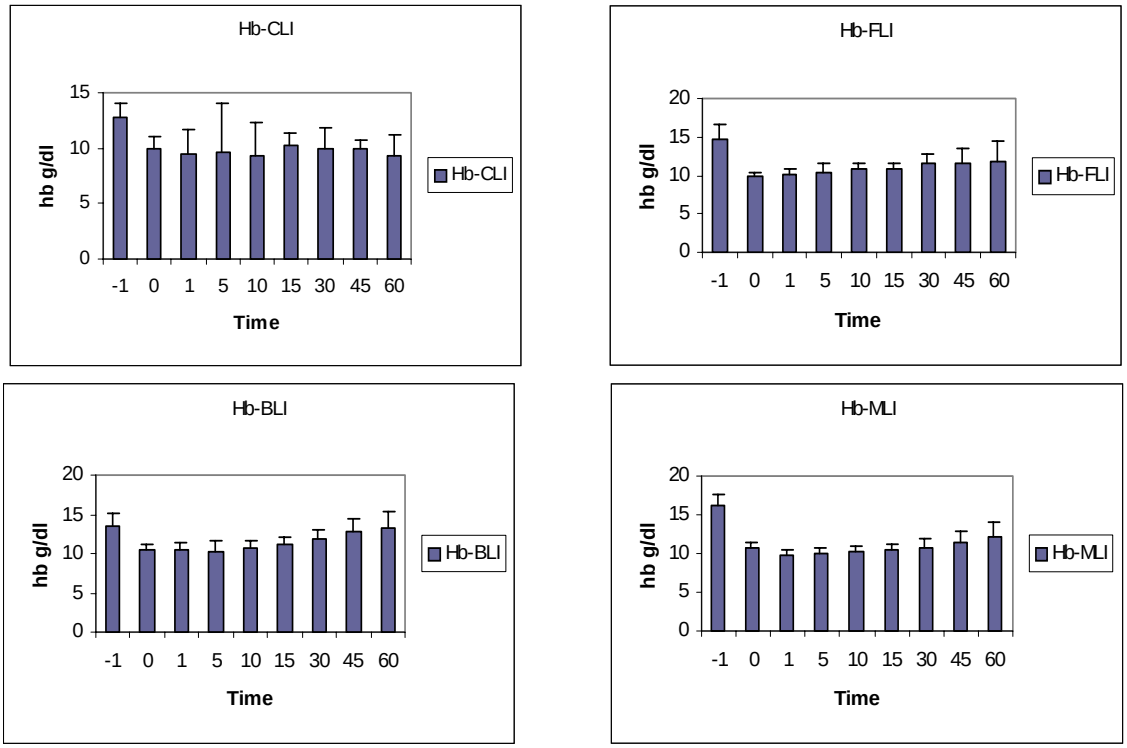


*Fig. 19:Hb (g/dl)-Long Ischemia: Amount of hb of the study groups after a LI period. Values are given as median and standard deviation. *Time point vs. Control [Time point 0] (p<0, 05)*

The haemoglobin values are substantially related to the values of erythrocytes and haematocrit. Therefore they present a similar behaviour after ATG treatment. The “ischemia time”, taken as variable alone, showed no significant differences between the ATG groups and the control groups. “Perfusion time” and “drug” presented statistically significant differences for p< 0,001. When we consider the variable “drug” , the values of haemoglobin in the ATG groups are higher than in the control groups, the same as happened before with erythrocytes and haematocrit. The differences between them after applying the Scheffe´s test are statistically significant (p<0, 05). There are no variations when we study the drug group dependent on the ischemia time. A multivariate model for haemoglobin values with smoothing is shown in the next figures (Figs. 20, 21).

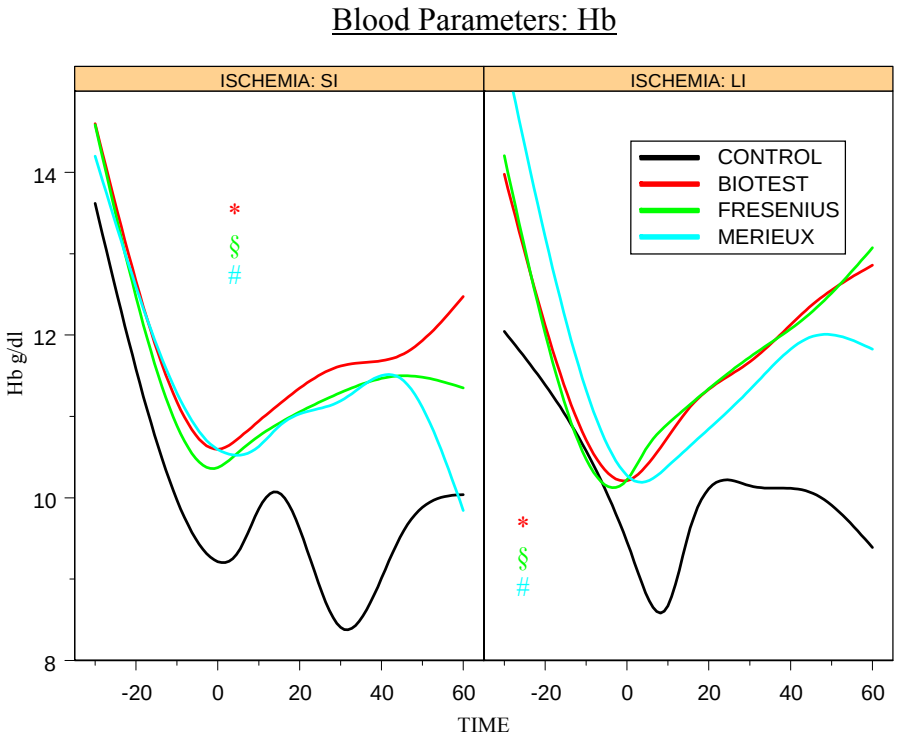


*Fig. 20: The haemoglobin amount (g/dl) in the three ATG groups was significantly higher than in the control group both in short and long ischemia times (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0,05).*

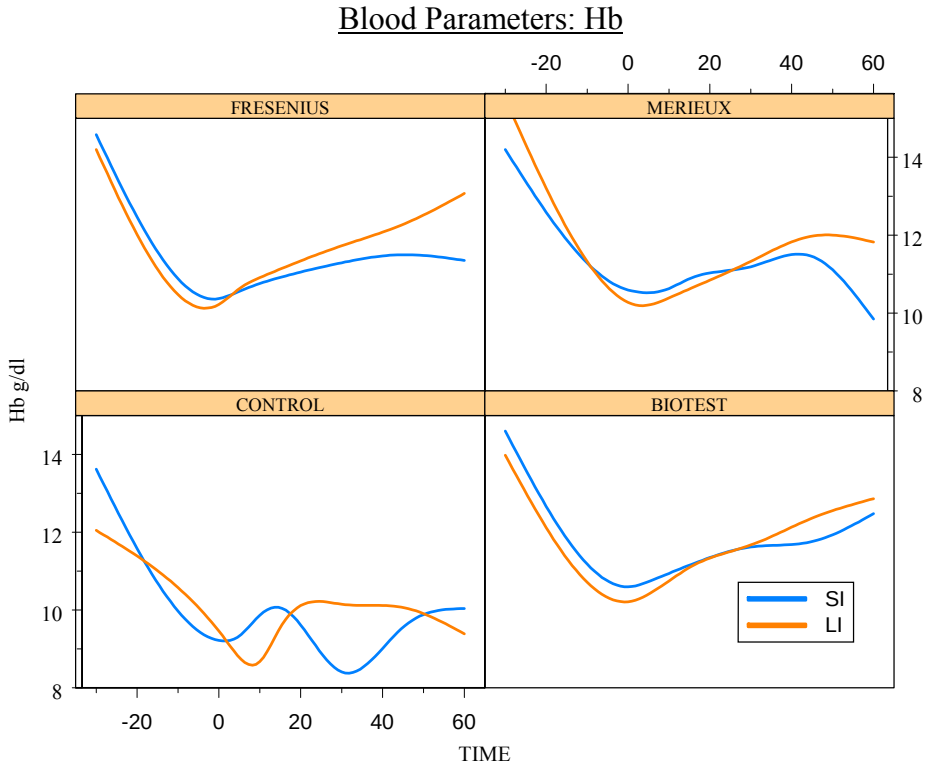


Fig. 21: Smoothing-Spline of haemoglobin amount: No statistically significant differences were found according to the length of the ischemia times within the study groups.

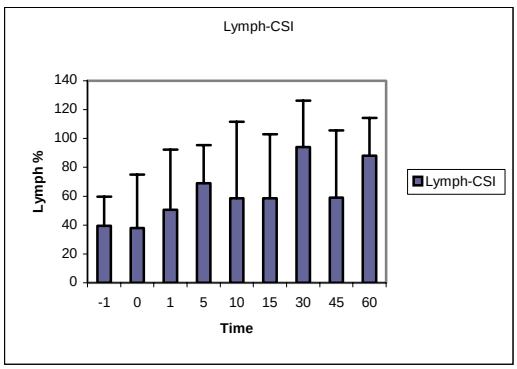
3.2 Smears

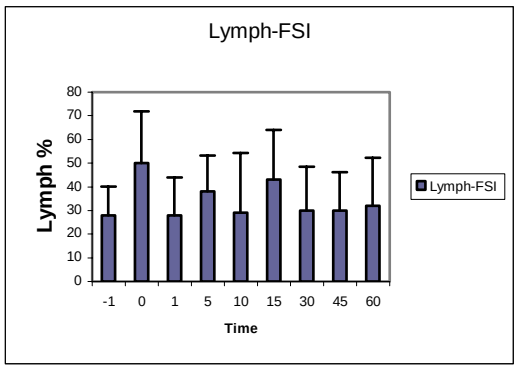
The percentages of lymphocytes, neutrophils and monocytes were measured as described in Material and Methods. Eosinophil and LGL percentages are not shown as their values are not significant for the study. Results for the different ATGs are assigned according to the time of ischemia. Short ischemia (SI) time was 60 ± 10 minutes and long ischemia (LI) 120 ± 10 minutes. Descriptive and comparative results for each group are expressed as median ± standard deviation in the graphics. The results are presented according to the following order: first the results of SI groups, then of the LI groups. Comparisons between groups and between different times of ischemia are shown at the end of each parameter.

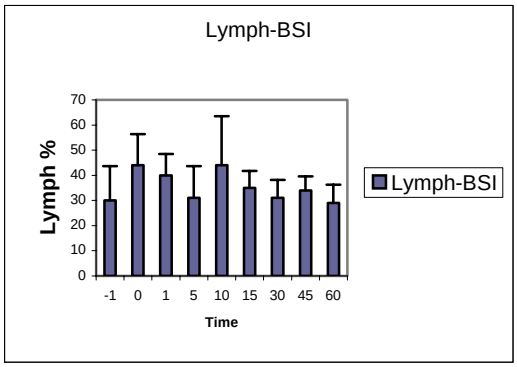
3.2.1 Lymphocytes

- Short Ischemia: BSI, FSI, MSI, CSI

Blood smears were performed with samples obtained from everyone of the four groups studied to evaluate the percentage of lymphocytes in PB and to compare it between the different ATG groups and control groups. In SI groups the samples were taken from the system after a period of short ischemia (60 ± 10 minutes). No significant differences in the percentage of lymphocytes during the reperfusion were found within the groups.







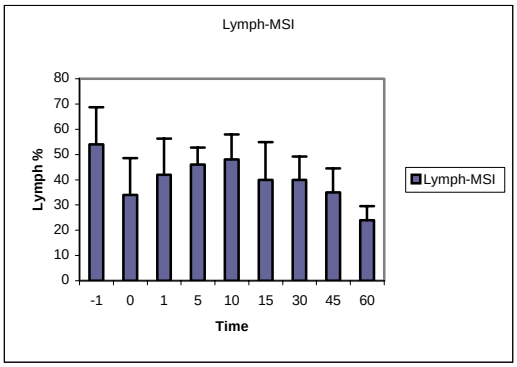
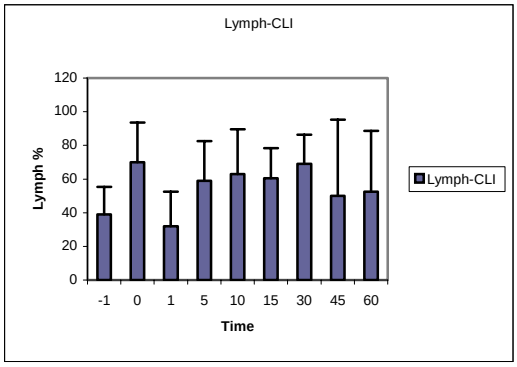
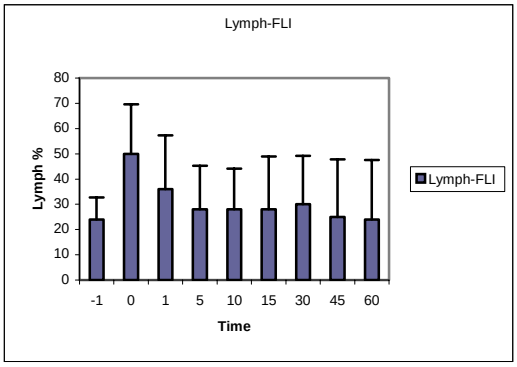


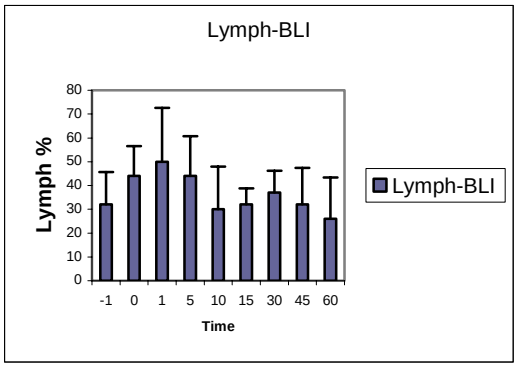
Fig. 22: Lymphocytes-Short Ischemia: Percentage of lymphocytes in PB smears during the reperfusion after a SI period (median and standard deviation are expressed in %).

- Long Ischemia: BLI, FLI, MLI, CLI

The percentage of lymphocytes in the LI groups is expressed in the following tables and figures. No significant differences in the percentage of lymphocytes during the reperfusion were found within the groups.







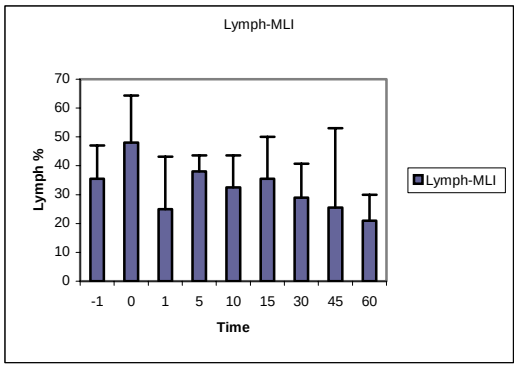


Fig. 23: Lymphocytes -Long Ischemia: Percentage of lymphocytes in PB smears during the reperfusion after a LI period (median and standard deviation are expressed in %).

The number of lymphocytes differentiated by means of cytology blood smears was substantially diminished in the ATG groups as compared to the control groups. Statistical tests with ANOVA were performed between groups according to the time of perfusion, the time of ischemia and the drug employed. After analysis, only the variable "drug" presented statistical significance, with a $p < 0.001$. Scheffe´s test was applied to further investigate this significant difference between groups. There is a statistically significant decrease in the number of lymphocytes in all the ATG groups when compared to the control group ($p<0,05$). "Ischemia Time" as variable did not show significant differences except for the Merieux group, presenting lower values of lymphocytes in the LI group ($p<0.05$). These results are expressed as a multivariate model of smoothing for ANOVA in the next figure.

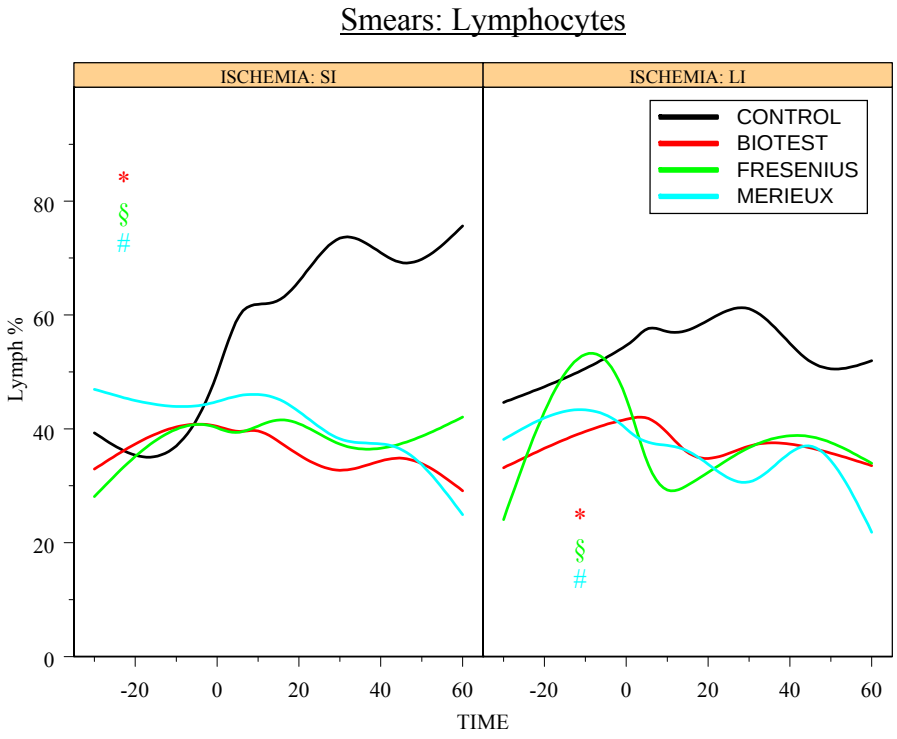


*Fig. 24: Smoothing-Spline of lymphocytes according to the drug and ischemia time: The percentage of lymphocytes in the three ATG groups was statistically lower than in the control group, both after SI and LI times (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0,05).*

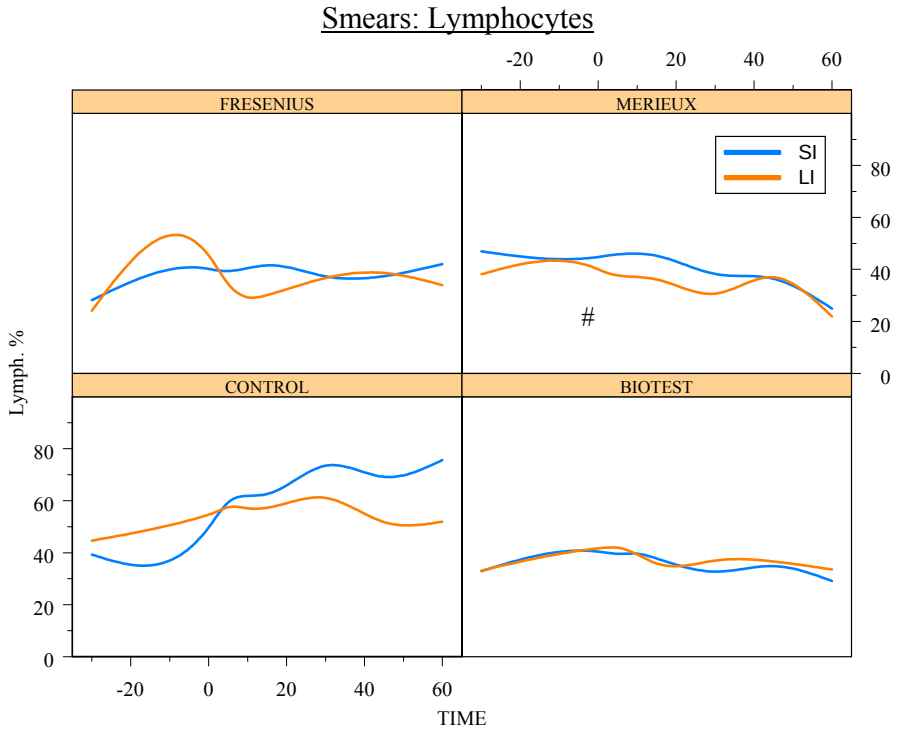
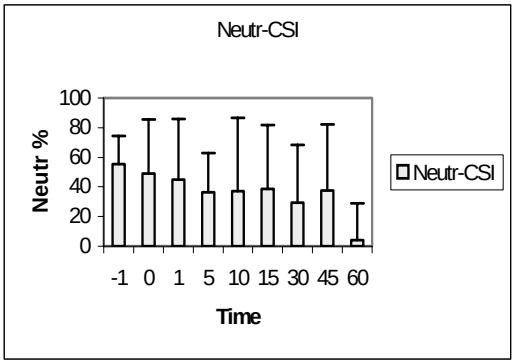


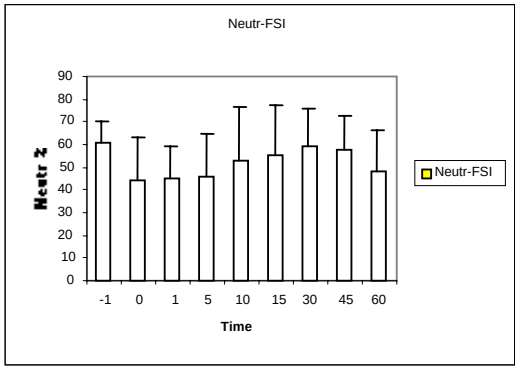
Fig. 25: Smoothing-Spline of lymphocytes according to the drug or ischemia time: Only Merieux-ATG groups experienced statistically significant differences between long and short ischemia percentage of lymphocytes, being significantly higher in SI time (# Merieux SI vs. Merieux LI p< 0.05). The other groups, including control, showed no statistical significance.

3.2.2 Neutrophils

- Short Ischemia: BSI, FSI, MSI, CSI

The percentage of neutrophils in PB was evaluated in SI groups throughout reperfusion with human blood. No significant differences were found within the study groups according to the time of perfusion.





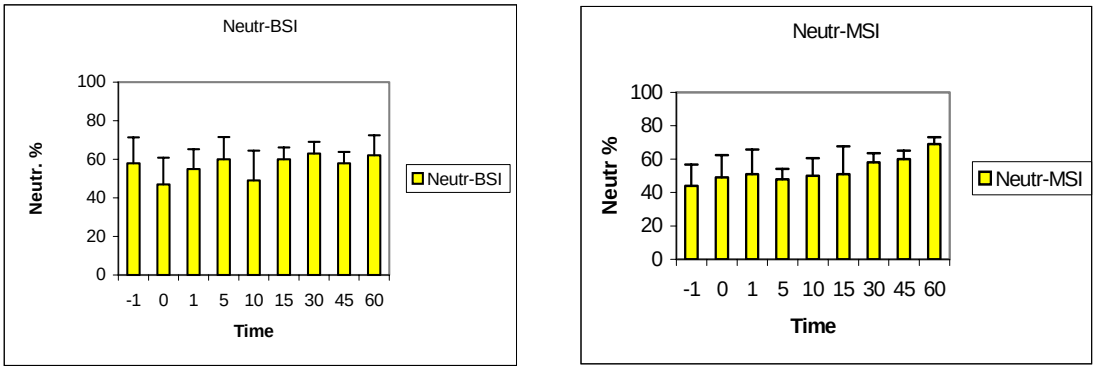


Fig. 26: Neutrophils-Short Ischemia: Percentage of neutrophils of the different study groups during the reperfusion (median and standard deviation are expressed in %). Note: the decrease of the % of neutrophils in the control group in comparison to the three ATG groups.

- Long Ischemia: BLI, FLI, MLI, CLI

The percentage of neutrophils in blood smears was also investigated in LI groups. No significant differences were found within the study groups according to the time of perfusion.

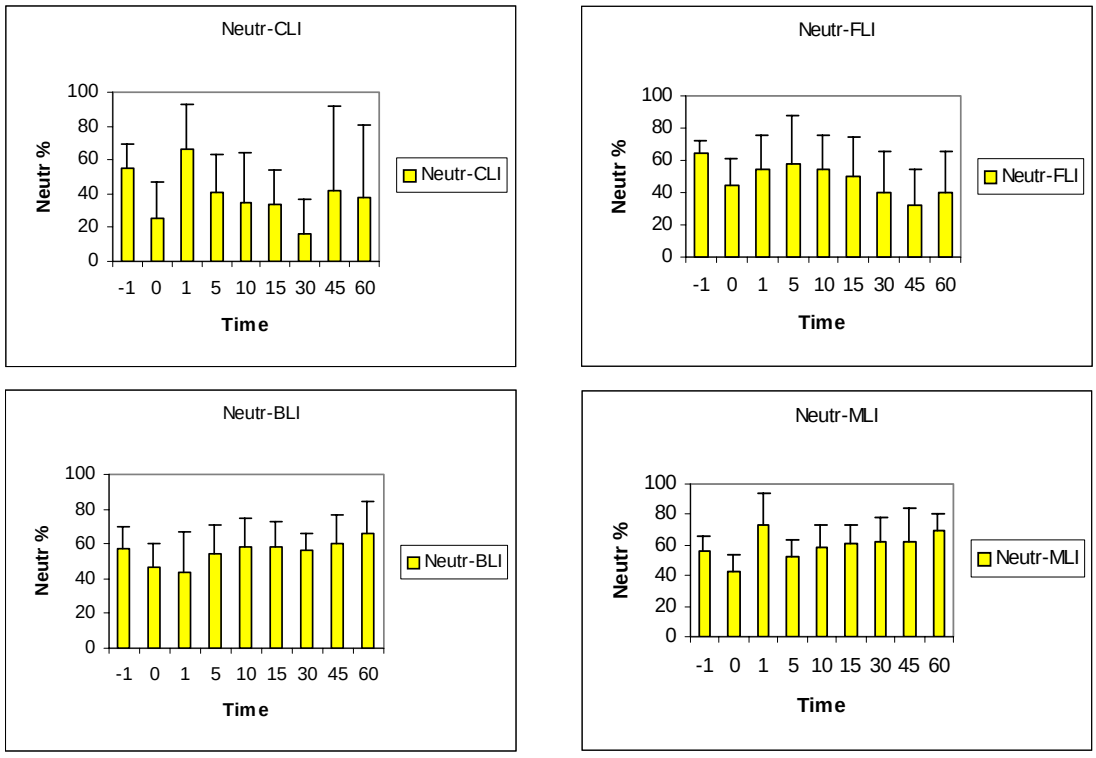


Fig. 27: Neutrophils-Long Ischemia: Descriptive graphics of the percentage of neutrophils of the different study groups during the reperfusion (median and standard deviation are expressed in %).

The percentage of neutrophil granulocytes in the different ATG and control groups was investigated. Higher percentages of neutrophils in the ATG groups in comparison to the control

groups were observed with the help of descriptive statistics. Further analysis was performed with ANOVA. Time of ischemia, drug and time of perfusion were selected as dependent variables. Time of perfusion and time of ischemia did not show any significant difference in the percentage of neutrophils in the general statistical study, while "drug" as a variable presented statistically significant differences of the % of neutrophils within the different groups with a p<0,001. Scheffe´s test to study the intragroup differences for a statistical significance was performed "a posteriori" taking "drug" as independent variable. Control groups presented significantly less neutrophils when compared to the ATG groups (p<0.05). However, considering ischemia time as an independent variable, there is no significance between control and the ATG-Fresenius group in the LI time, while the other groups results remain unaltered.

Smears: Neutrophils

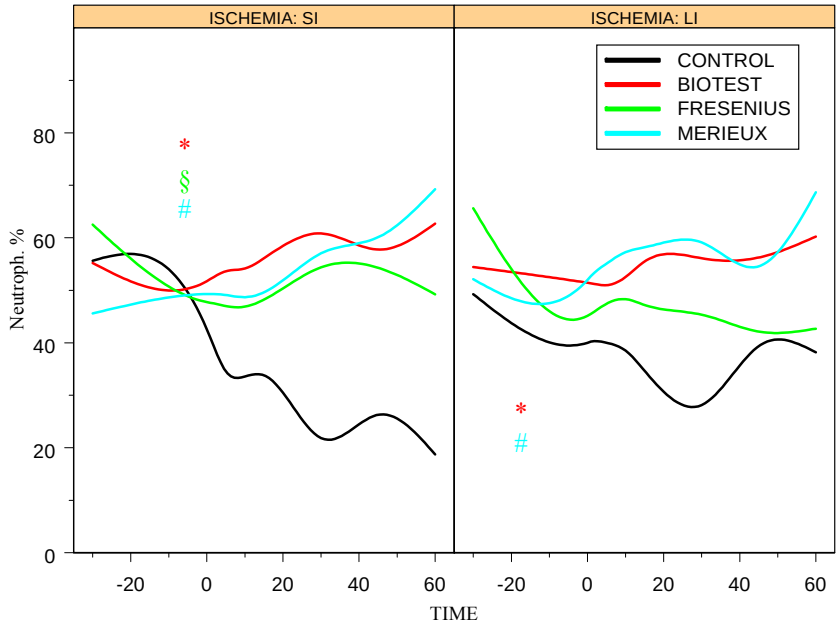


*Fig. 28: Smoothing-Spline of neutrophils according to the drug or ischemia time: The % of neutrophils in the three ATG groups was significantly higher than in the control group for SI time. Percentage of neutrophils in the Biotest-ATG and the Merieux ATG groups was significantly higher than in the control group after LI time (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0, 05).*

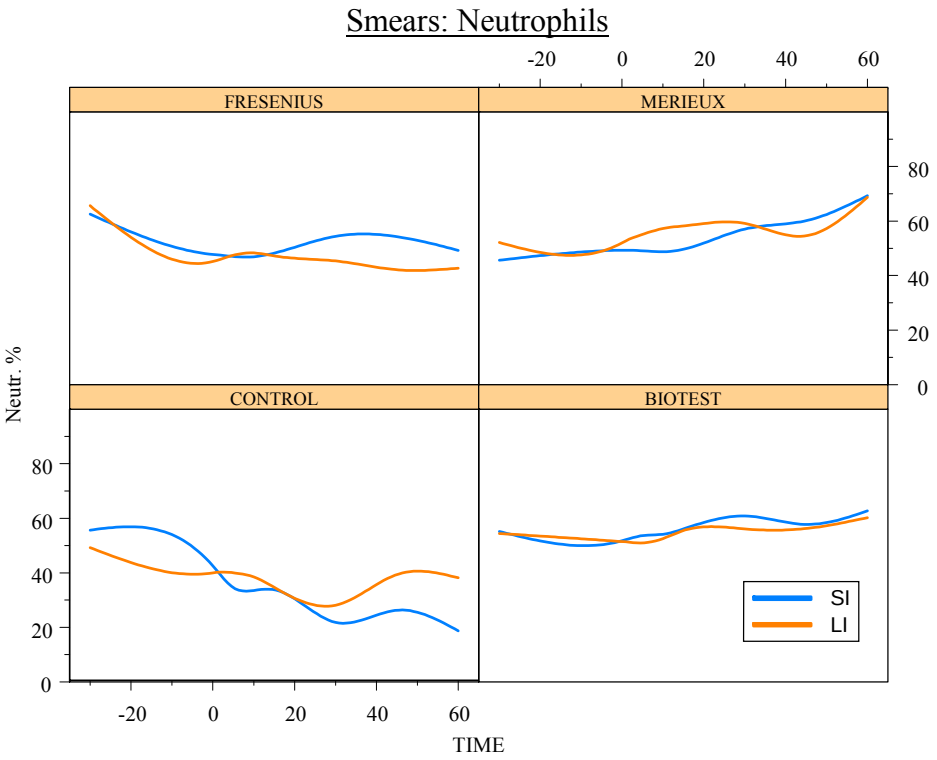
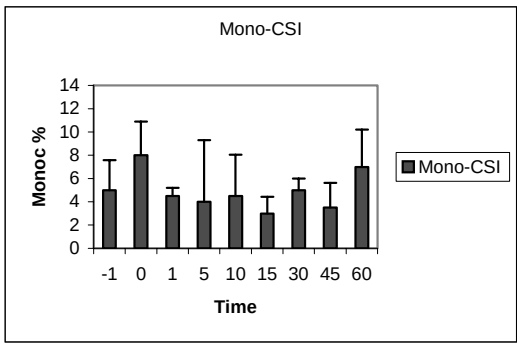


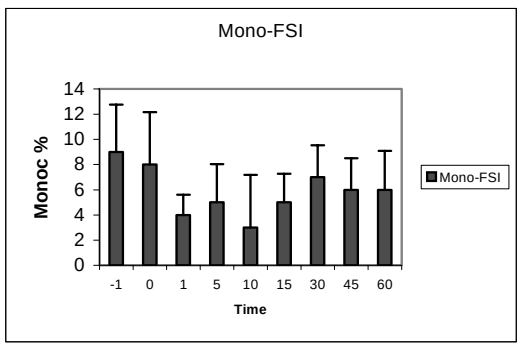
Fig. 29: Smoothing-Spline of neutrophils: No statistically significant differences were found according to the length of the ischemia times within the study groups.

3.2.3 Monocytes

- Short Ischemia: BSI, FSI, MSI, CSI

Blood smears were performed to evaluate the percentage of monocytes in PB and compare the values of the different ATG and the control groups. The percentage of monocytes is much lower than those of lymphocytes and neutrophils. That is the reason why a different scale is used for the graphics. No significant differences were found within the study groups according to the time of perfusion.





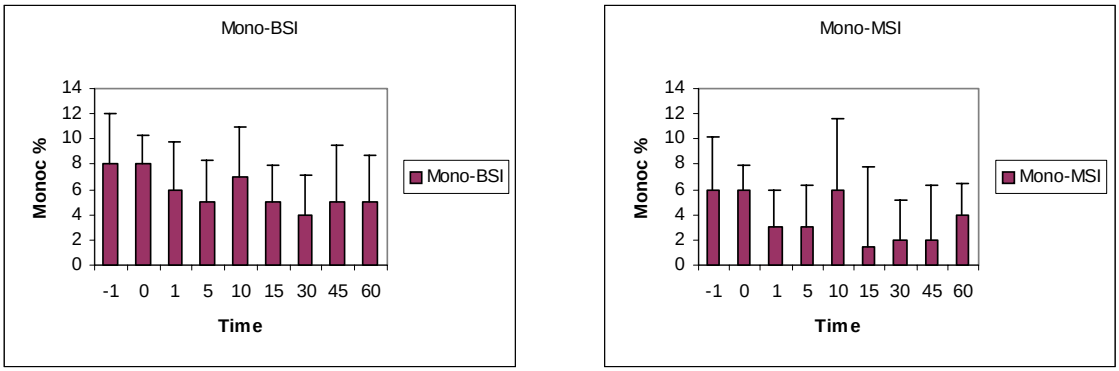


Fig 30: Monocytes-Short Ischemia: Percentage of monocytes during the reperfusion after a SI period (median and standard deviation are expressed in %).

- Long Ischemia: BLI, FLI, MLI, CLI

The percentage of monocytes in the different groups was assessed. A different scale has been employed also in the long ischemia groups. No significant differences were found within the study groups according to the time of perfusion.

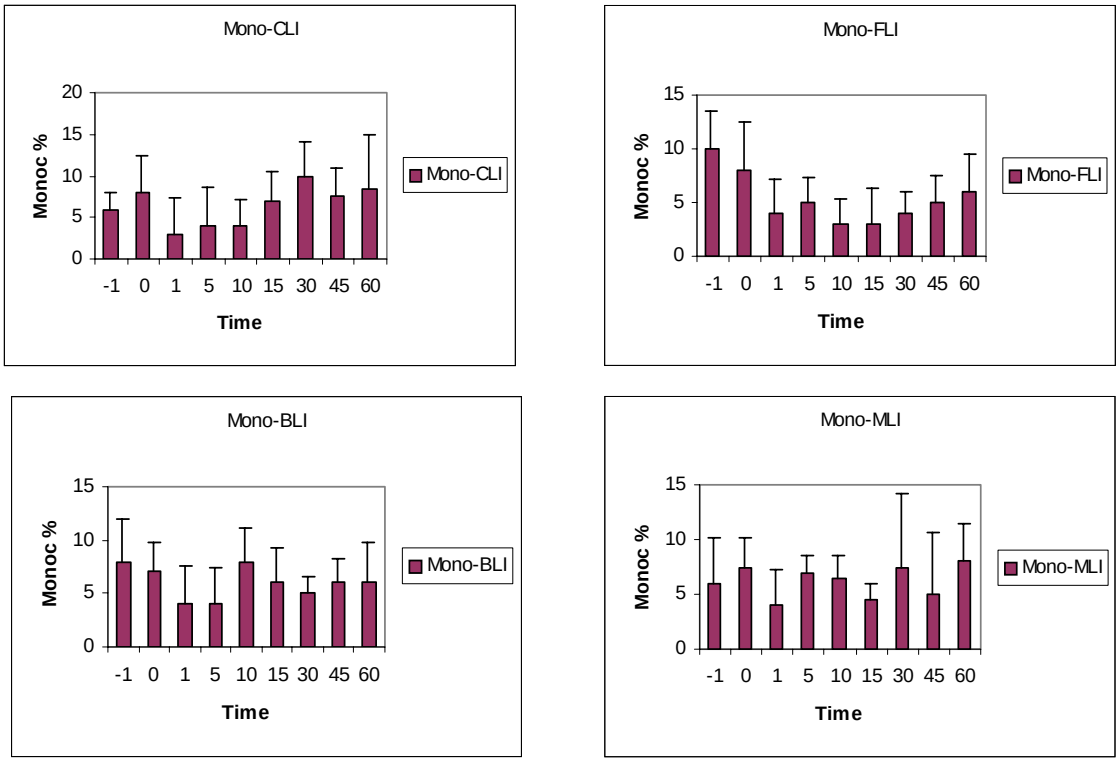


Fig. 31: Monocytes-Long Ischemia: Percentage of monocytes during the reperfusion after LI time (median and standard deviation are expressed in %).

The percentage of monocytes did not present much variation between groups in the cytological studies. ATG-Merieux group presented a significant increase of monocytes in LI, considering Ischemia as independent variable.

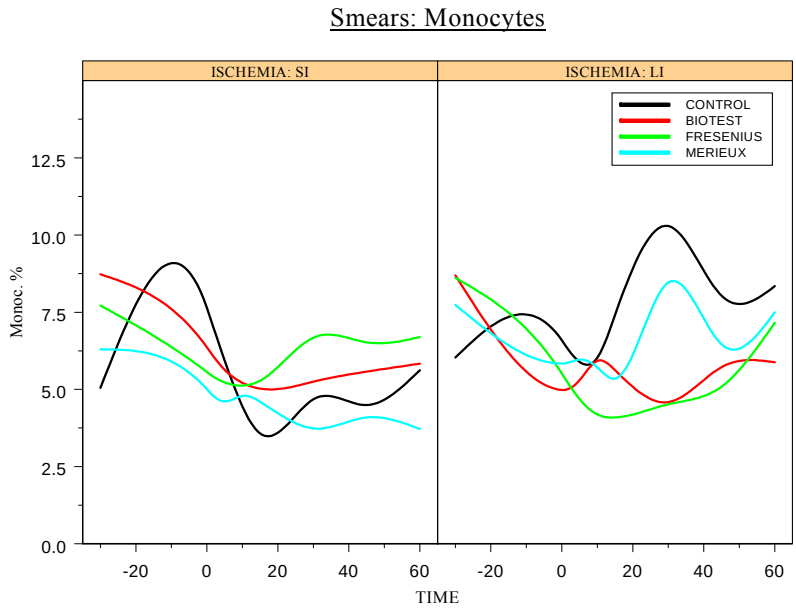


Fig. 32: Smoothing-Spline of monocytes: No significant differences were found after statistical analysis.

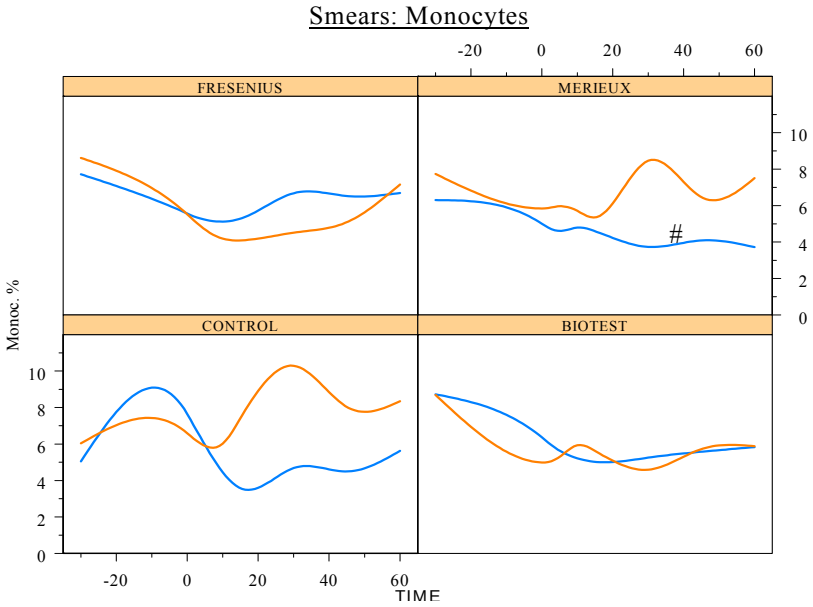


Fig. 33: Smoothing-Spline of monocytes according to ischemia time: Only Merieux-ATG groups experienced statistically significant differences between SI and LI percentage of monocytes, being significantly higher in LI times (# Merieux SI vs. Merieux LI $p < 0.05$).

3.2.4. Microphotographs

The following figures are microphotographs obtained from the smears performed with every blood sample. The high number of smears evaluated does not allow us to present all the images. These pictures try to reflect the standard behaviour of both the control and study groups. As a general rule, more mononuclear cells in whole blood can be observed in the control groups and more polymorphonuclear cells are to be seen in the study groups. All pictures are shown as microscope magnifications.

1. Biotest Short Ischemia: BSI

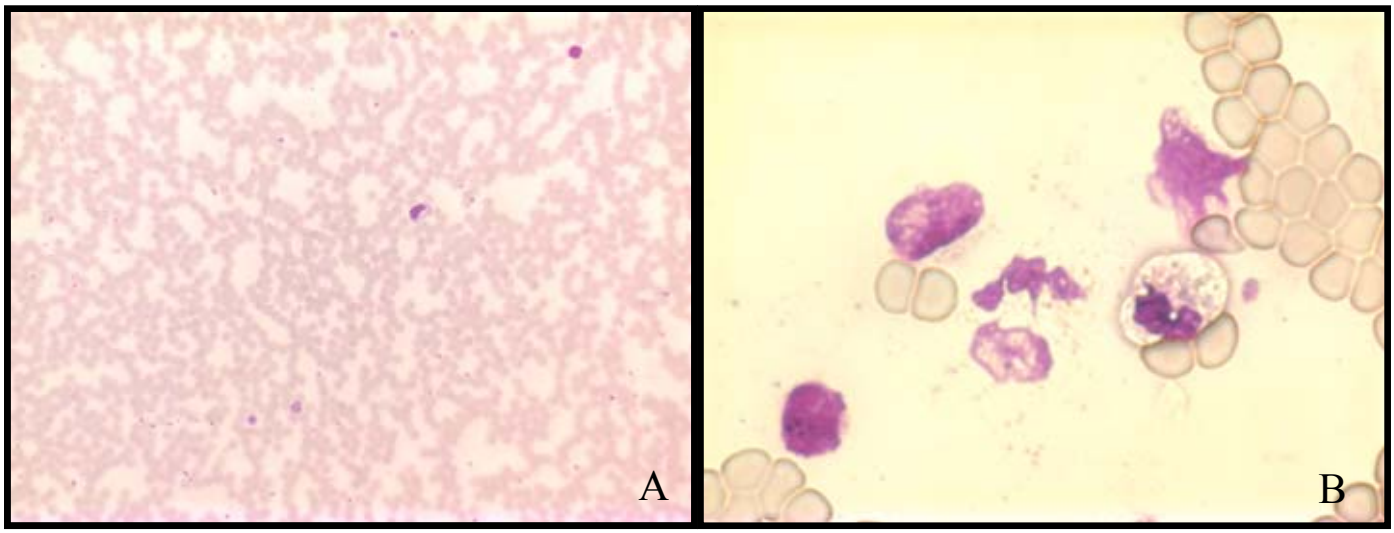


Fig. 34: Higher proportion of polymorphonuclear cells was observed in the ATG groups. However, mononuclear cells were also seen (Fig. 34-A x20). Damaged WBC can be observed at a higher magnification after treatment with ATG (Fig. 34-B x100)

2. Biotest Long Ischemia: BLI

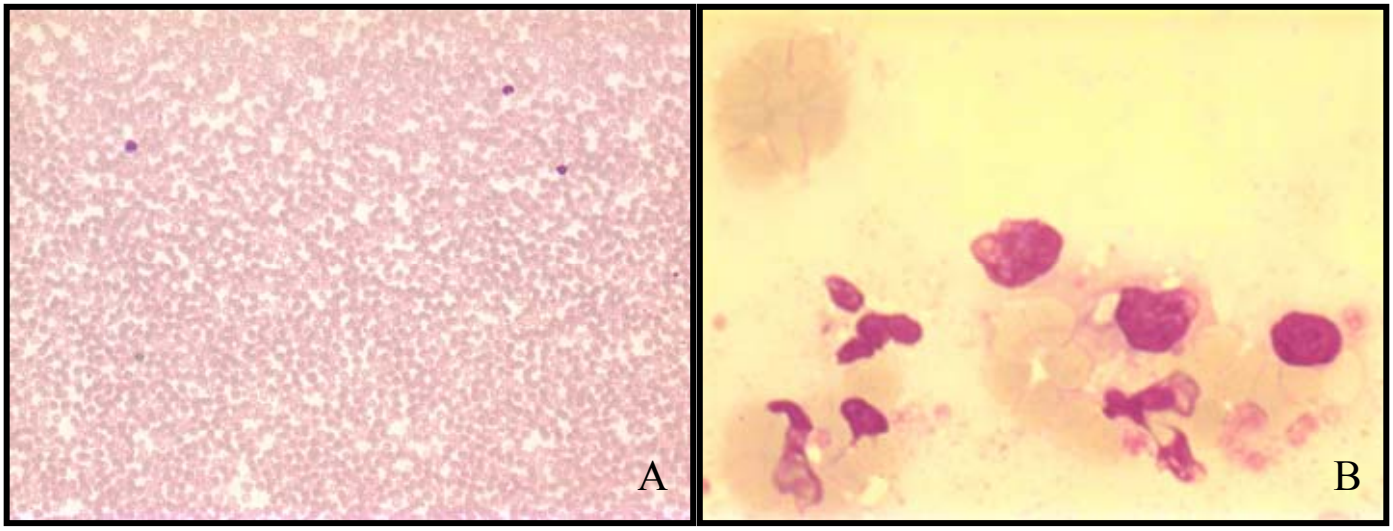


Fig. 35: In the BLI group, a higher percentage of lymphocytes than in other ATG groups was observed at the beginning of the reperfusion (Fig. 35-A x20). No significant differences were detected between the ATG groups. Segmented granulocytes were seen at a higher magnification (Fig. 35-B x100)

3. Fresenius Short Ischemia: FSI

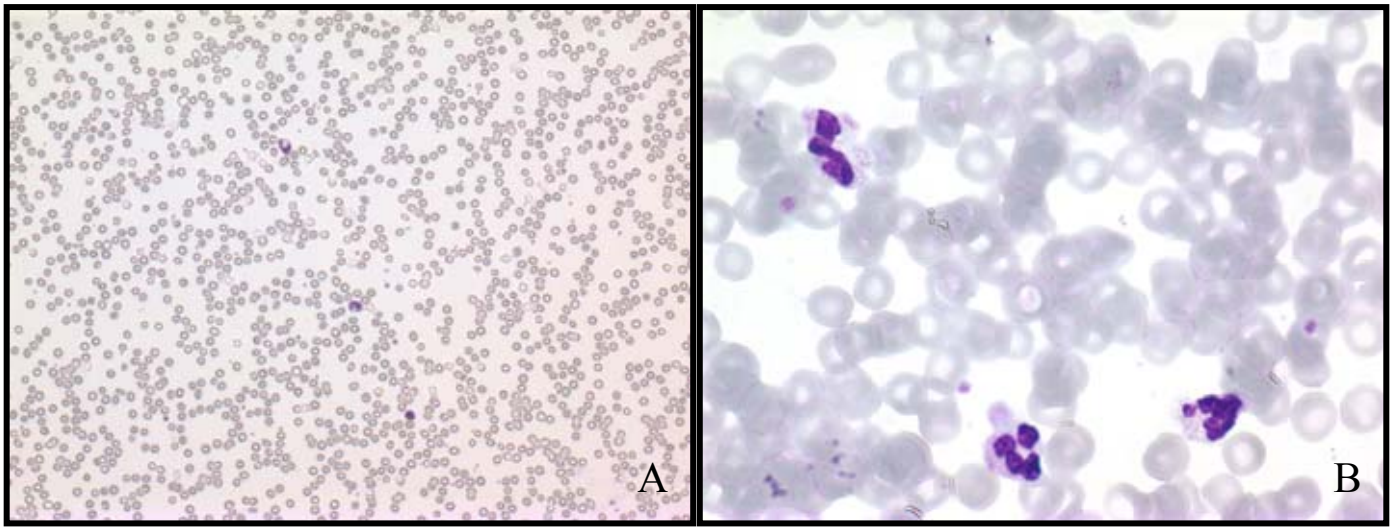


Fig. 36: In the FSI group, few mononuclear cells were observed both at low and high magnification (Fig. 36-A x20, Fig. 36-B x100)

4. Fresenius Long Ischemia: FSI

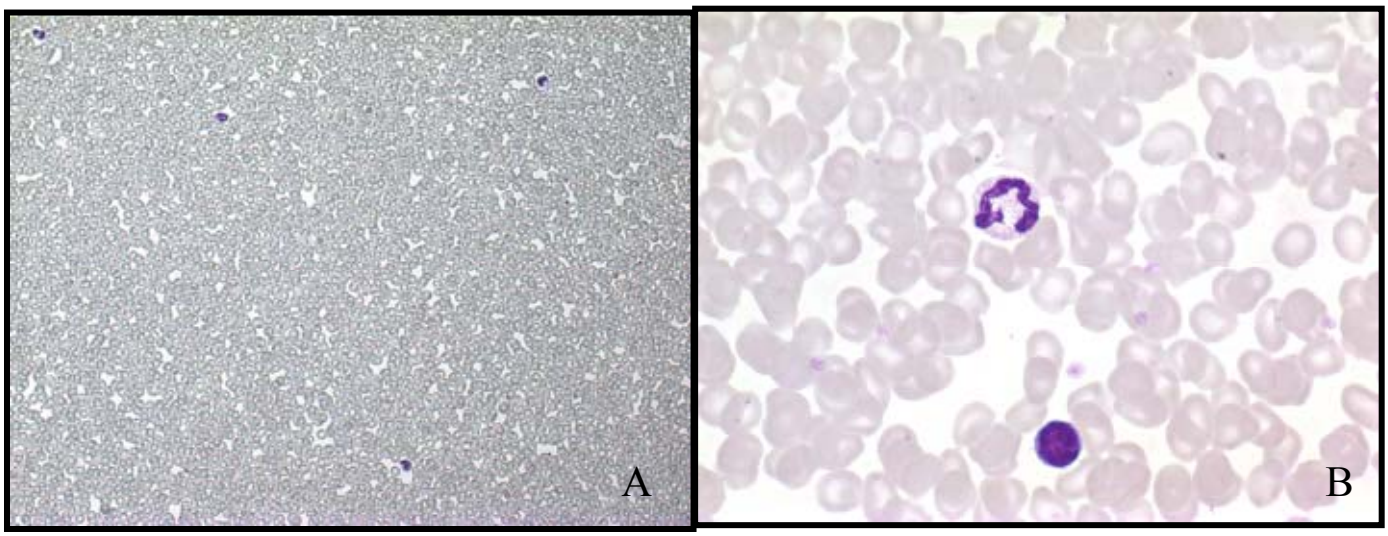


Fig. 37: More cells were seen in the FLI group in comparison to FSI group, although no statistically significant difference was observed. Presence of mononuclear cells was low in comparison to polymorphonuclears. (Fig-37-A x20, Fig. 37-B x100)

5. Merieux Short Ischemia: MSI

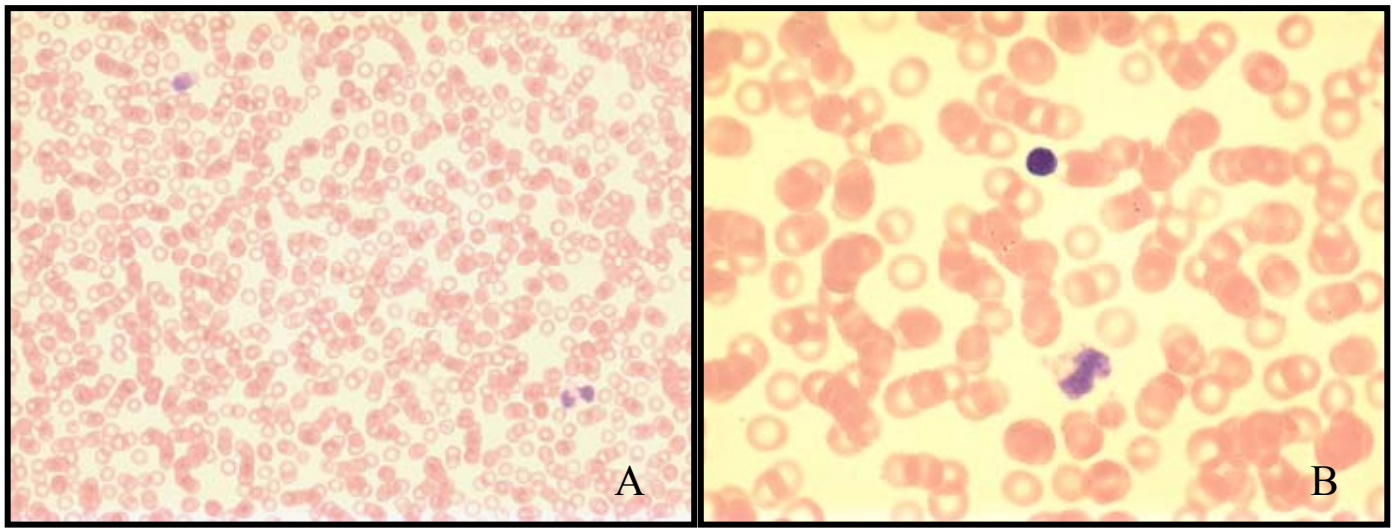


Fig. 38: A lower amount of cells was seen in the MSI group in comparison to the control groups. There were more polymorphonuclear cells (Fig. 38-A x40) although lymphocytes were also observed (Fig. 38-B x100)

6. Merieux Long Ischemia: MLI

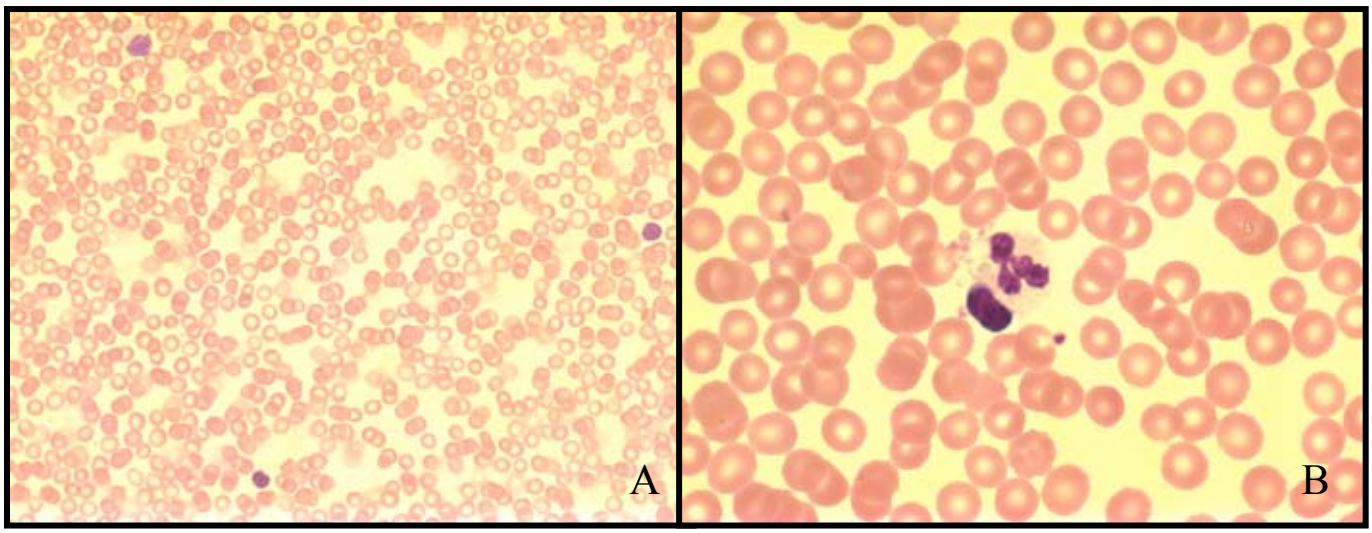


Fig. 39: MLI group presented fewer lymphocytes than the MSI group, but a higher percentage of monocytes (Fig. 39-A x40). At a higher magnification, a segmented polymorphonuclear cell is shown (Fig. 39-B x100)

7. Control Short Ischemia: CSI

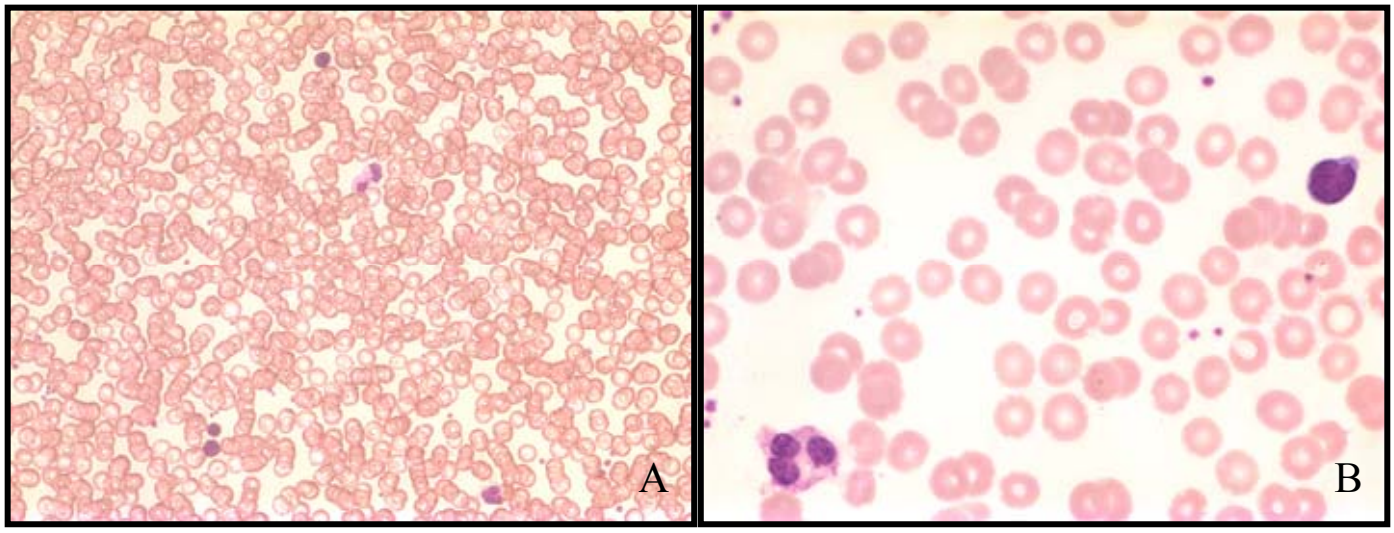


Fig. 40: CSI group presented a higher number of mononuclear WBC (Fig. 40-A x40, Fig. 40-B x100)

8. Control Long Ischemia: CLI

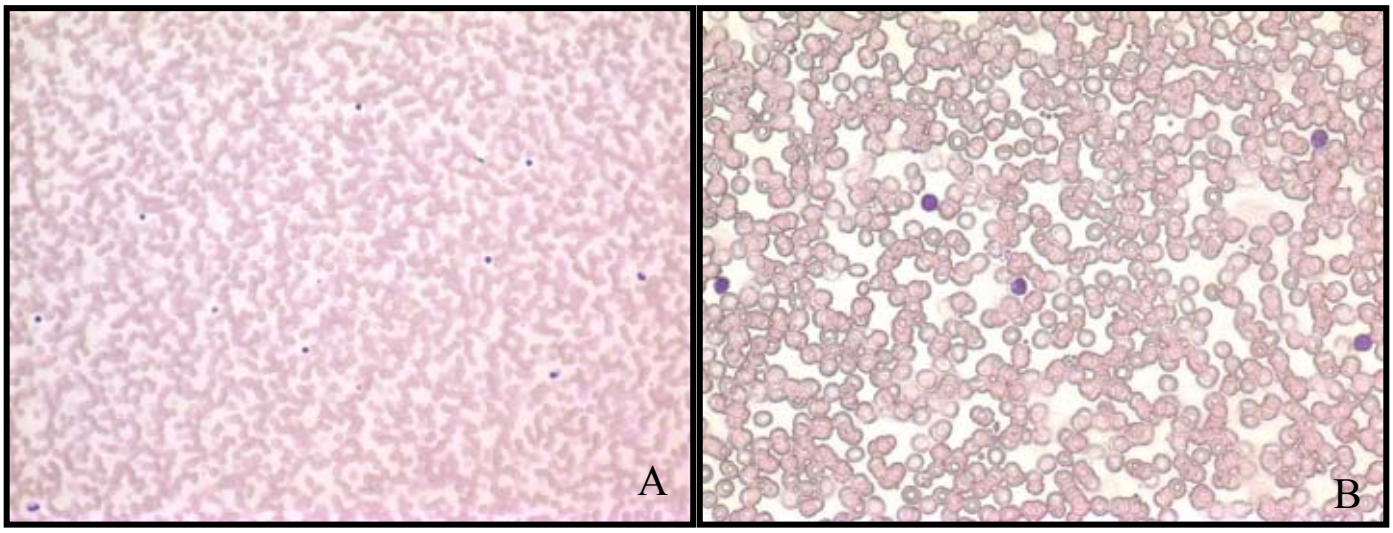


Fig. 41: At the end of the reperfusion, the CLI group presented a high number of cells per smear. The percentage of lymphocytes was much higher than other WBC in comparison to the ATG groups. (Fig. 41-A x20, Fig. 41-B x40)

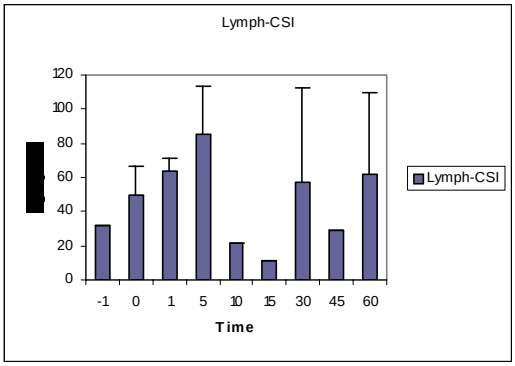
3.3. CIM

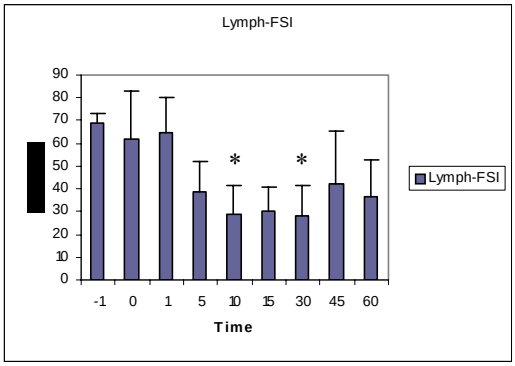
Descriptive results for each group are presented as median ± standard deviation in the graphics. Comparisons between groups are described in the text and shown in the figures. The results are expressed according to the following order: first results of the SI groups are shown, then of the LI groups. Analysis of the time of perfusion is performed taking 0 as control point (blood diluted to 30% Hct). Comparisons between groups and between different times of ischemia are also shown.

3.3.1 Lymphocytes

- Short ischemia: BSI, FSI, MSI, CSI

CIM of the four groups studied evaluated the percentage of lymphocytes differentiated in WBC subpopulations. The results were compared between the ATG and control groups. In SI groups the samples were taken from the system after a period of short ischemia (60 ± 10 minutes). CSI and MSI showed no significant differences according to time of perfusion. In contrast to this, the percentage of lymphocytes decreased in the BSI and FSI ATG groups throughout the reperfusion. ($p<0,05$; BSI: Dunn's test; FSI: Tukey's test).





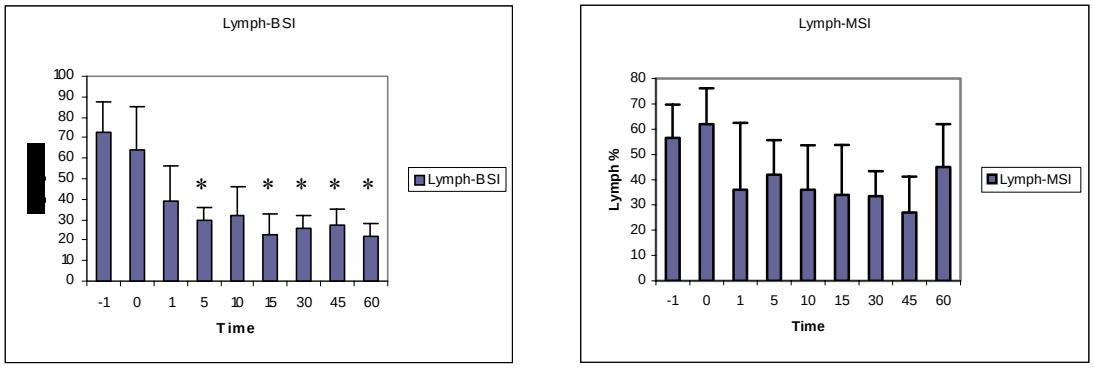
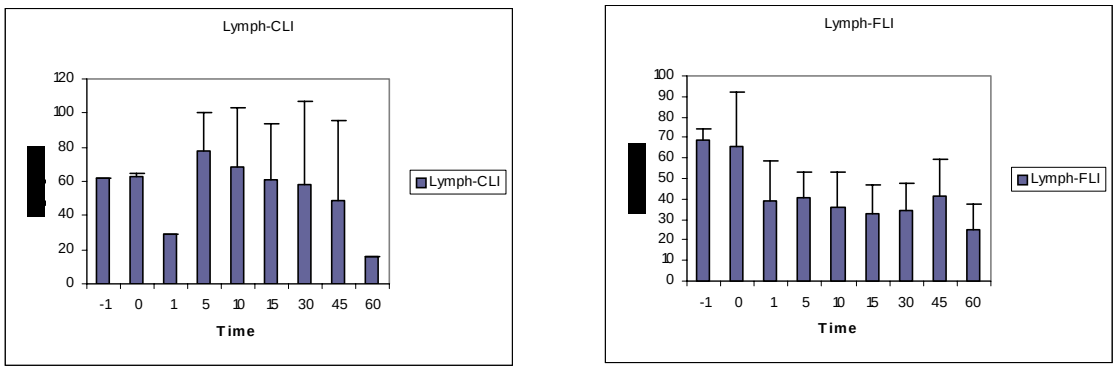


*Fig. 42: Lymphocytes-Short Ischemia: Percentage of lymphocytes in CIM in the different study groups after a SI period (median and standard deviation are given in %). *Time point vs. Control [Time point 0] (p<0, 05).*

- Long Ischemia: BLI, FLI, MLI, CLI

CIM was performed in samples obtained from every of the four groups studied to evaluate and compare the percentage of lymphocytes. In these groups the samples were taken from the arterial branch of the system after a LI period. MLI presented a statistically significant decrease of the percentage of lymphocytes throughout the reperfusion (p<0,05; MLI: Tukey's test). In contrast to this, no significant differences were observed in CLI, BLI and FLI groups.



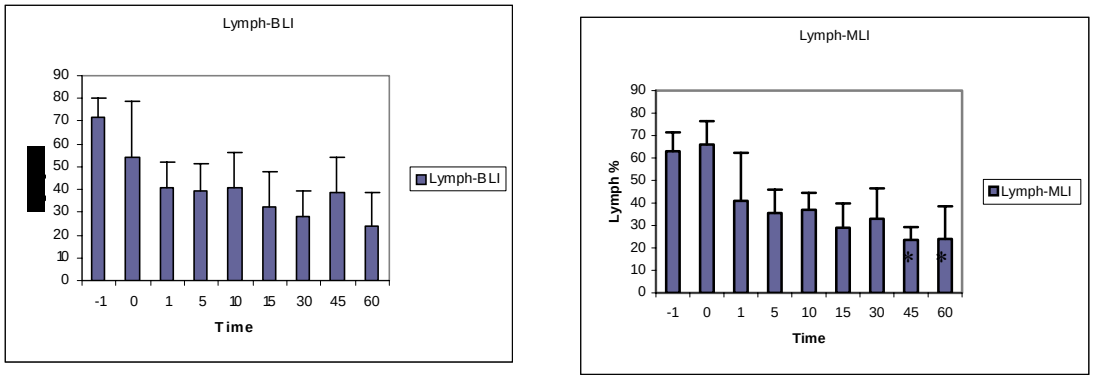


*Fig. 43: Lymphocytes-Long Ischemia: Percentage of lymphocytes with CIM after a LI period (median and standard deviation are given in %). *Time point vs. Control [Time point 0] (p<0, 05).*

The percentage of lymphocytes as WBC subpopulation was investigated according to three different variables: "time of perfusion", "drug" and "time of ischemia. Differences between the groups were studied showing that both the variables "drug" and "perfusion time" presented statistical significance, with a $p < 0.001$ (as shown in the previous graphics). Factor "ischemia time" showed no significance in the general test. Scheffe´s test was applied to further investigate this significant difference between groups for the "drug" variable. The percentage of lymphocytes of the three ATG groups presented a statistically significant decrease when compared to the control group throughout the reperfusion process (p<0, 05). This significance remained unaltered when the groups were compared according to the time of ischemia. In the particular tests between groups, no significance for "ischemia time" was found.

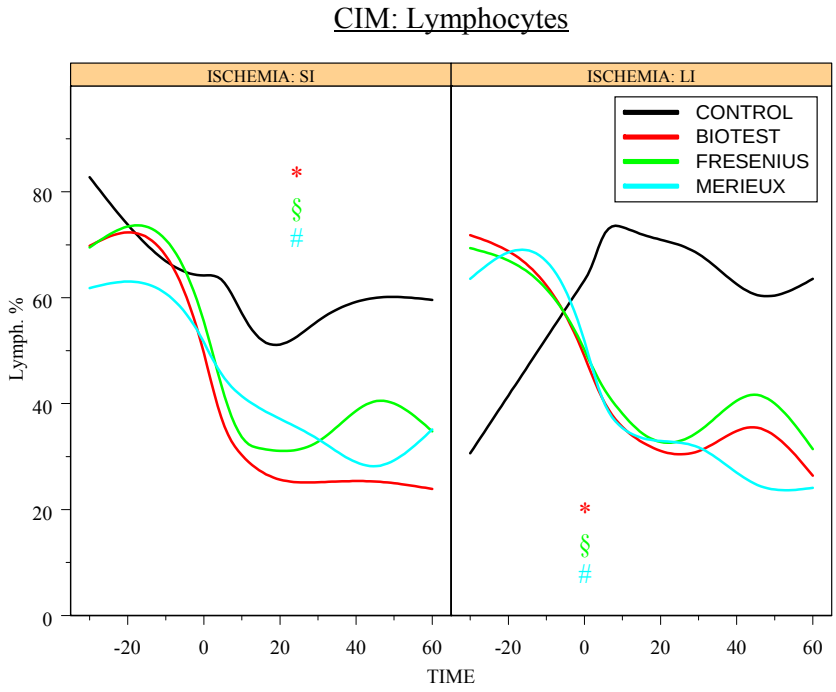


*Fig. 44: Smoothing-Spline of lymphocytes (CIM): The percentage of lymphocytes among WBC in the three ATG groups was significantly lower than in the control group both in SI and LI times (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0,05).*

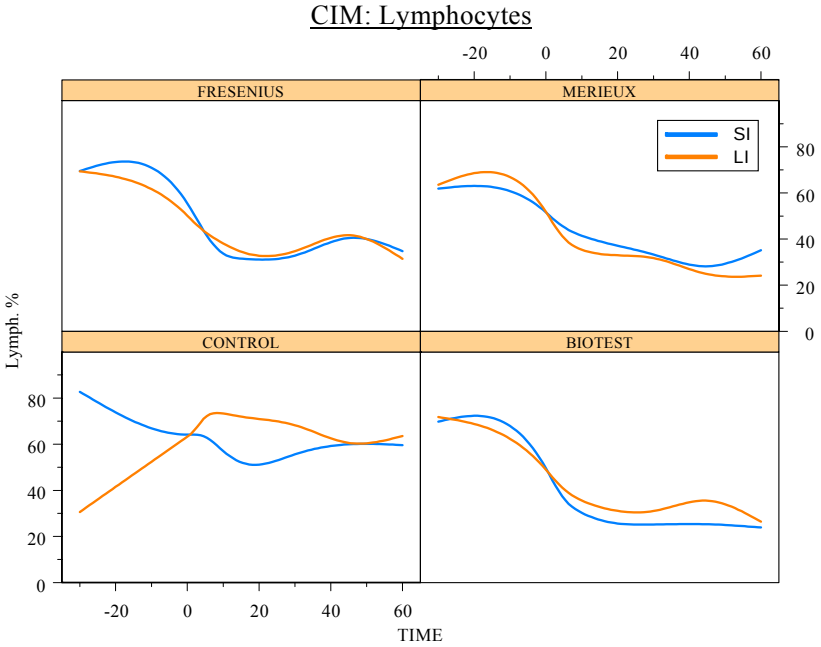


Fig. 45: Smoothing-Spline of lymphocytes according to ischemia time: No statistically significant differences were found according to the length of the ischemia times within the study groups.

3.3.2 Neutrophils

- Short Ischemia: BSI, FSI, MSI, CSI

The percentage of neutrophils in differentiated WBC subpopulation was compared within the different ATG groups and the control groups. The samples were taken from the arterial branch of the system after a SI period (60 ± 10 minutes) and processed as described before. BSI and FSI groups experienced a significant increase of the percentage of neutrophils ($p<0,05$; Tukey's test). MSI and CSI, however, showed no statistically significant differences.

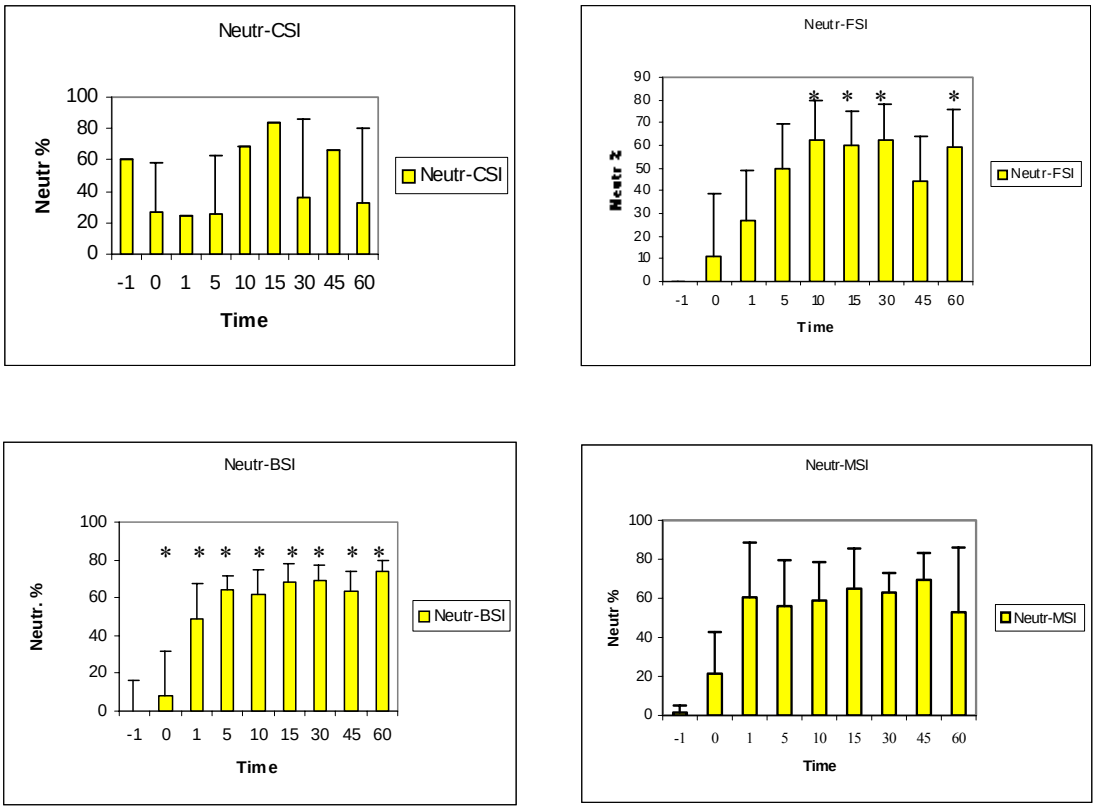


*Fig. 46: Neutrophils-Short Ischemia: Percentage of neutrophils after a SI period (median and standard deviation are given in %). *Time point vs. Control [Time point 0] ($p<0, 05$).*

- Long Ischemia: BLI, FLI, MLI, CLI

CIM was performed in samples obtained from each of the four groups studied to evaluate the percentage of neutrophils within the different groups and compare the obtained results. MLI

presented a significant intragroup increase of the percentage of neutrophils throughout the reperfusion. In contrast to this, BSI, FSI and CSI showed no statistically significant difference.

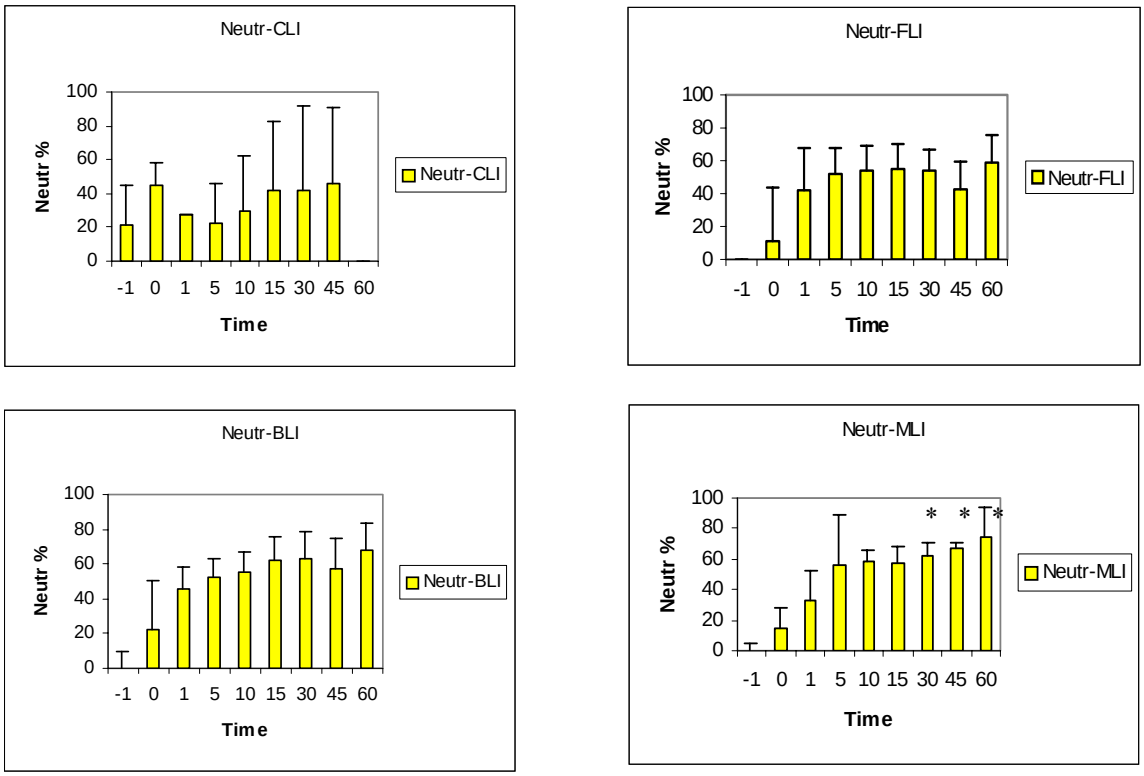


*Fig. 47: Neutrophils-Long Ischemia: Percentage of neutrophils in the different study groups after a LI period(median and standard deviation are given in %). *Time point vs. Control [Time point 0] (p<0, 05).*

The relative number of neutrophils was investigated in the different experiment groups and the results obtained were compared according to three different variables: “time of perfusion”, “drug” and “time of ischemia”. Statistical tests with ANOVA were performed showing statistical significance for “time of perfusion” and “drug” with a p<0,001. “Time of ischemia” presented a significance of p=0,084. Taking “drug” as independent variable, the Scheffe´s test showed that there is statistical significance between the percentage of neutrophils in ATG-Fresenius and ATG-Biotest groups, this being higher in the ATG-Biotest groups (p<0,05). No drug showed statistical differences for the percentage of neutrophils dependent on the length of the ischemia

time. However, the difference between the ATG-Fresenius and ATG-Biotest groups could only be demonstrated for the short ischemia time when we accepted "time of ischemia" as independent variable and drug as dependent variable in the Scheffe´s test ($p<0,05$). No significance for this difference was accepted in the LI time between both groups while the percentage of neutrophils in the control group was lower than in the three ATG groups in LI time.

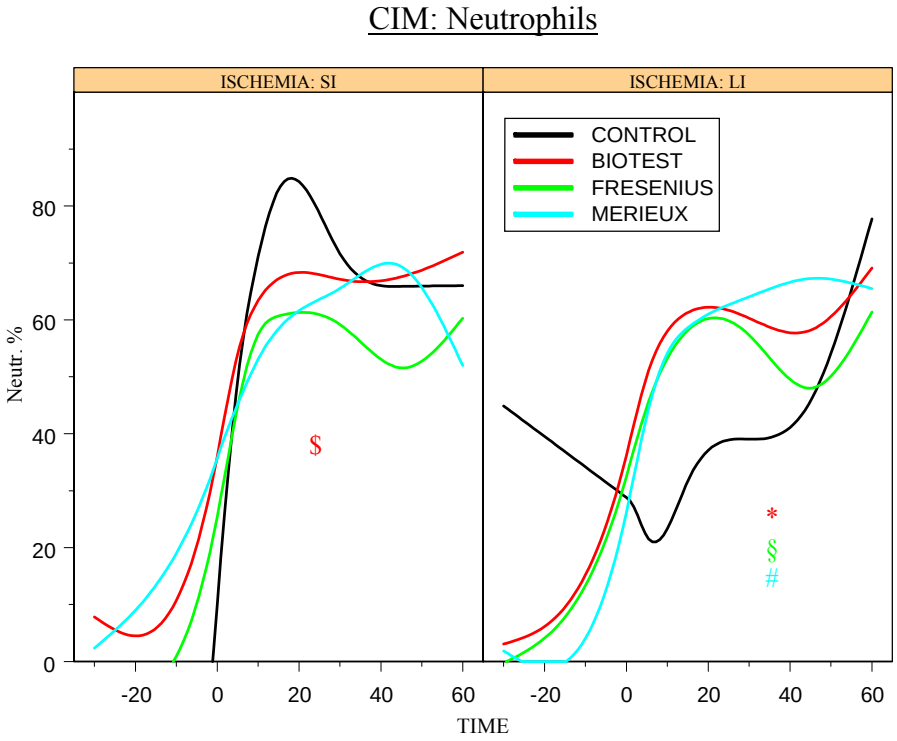


*Fig. 48: Smoothing-Spline of neutrophils (CIM): The percentage of neutrophils of the Biotest-ATG group was significantly higher than the percentage of neutrophils in the Fresenius-ATG group only in the SI time. The percentage of neutrophils in the control group was lower than the three ATG groups in LI time ($ Fresenius-ATG group vs. Biotest-ATG group; *Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: $p<0,05$).*

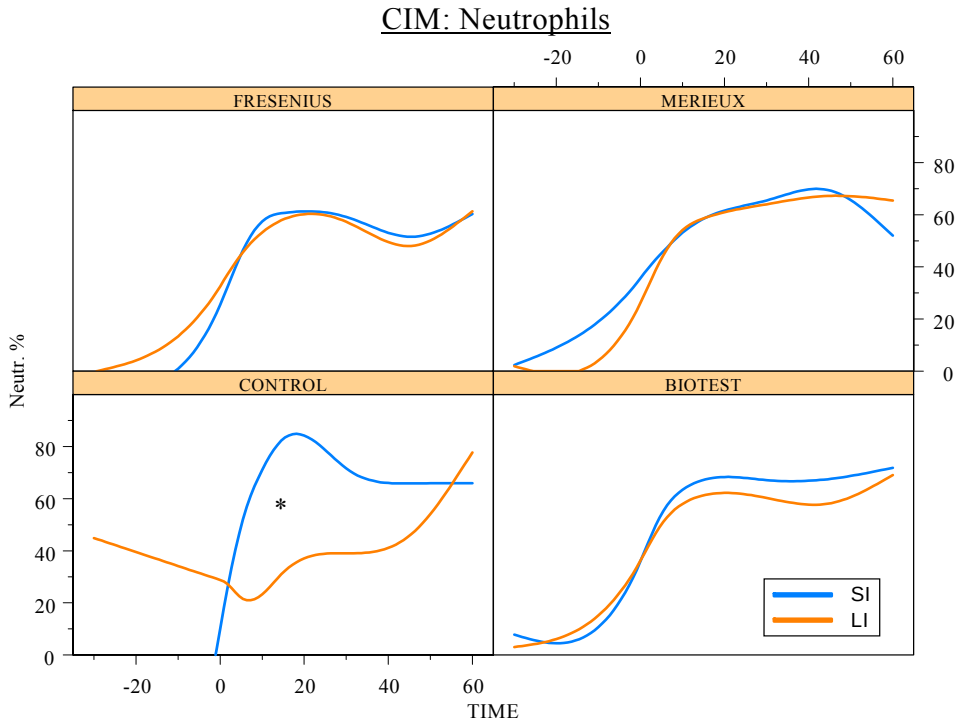


Fig. 49: Smoothing-Spline of neutrophils: Only the control groups showed significant differences between SI and LI times, being the percentage of neutrophils higher in the SI time. (Control SI vs. Control LI p < 0, 05)*

3.3.3. Monocytes

- Short Ischemia: BSI, FSI, MSI, CSI

The relative percentage of monocytes within the absolute number of WBC was evaluated with CIM in the SI groups. Median and standard deviation values for every time point are expressed in %. Due to the reduced percentage of monocytes in relation to the absolute to percentage of WBC, the scale in the graphics is limited 50%. BSI and MSI groups showed a statistically significant decrease of the percentage of monocytes after time point 0 ($p<0,05$; Dunn's test). In contrast to this, FSI and CSI presented no significant differences.

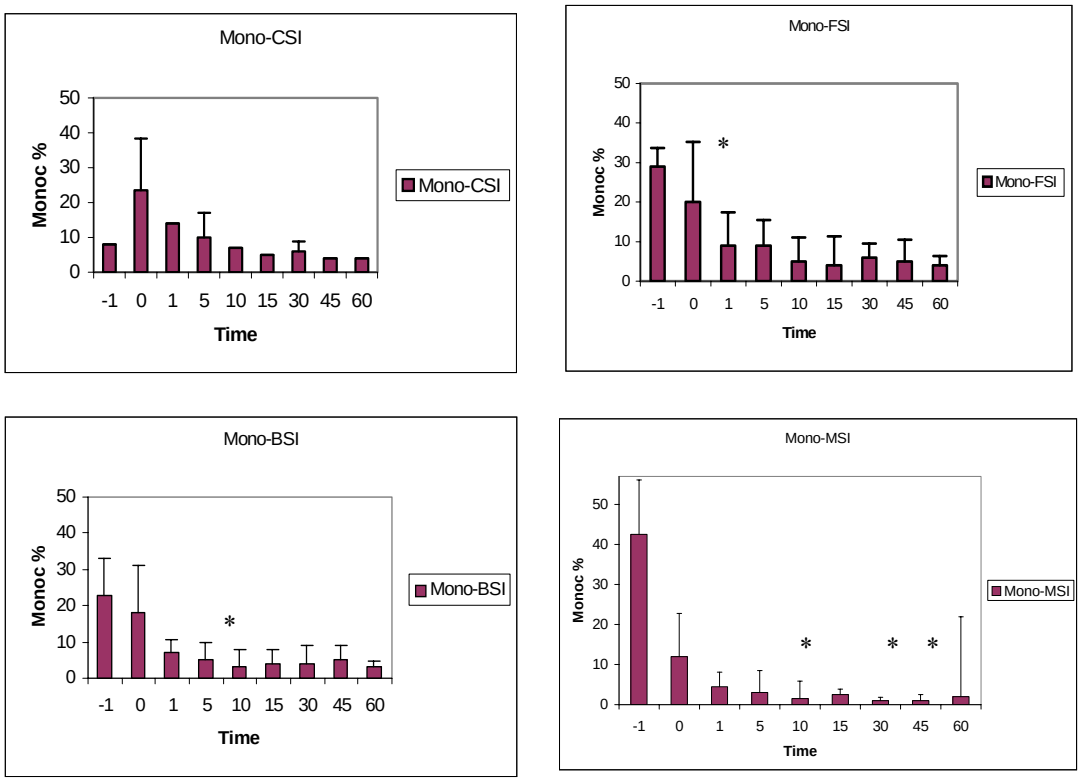
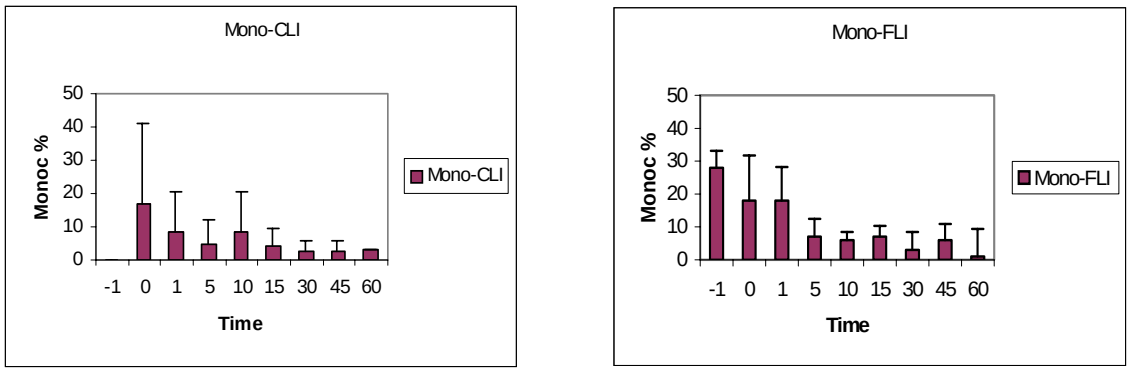


*Fig. 50: Monocytes -Short Ischemia : Percentage of monocytes in the different study groups after a SI period (median and standard deviation are given in %). *Time point vs. Control [Time point 0] (p<0, 05).*

- Long Ischemia: BLI, FLI, MLI, CLI

Percentage of monocytes in differential counting of WBC was performed for LI groups. Median and standard deviation values for every time point are expressed in %. BLI and MLI groups presented a significant decrease of the percentage of monocytes after time point 1. FLI and CLI, however, showed no statistically significant differences.



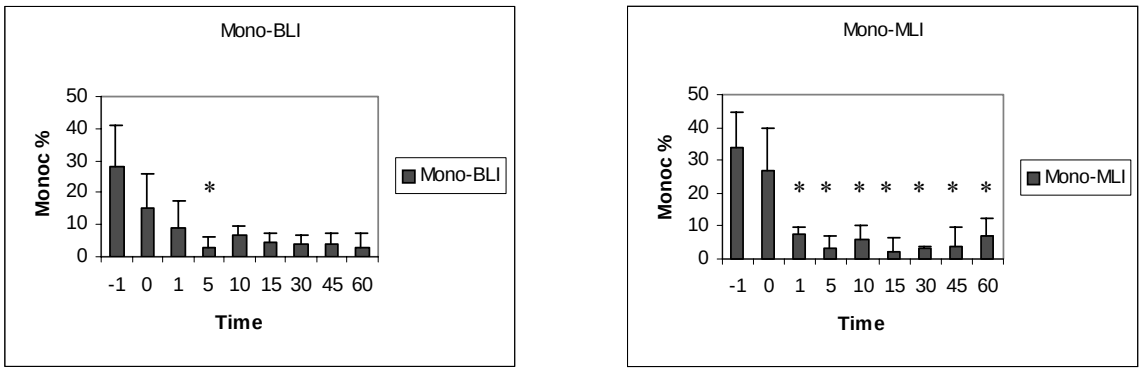


*Fig. 51: Monocytes-Long Ischemia: percentage of monocytes (CIM) after LI period (median and standard deviation are given in %). *Time point vs. Control [Time point 0] (p<0, 05).*

Analytical study of the differences between ATG groups and control groups showed no significances according to the variable "time of ischemia" while statistical significance was obtained for "drug" and "time of perfusion" (p<0.01). Applying Scheffe´s test and using "drug" as independent variable, significance was found between the lower values of the ATG groups and the higher values of the control groups. Statistical differences between ATG groups and control groups were the same in LI groups. However, in SI groups only ATG-Biotest showed statistically significant lower values than the control group.

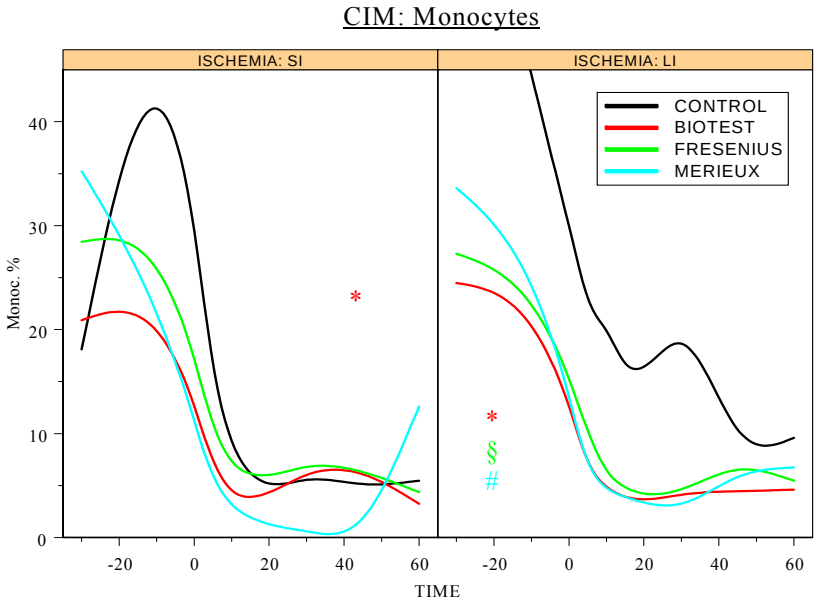


*Fig. 52: Smoothing-Spline of monocytes: The percentage of monocytes in the three ATG groups was statistically lower than the number of WBC in the control group in LI times, while only Biotest-ATG group presented lower values in the SI time. (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG: p<0, 05).*

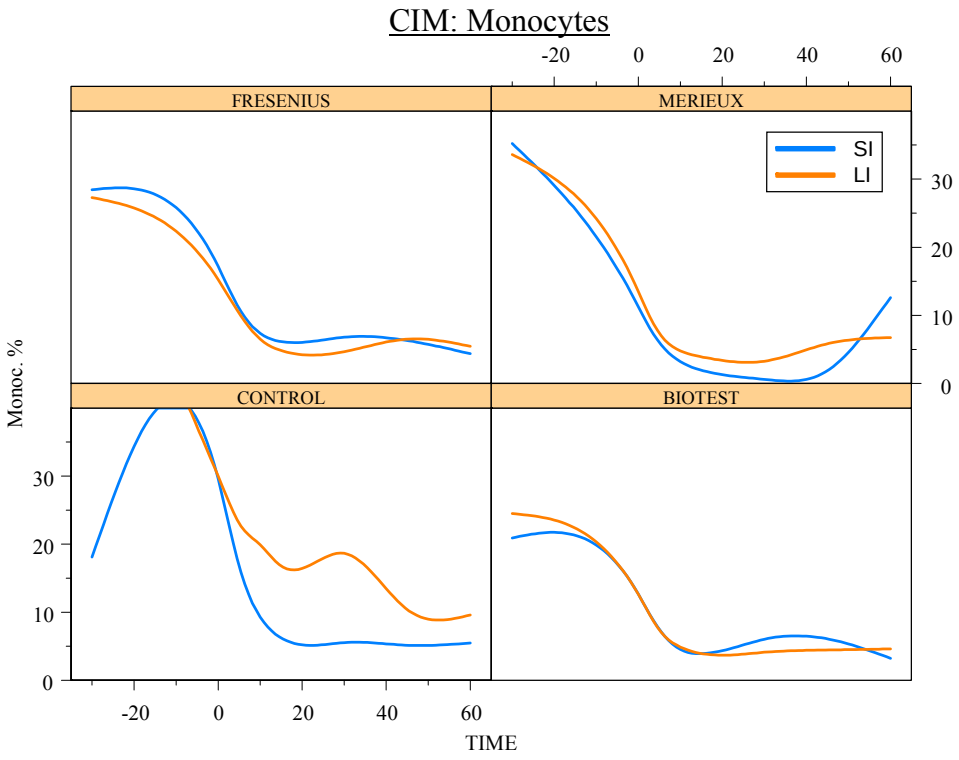


Fig. 53: Smoothing-Spline of monocytes: No statistically significant differences were found according to the length of the ischemia times within the study groups.

3.3.4. Microphotographs

The following microphotographs were taken from the analysed CIM slides. Although it is not possible to show pictures from all the samples performed due to the place restrictions we think they reflect the general behaviour of the groups studied. Note the relative higher number of mononuclear cells in the control group when compared to the treated groups and the higher number of polymorphonuclear cells present in the study groups. All the microphotographs are given as microscope magnifications.

1. Biotest Short Ischemia

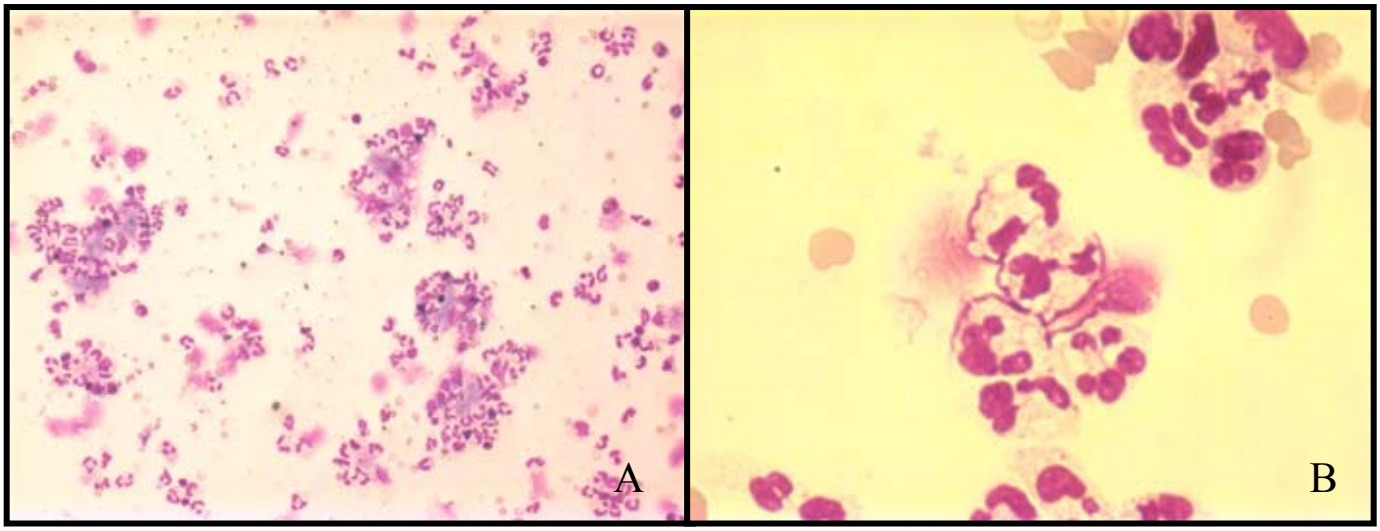


Fig. 54: Biotest SI group presented a high number of neutrophils in comparison to the other ATG groups. Polymorphonuclear cells are shown in the pictures (Fig. 54-A x20, 54-B x100).

2. Biotest Long Ischemia

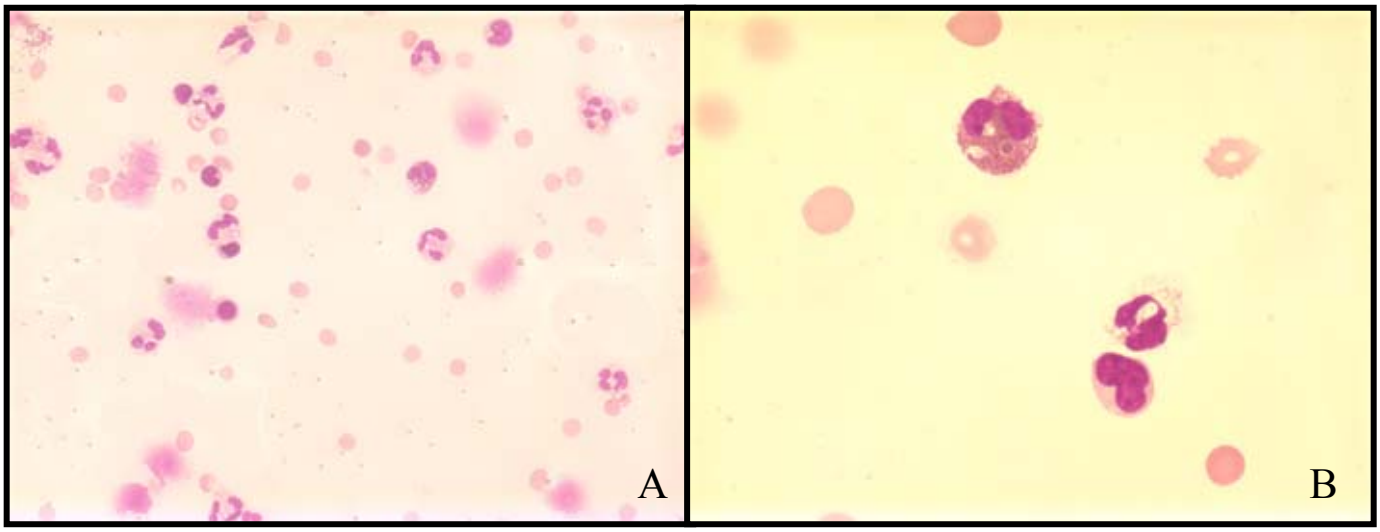


Fig. 55: Biotest LI group presented a high number of neutrophils in comparison to other WBC. Polymorphonuclear cells are shown in the pictures (Fig. 55-A x40, 55-B x100).

3. Fresenius Short Ischemia

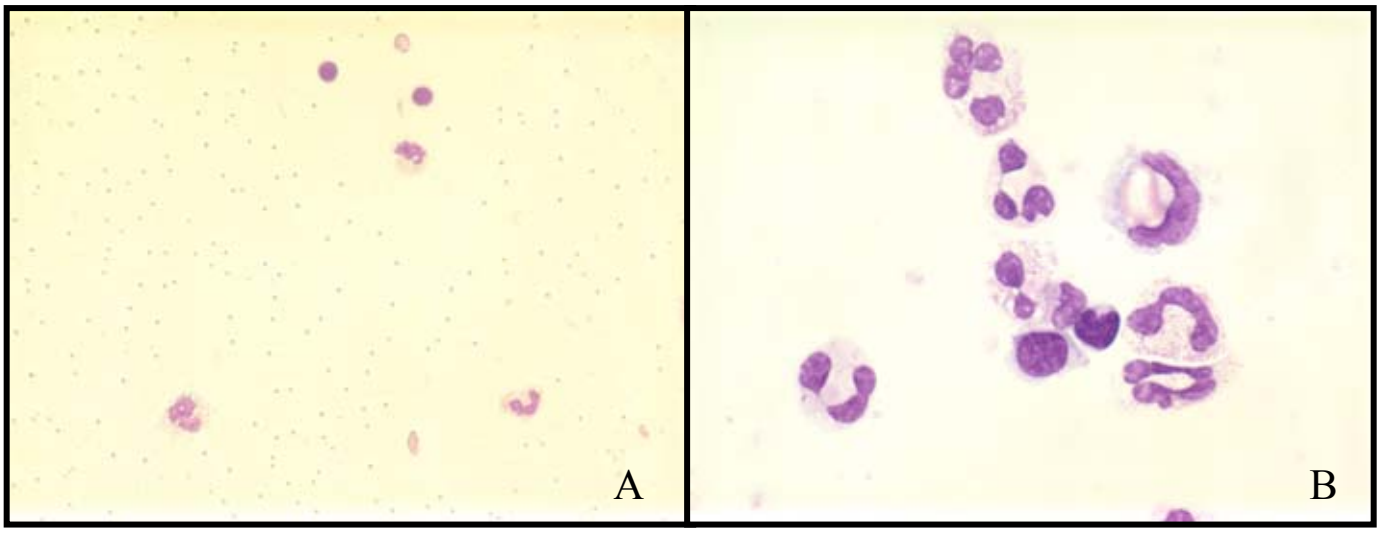


Fig. 56: Fresenius SI group presented a lower percentage of neutrophils than Biotest-ATG group in CIM. However, more neutrophils than other mononuclear cells were observed (Fig. 56-A x20, Fig. 56-B x100).

4. Fresenius Long Ischemia

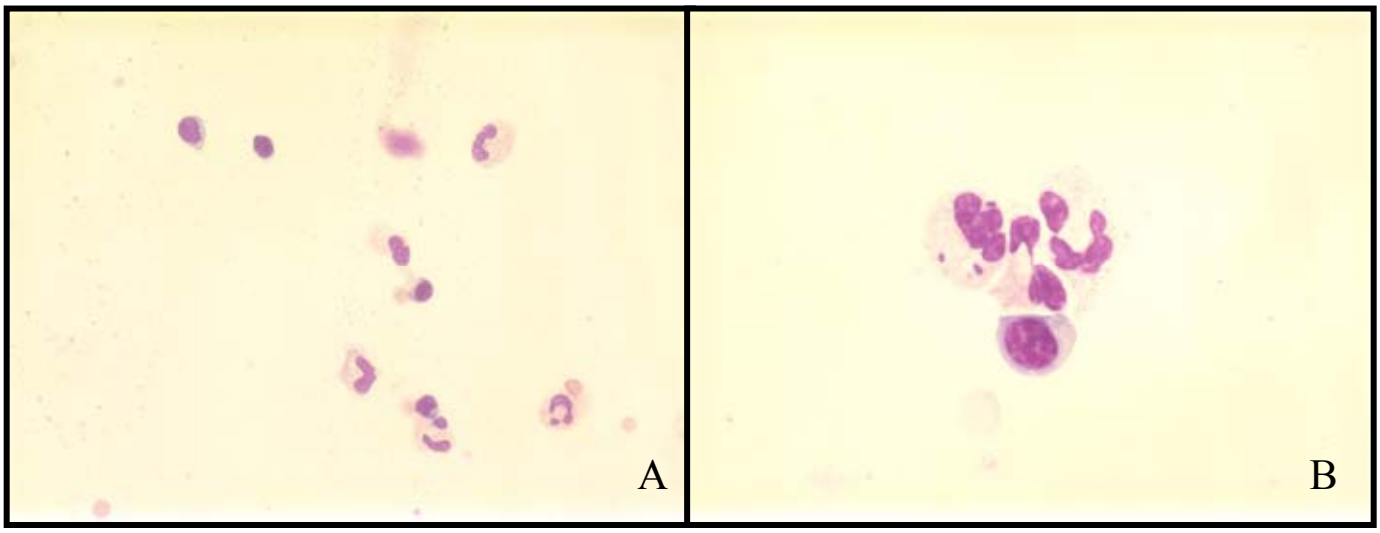


Fig. 57: Fresenius LI presented more polymorphonuclear cells than mononuclear cells, however, mononuclear cells were observed (Fig. 57-A x40, Fig. 57-B x100).

5. Merieux Short Ischemia

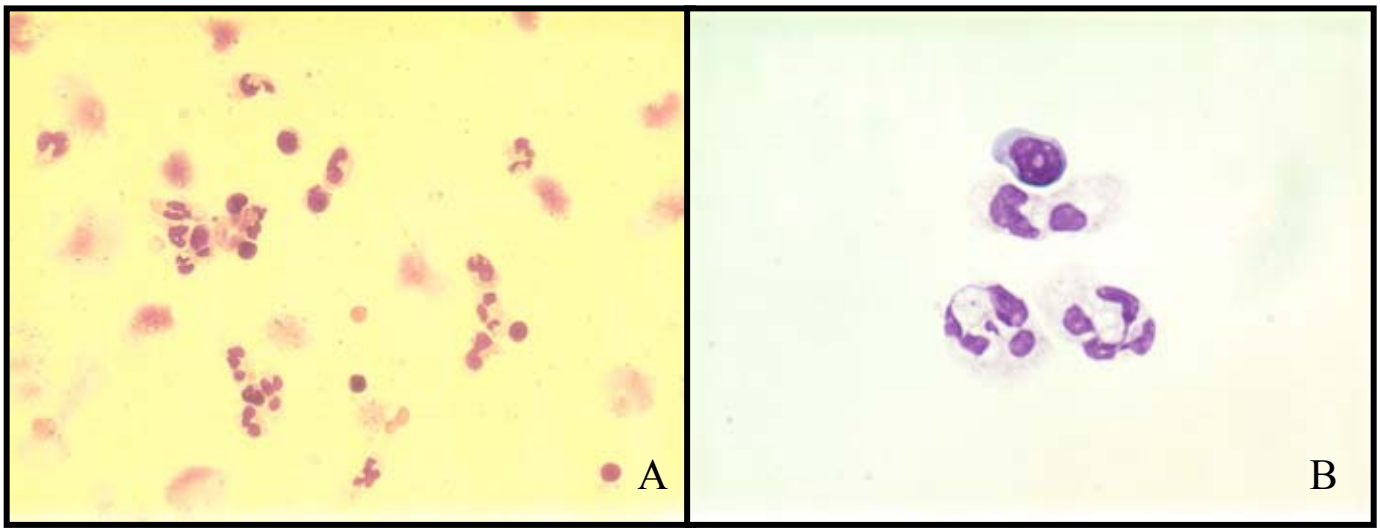


Fig. 58: Merieux SI showed, like the other ATG groups, a lower amount of cells in the preparations studied. This group also presented more neutrophils than mononuclear cells (Fig. 58-A x40, Fig. 58-B x100).

6. Merieux Long Ischemia

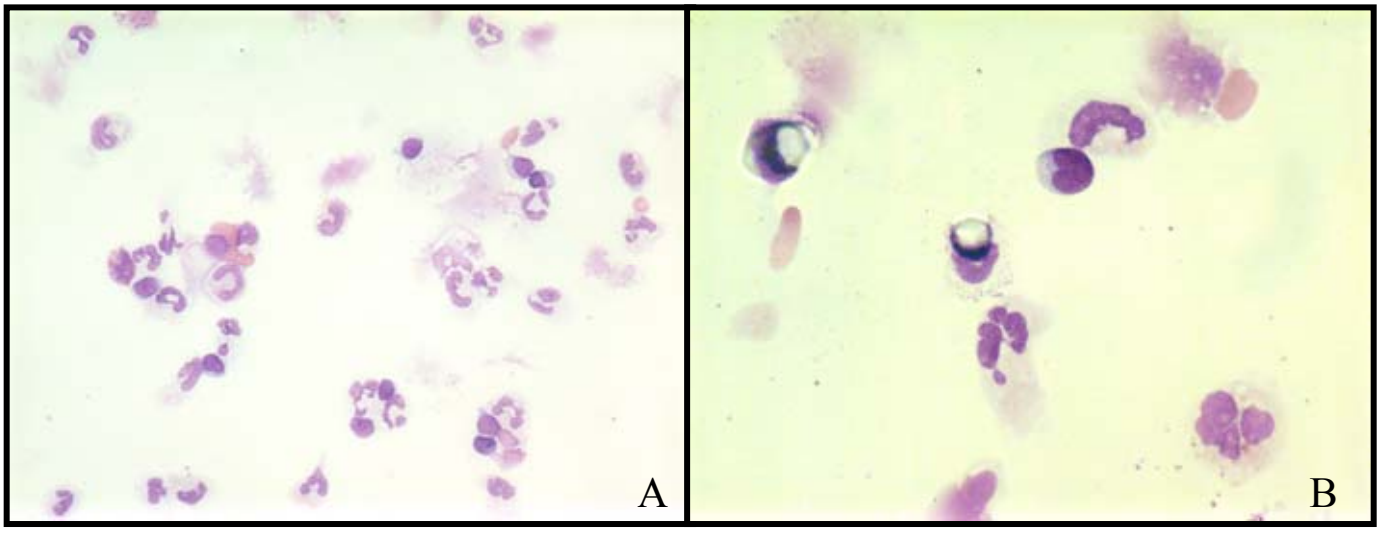


Fig. 59: MLI group presented a higher amount of polymorphonuclear cells in comparison to other WBC (Fig. 59-A x40, 59-B x100).

7. Control Short Ischemia

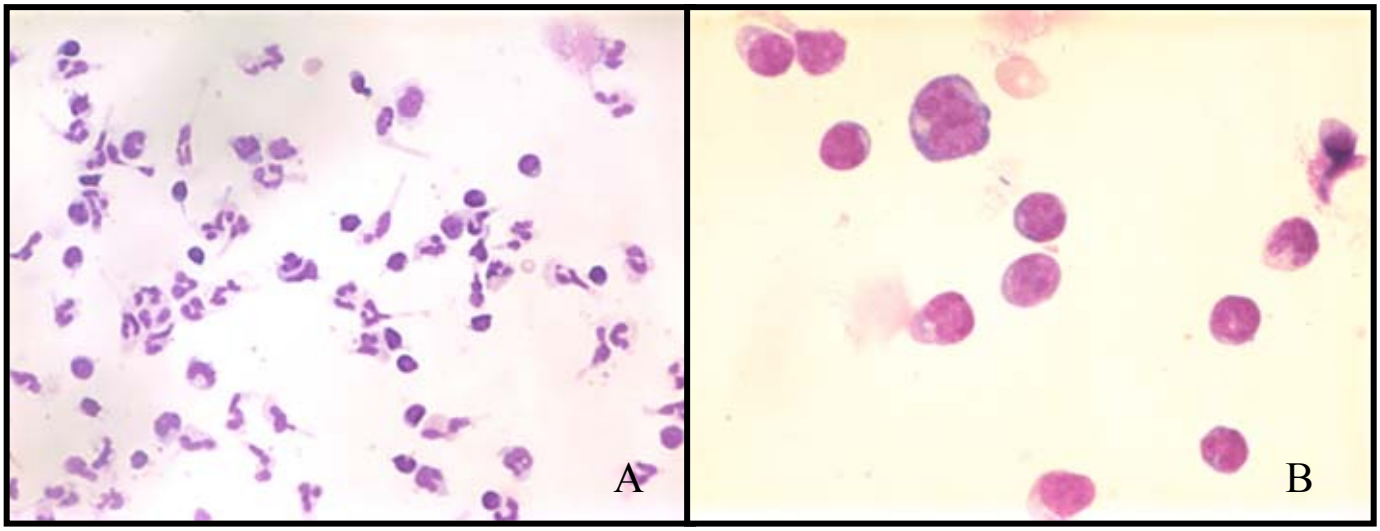


Fig. 60: CSI group presented a higher density of cells in the preparation. At the same time, there were more mononuclear cells than in the ATG groups, mostly lymphocytes (Fig. 60-A x40, 60-B x100).

8. Control Long Ischemia

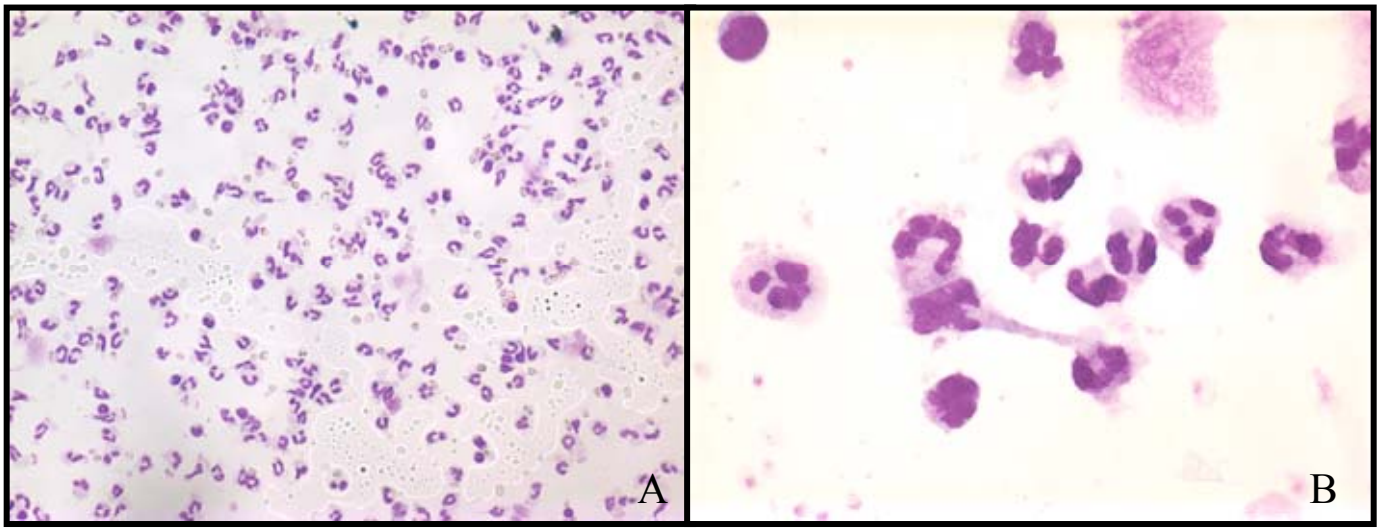


Fig. 61: A higher amount of mononuclear cells was also observed in the CLI group. Cellular density was higher than in the ATG-groups, as observed in the pictures (Fig. 61-A x20, Fig. 61-B x100) .

3.4.Histology and Immunohistochemistry

3.4.1 Results

Histological and immunohistochemical techniques were performed to assess the muscle damage after different times of ischemia and after reperfusion. In addition, samples were analysed for leukocyte infiltration in connective, vascular, perivascular and muscular tissue as well as for the vascular presence of fibrin.

Results are expressed as the mean result ± standard deviation obtained from the different fields studied. The preparations were evaluated randomly and blind. The criteria were arbitrarily established although based on previous works in the literature (17, 38, 74, 149):

- Muscle damage: loss of architecture, necrosis or signs of ischemia were considered:
    - normal architecture: 0
    - light damage: 1
    - moderate damage: 2
    - severe damage: 3
- Soft tissue infiltration: Connective tissues present in the biopsy as well as connective perimisial structures were considered.
    - any WBC in these structures: 1
    - more than 10 cells per field: 2
    - more than 25 cells per field: 3
- Vascular infiltration: Cells present in the vascular spaces were considered, free or attached to the endothelial walls.
    - free cells in the vessels: 1
    - free and attached to the endothelial cells: 2

- free cells, attached cells, granulocytes, occluding or almost occluding the vessel lumen: 3

- Perivascular infiltration: Presence of WBC in any tissue adjacent to the vessels:
  - presence of cells attached to endothelial walls: 1
  - presence of cells in perivascular connective tissue: 2
  - presence of cells in perivascular connective tissue and muscular tissue: 3

- Muscular infiltration:
  - no cells in muscle fibres: 0
  - 1-5 cells pro muscular fibre: 1
  - >10 cells pro muscular fibre: 2
  - >25 cells pro muscular fibre: 3

Results are expressed as the mean values obtained from the different fields studied. The preparations were labelled with a code and evaluated blindly.

Fibrin, Masson's T and CD31 immunostaining were performed to localise fibrin and thrombus formation and the criteria were established as follows:

- Presence of fibrin or thrombocytes in the vascular spaces:
  - presence of fibrin or thrombocytes without occlusion: 1
  - occlusion of the vessel: 2
  - occlusion of the vessel and destruction of the vascular or endothelial integrity: 3

The immunoreaction with IL4 was classified as positive or negative depending on the presence or absence of this molecule in the sections studied.

Muscle damage, connective and muscle tissue infiltration as well as presence of fibrin in the vessels are presented in the following table (table 5). The values are expressed in mean ± standard deviation. ANOVA was employed to perform the statistical analysis.

	CSI		FSI		BSI		MSI		CLI		FLI		BLI		MLI	
	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D
Muscle Damage	2,6	0,51	0,9	0,73	1,1	0,73	1,2	0,63	2,8	0,42	1	0,66	1,6	0,69	1,4	0,51
Connect. Infiltration	2,5	0,52	1,1	0,31	1,2	0,42	1,3	0,48	2,3	0,67	1,3	0,48	1,4	0,51	1,3	0,48
Vascular Infiltration	2,3	0,48	1,1	0,31	1,3	0,48	1,4	0,51	2,4	0,69	1,5	0,52	1,5	0,52	1,3	0,48
Perivasc. Infiltration	2,3	0,67	1,2	0,42	1,3	0,48	1,3	0,48	2,3	0,67	1,2	0,42	1,4	0,51	1,5	0,52
Muscular Infiltration	2,4	0,69	0,5	0,52	0,4	0,51	0,4	0,51	2,7	0,48	0,4	0,51	0,6	0,51	0,9	0,79
Presence Fibrin	1,9	0,56	0,3	0,48	1,2	0,63	0,8	0,63	2,2	0,63	0,8	0,63	1,3	0,67	1,1	0,56

Table 5: Histological criteria: Values for Control and ATG groups

These results are also expressed in the following figures to illustrate the differences between the studied groups. Morphological differences were typical within the control and ATG groups. Control groups´ muscle presented more necrosed fibres, loss of normal muscular tissue architecture, areas of haemorrhage and massive and diffuse infiltration of leukocytes in almost all the biopsies studied. On the other hand, the ATG groups presented less muscle damage, with slight infiltration of the perivascular areas and almost no presence of WBC in the muscular tissue.

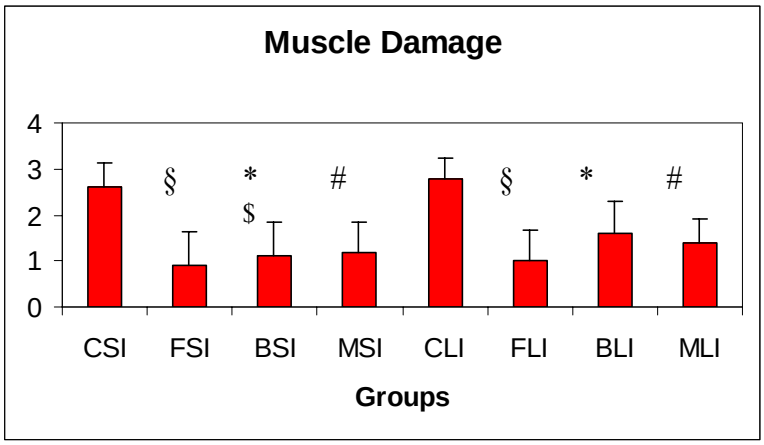


*Fig. 62: Muscle damage: ATG groups showed less muscle damage than control groups in short and long ischemia times. Biotest groups showed differences between different ischemia times. (*Control Vs. Biotest-ATG; § Control Vs. Fresenius-ATG; # Control Vs. Merieux-ATG; $ BSI vs. BLI: p<0,05).*

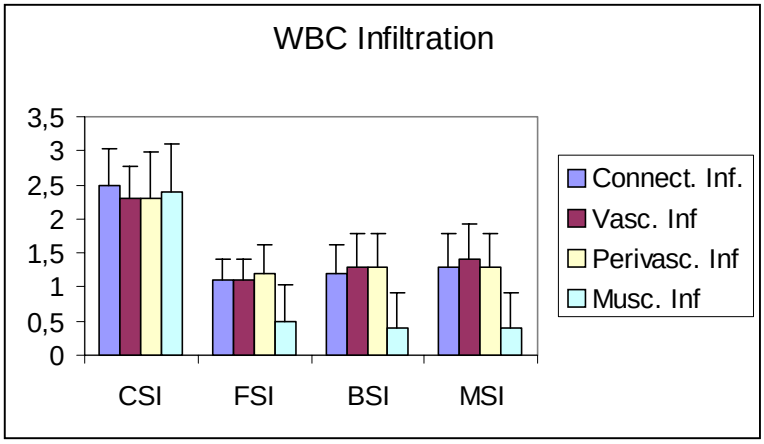


Fig. 63: Leukocyte infiltration in the SI groups

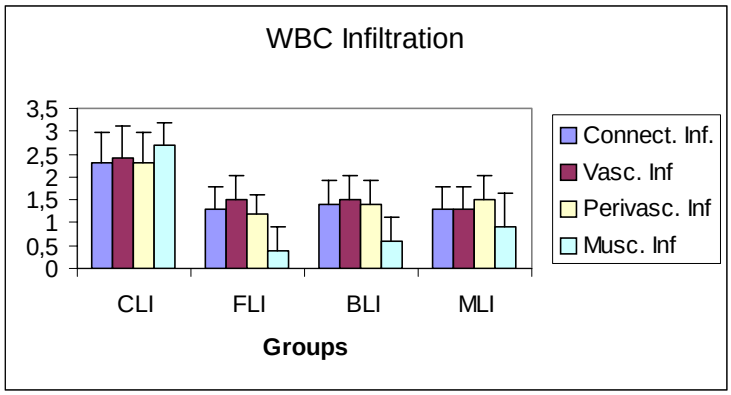


Fig. 64: Leukocyte infiltration in the LI groups

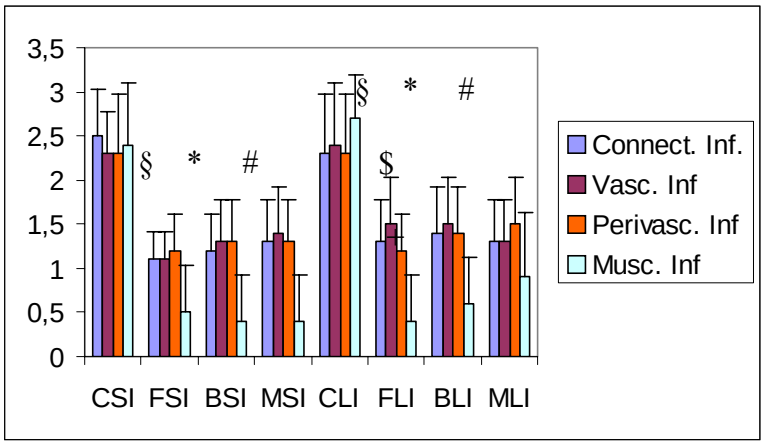


*Fig. 65: ATG groups showed lower infiltration than the control groups for both ischemia times. Fresenius-ATG group showed less perivascular and muscular infiltration than the other ATG- groups after LI time. (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG; $, +, FLI vs. BLI and MLI: p<0.05).*

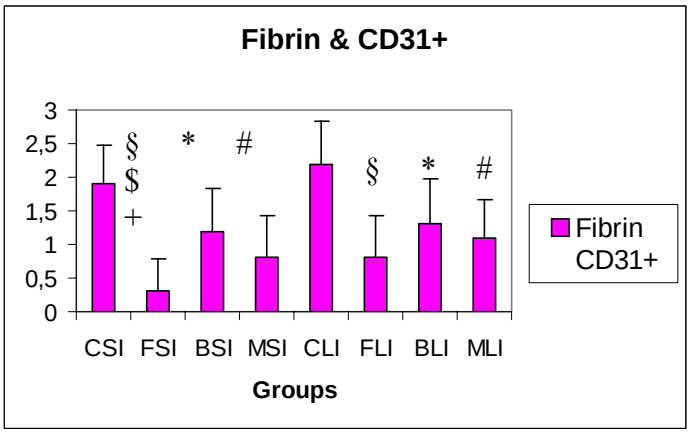


*Fig. 66: Fibrin and CD31+: ATG groups presented lower fibrinoid aggregates and CD-31 positive reaction than the control groups for both ischemia times. Fresenius-ATG group and Merieux-ATG group showed more intense reaction in LI times. Fresenius-ATG group showed less intense reaction than the other ATG-groups in SI time (*Control vs. Biotest-ATG; § Control vs. Fresenius-ATG; # Control vs. Merieux-ATG; $, +, FSI vs. BSI and MSI: p<0.05).*

Comparisons within the different groups using the above mentioned criteria presented statistical differences on the muscle damage, leukocyte infiltration and presence of fibrin between the ATG groups and the control groups. ATG groups showed less muscle damage, leukocyte infiltration and vascular presence of fibrin compared to the untreated groups together and taken one by one in separate comparisons. Individual comparisons were performed “a posteriori” to detect further significances between the groups.

All treated groups presented less muscle damage than the control groups. When considering ischemia time as independent variable, statistical analysis showed differences only between the Biotest groups, with more muscle damage being observed in the LI biopsies. No statistical differences in muscle damage were observed within Biotest and Merieux groups both in short and long times of ischemia. In contrast to this, Fresenius presents less muscle damage than the other ATG groups both in SI and LI times.

When leukocyte infiltration was analysed according to the explained criteria, ATG groups showed statistically significant diminution of infiltration in comparison to the control groups in every parameter studied: connective, perivascular, vascular and muscle tissue infiltration both in SI and LI times ($p<0.05$).

The ATG groups were compared one-by-one to detect differences in the various criteria studied. No differences were found between the study groups according to connective and vascular tissue infiltration, both in SI and LI times. No differences were found in SI time while studying perivascular tissue infiltration. However, Fresenius-ATG groups presented statistically significant lower values than the other study groups in perivascular tissue infiltration in the LI time ($p<0.05$). Muscle tissue infiltration presented no significant variances in the SI time groups although these differences were present in the LI groups, where the Fresenius-ATG group showed less muscle infiltration than the other study groups.

The differences between study groups treated with the same drug, only varying the ischemia time, were studied, these differences only being statistically significant in the case of the Merieux-ATG group, with significant higher values of leukocyte infiltration in the LI group.

Presence of fibrin and activated platelets as well as endothelial cells (CD31+) was studied. Control groups showed statistically significant higher values than the study groups both in long and short ischemia times ($p<0.05$). Intragroup differences according to the length of ischemia time were only significant in the Fresenius-ATG and Merieux-ATG groups, both presenting

higher values of CD31-like immunoreaction and fibrin in the LI groups. After one-to-one comparison between the drug groups, Fresenius-ATG group showed statistically significant lower values than the other groups in SI time, while no statistically significant differences were detected between the study groups within the LI time.

Presence or absence of IL-4 was studied as described before, without considering the different ischemia times. Statistical significance between the control groups and the ATG groups was detected with student's t-test. Expression of IL-4 measured by immunohistochemical means was significantly higher in the control groups than in the study groups.

Biopsy	Control	Biotest	Fresenius	Merieux
+++	11/12	1/12	0/12	0/12
++	1/12	1/12	1/12	0/12
+	0/12	3/12	1/12	1/12
0	0/12	7/12	10/12	11/12

Table 6: Positive immunoreaction for IL-4 was found mostly in the control group.

In the following pages, microphotographs of the control and study groups with the performed histological and immunohistochemical techniques will be shown. The pictures will be commented after each group of images. All pictures are shown as microscope magnifications.

3.4.2. Hematoxiline/Eosin

3.4.2.1 Control Short Ischemia

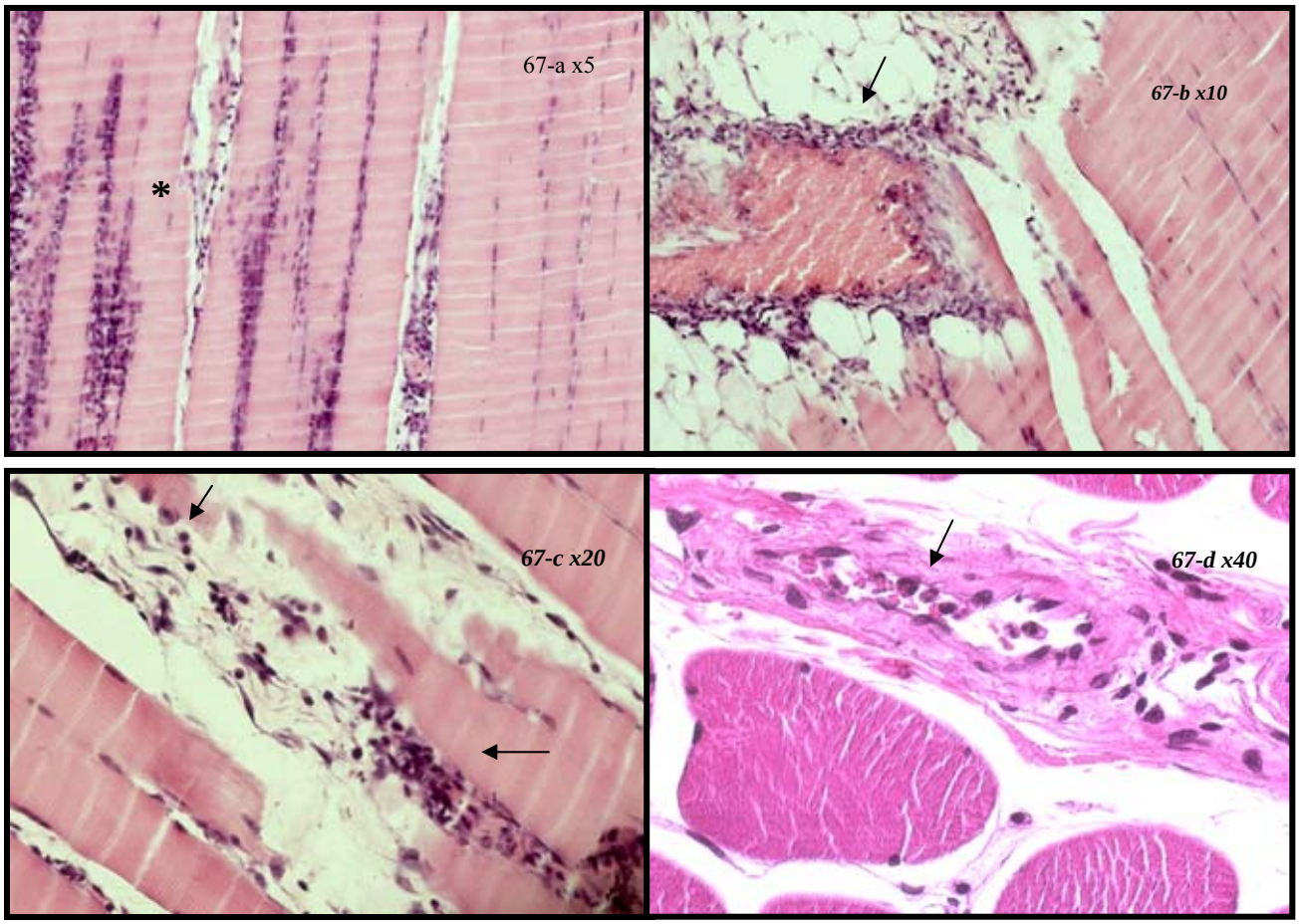


Fig. 67:In the control group, massive and diffuse infiltration and inflammation phenomena can be observed. Note the extensive infiltration on the picture 67-a. Necrosis and hemorrhagic features as well as tissue infiltration are to be seen in the second picture. Infiltration of the connective perivascular tissue is described in the microphotograph 67-c (arrow). Morphological characteristics of these infiltrates are shown in the picture 67-d. Note: the segmented nucleus of the neutrophils adherent to the endothelial cells (67-d, arrow).

3.4.2.2: Biotest Short Ischemia

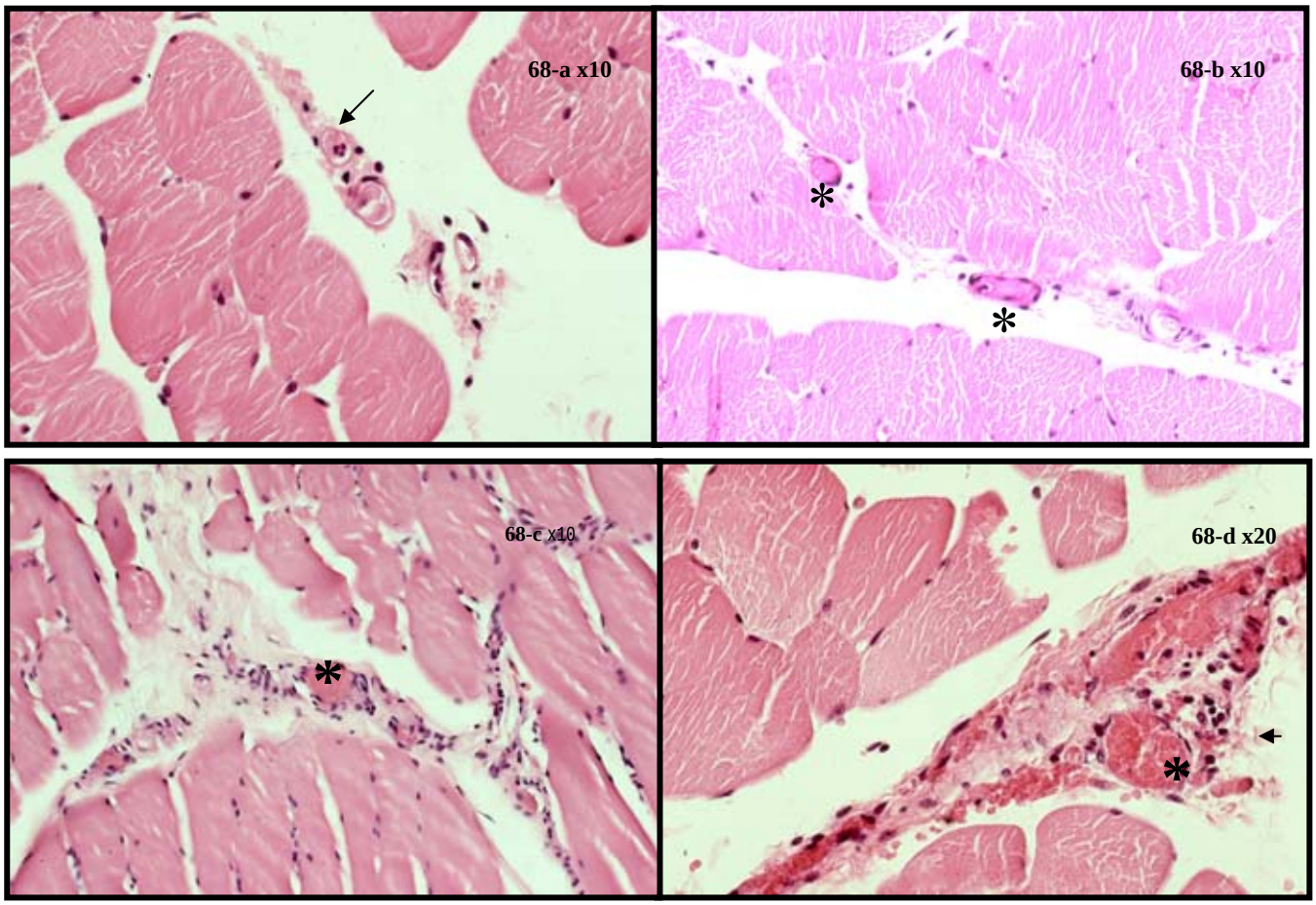


*Fig. 68: BSI group presents a well conserved muscular structure and low tissue infiltration. However, the aggregation of platelets and fibrin formation in the small vessels is characteristic. These aggregates usually include WBC (Fig.68-a). Vessel occlusion is signaled with a * symbol. In the picture 68-d, hemorrhagic phenomena can be observed, with extra-vasated erythrocytes in the histological preparation.*

3.4.2.3: Fresenius Short Ischemia

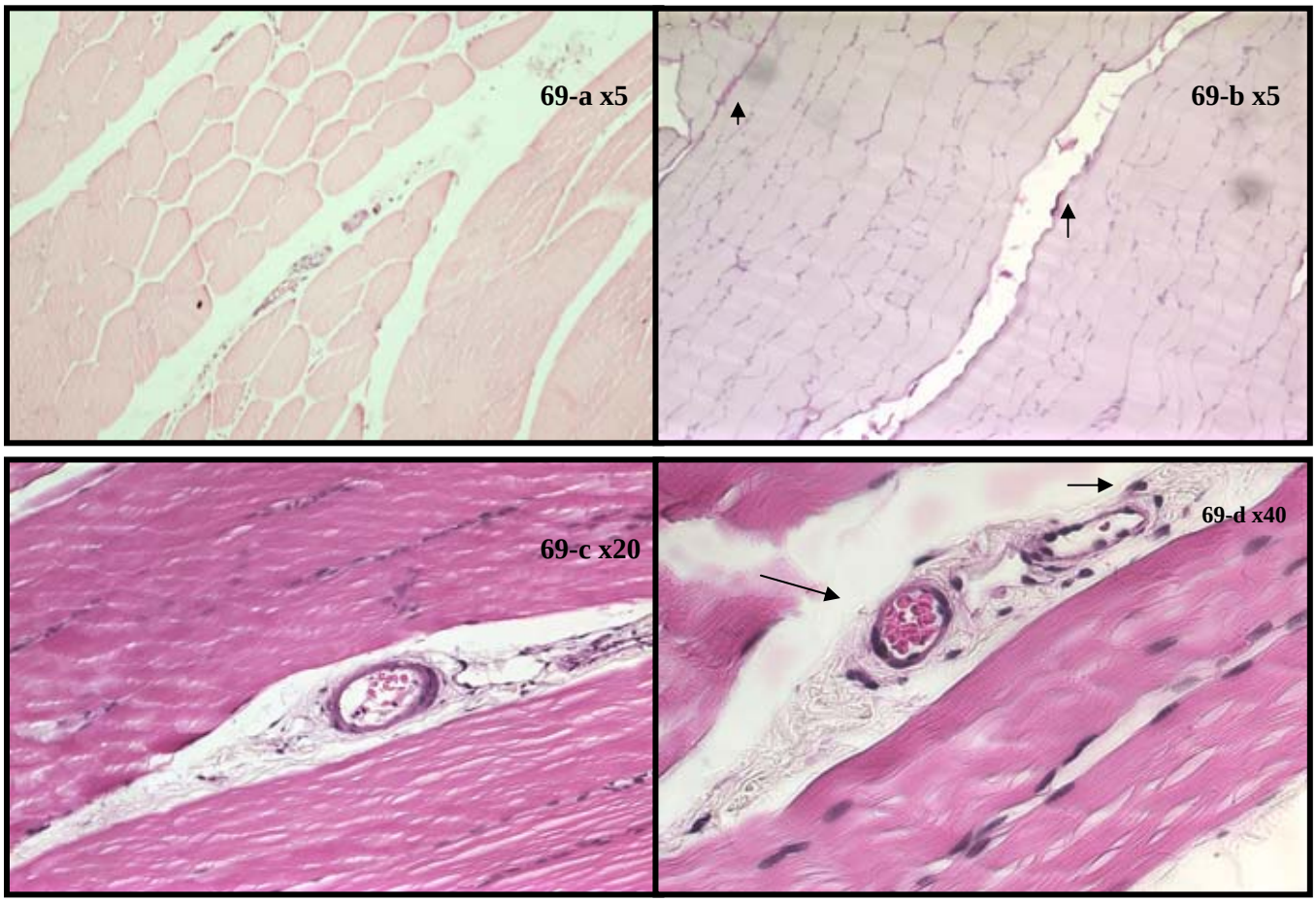


Fig. 69: Lower muscle damage than in the control group was detected in the FSI group. The muscle structure is well conserved (69-a, 69-b). However, areas of ischemic tissue can be observed in the external perimeter of some muscular fibers (arrows, 69-b). Leukocyte infiltration into connective, vascular, perivascular and muscular tissue is slight and comparable to untreated human muscle sections (69-d). Most of the endothelial structures show neither loss of structure nor adherence of WBC. (x 10, 20: microscope magnifications)

3.4.2.4: Merieux Short Ischemia

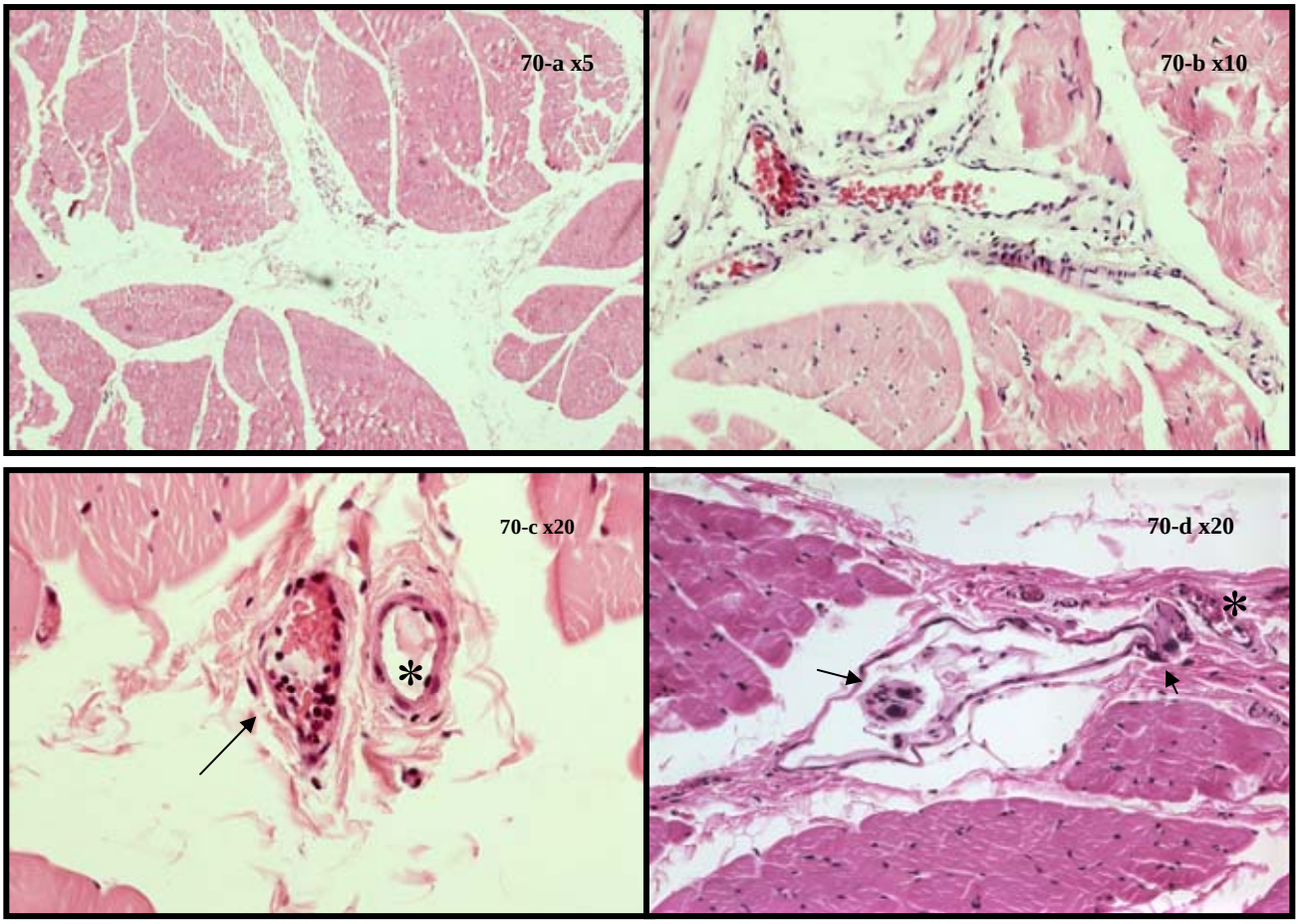


Fig. 70: Biopsies of the Merieux Short Ischemia group present a well defined muscular and vascular structure. Only slight damage was found. However, perivascular and vascular infiltration was observed in some of the biopsies, showing cellular presence on the connective perivascular tissue (70-c) or included in aggregates on the vascular lumen (70-d). Some of the vessels presented accumulation of fibrin or degradation materials without occluding the vascular lumina, as shown in the figures 70-c and 70-d. (, x 10,20 : microscope magnifications)*

3.4.2.5: Control Long Ischemia

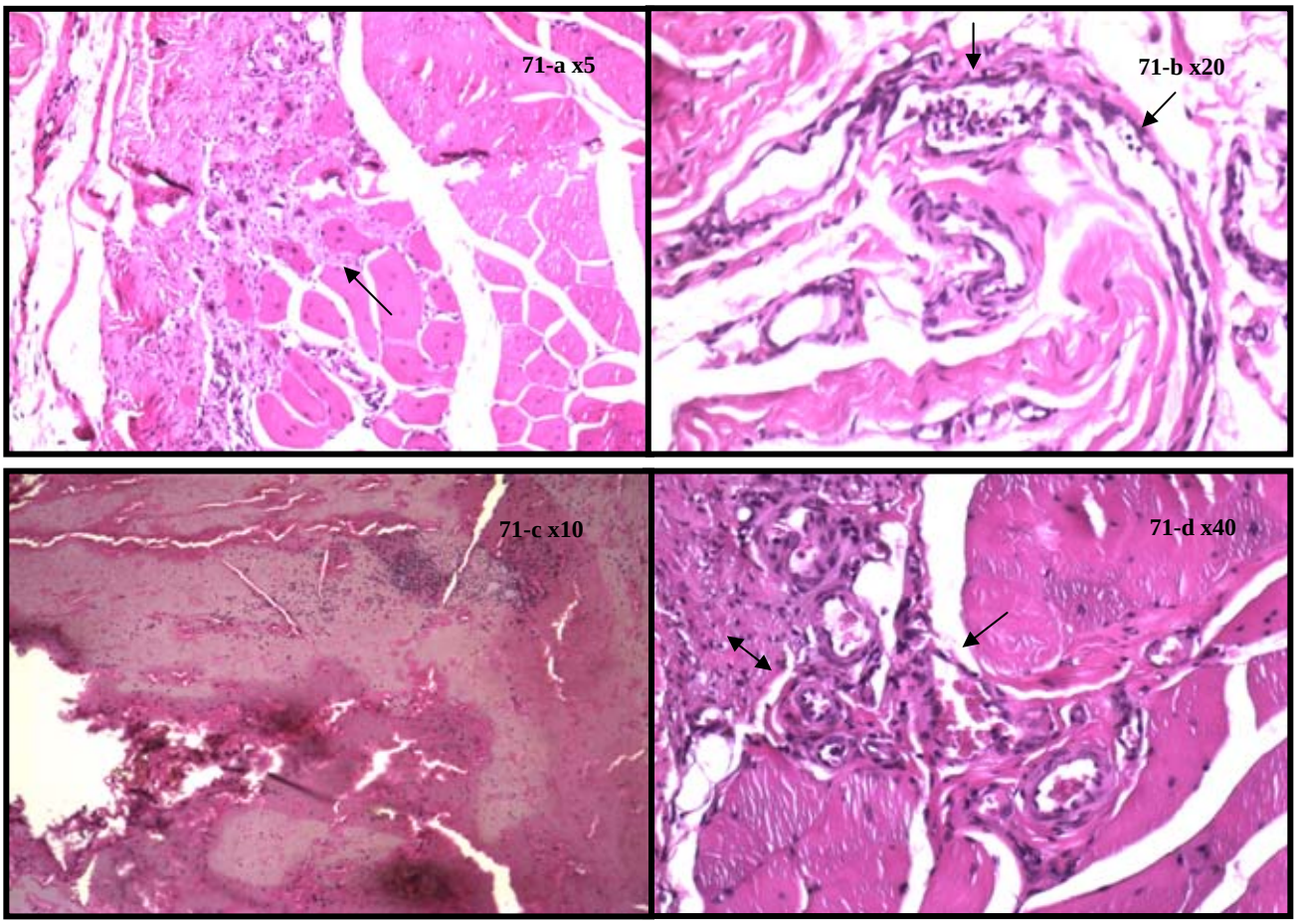


Fig. 71: Biopsies obtained from CLI group show massive loss of structure, fiber necrosis and hemorrhagic phenomena of muscle. In the picture 71-a, necrosis of muscle fibers can be observed. Infiltration of perimisial tissue is shown on the second picture (71-b). Necrosis and ischemic features can be observed at a low magnification on the third image (71-c). Please note the diffuse infiltrate present on the muscle surface. The photograph 71-d shows some vessels situated between damaged muscle fibers.

3.4.2.6: Biotest Long Ischemia

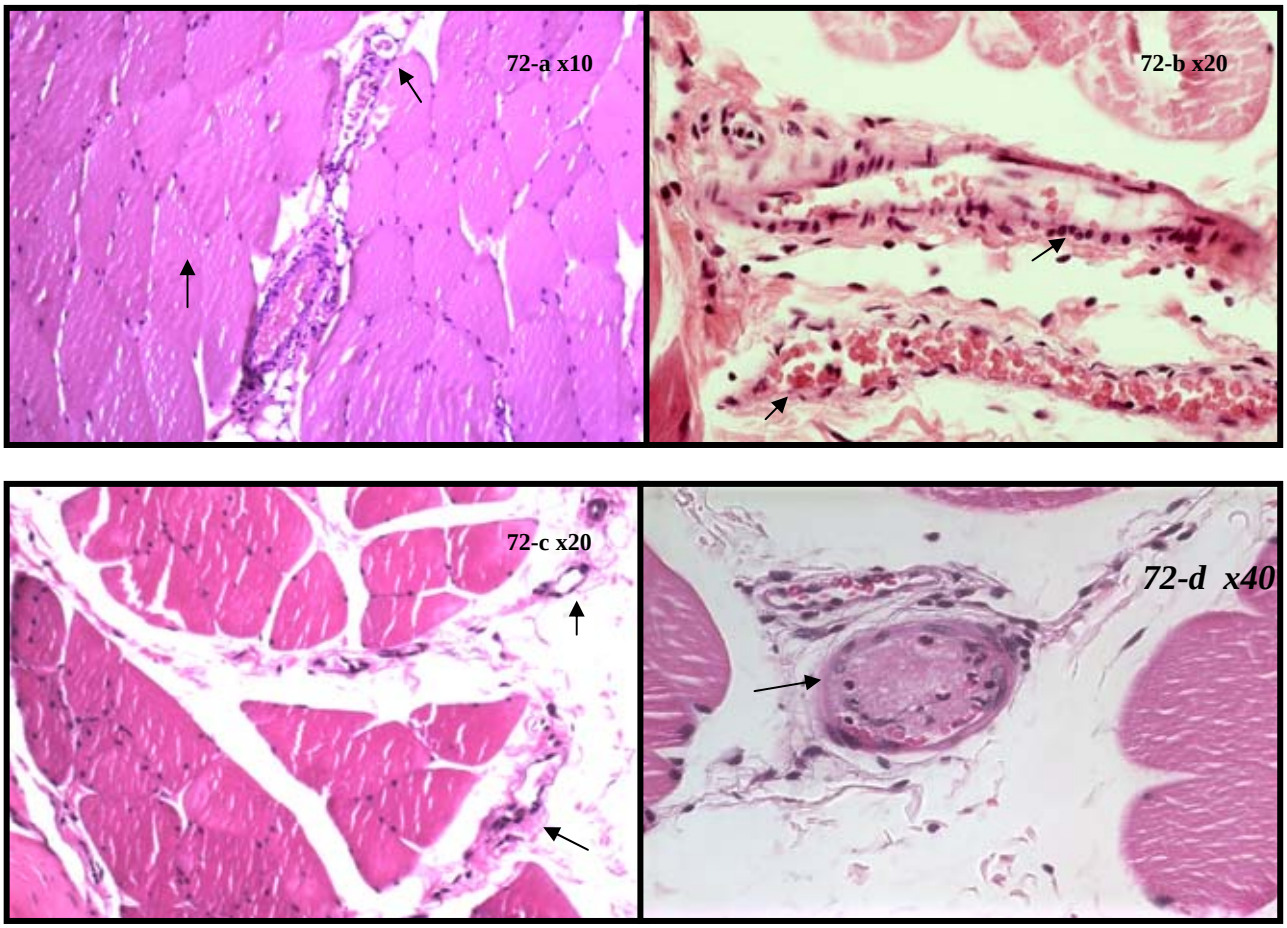


Fig. 72: These microphotographs from the BLI group show a well conserved muscle structure and no muscle infiltration (Fig. 72-a, arrows). However, there are WBC present on vascular and perivascular structures (Fig. 72-b superior arrow, Fig. 72-c). WBC included in an aggregate adherent to the endothelial wall can be observed in the picture 72-b (inferior arrow). Some vessels, mostly venous, present fibrinoid aggregates even occluding the vascular lumen, as shown in the picture 72-d (arrow).

3.4.2.7: Fresenius Long Ischemia

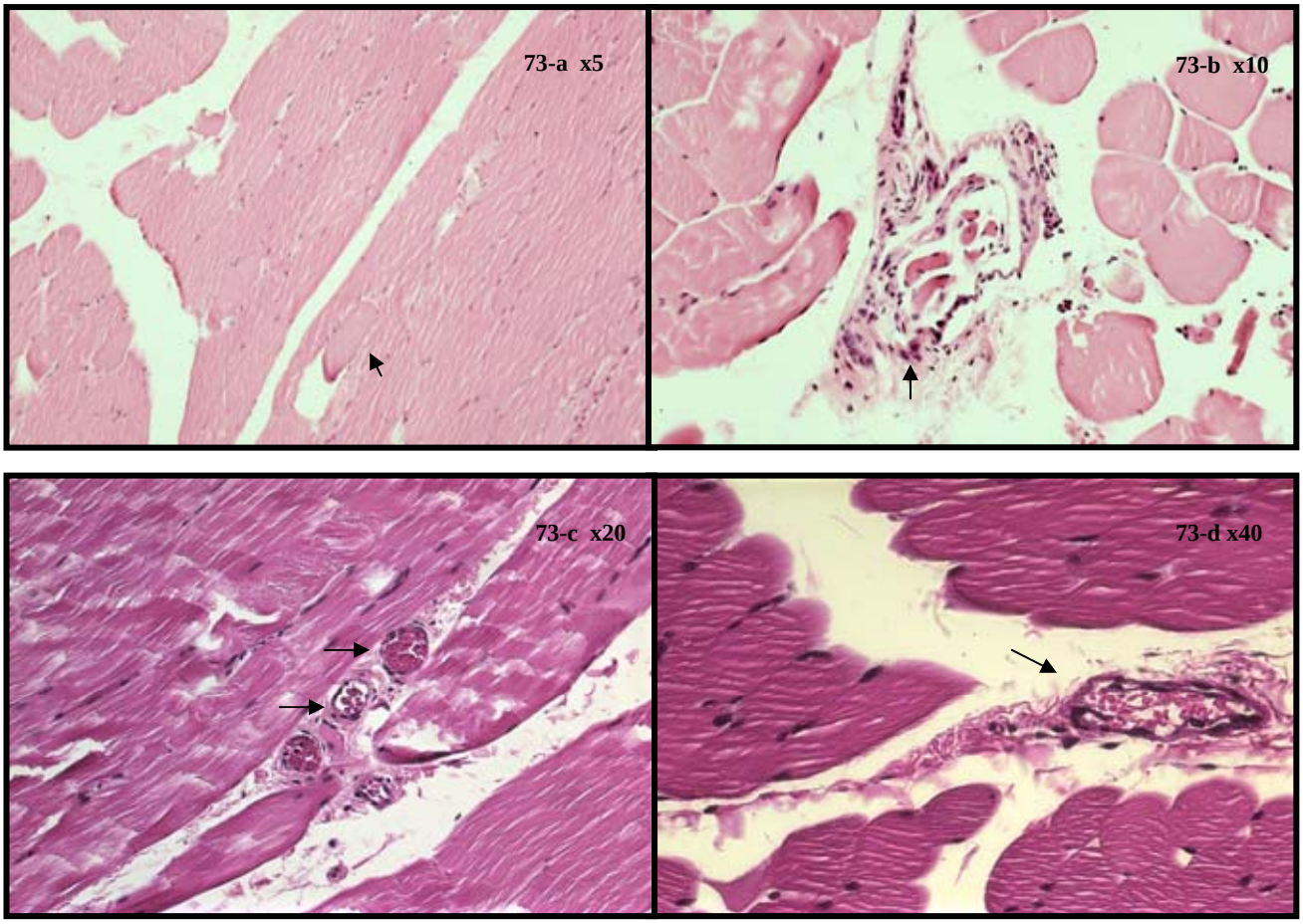


Fig. 73: FLI group presents a well-conserved muscle structure with slight tissue damage (fig. 73-a, 73-b). Necrosis is observed in isolated fibers (73-a, arrow). Vascular lumen are not occluded (73-c, 73-d, arrows) and show no or slight infiltration or WBC adherent to endothelia (73-c, 73-d). Perivascular and perimisial connective tissue show low WBC presence.

3.4.2.8: Merieux Long Ischemia

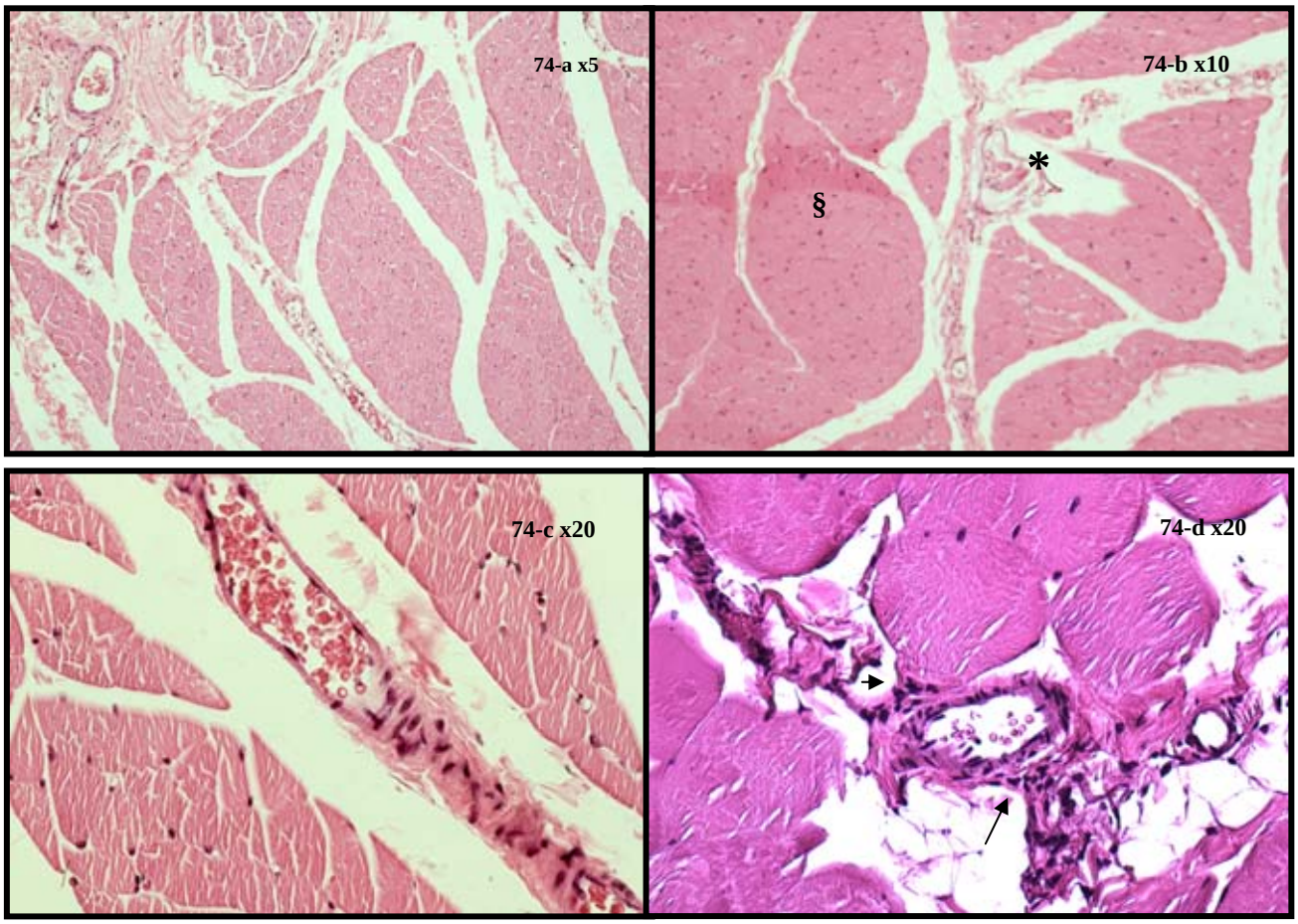


Fig. 74: MLI group shows no leukocyte infiltration in most of the biopsies. The muscular tissue is well structured (74-a), although ischemic damage of the muscle is to be seen in some of the histological sections (74-b, §). There is slight connective tissue and vascular infiltration in comparison to control groups although presence of WBC in some perivascular spaces can be observed (74-d, arrow). MLI presents, however, a higher incidence of fibrinoid aggregates in vascular spaces (74-b, 74-c) than the SI group of the same drug.*

3.4.3 Leukocyte infiltration: Immunohistoreaction to CD45 and granulocyte esterase

Immunostaining with granulocyte esterase and CD45 is presented in the following microphotographs. The upper images correspond to granulocyte esterase while the lower represent the CD45 immunoreaction. All pictures are shown as microscope magnifications.

3.4.3.1: Control Short Ischemia

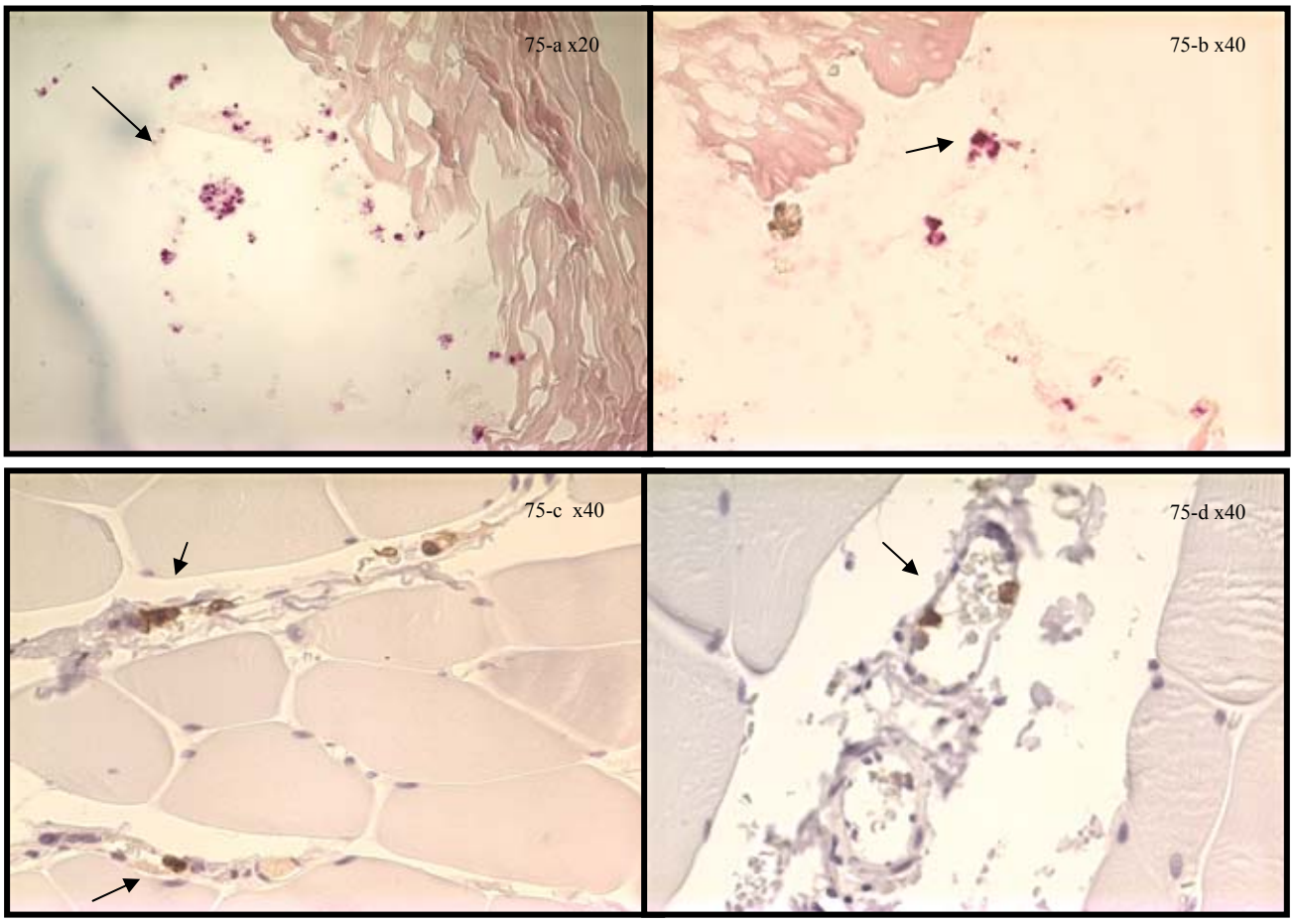


Fig. 75: In the upper images, belonging to the CSI group, perimisial and perivascular presence of neutrophils can be observed. PMN aggregates are present on both images. Some neutrophils are present in muscular tissue, as shown at a low magnification on the left hand side picture. Positive immunoreaction for CD45 was found in the CSI group in all previously defined structures (75-c, 75-d, arrows). On the left hand side, CD45 stained cells can be seen in perivascular and connective tissue (arrow). On the right hand side, positive cells adhering to the endothelial wall can be observed (arrow).

3.4.3.2: Biotest Short Ischemia

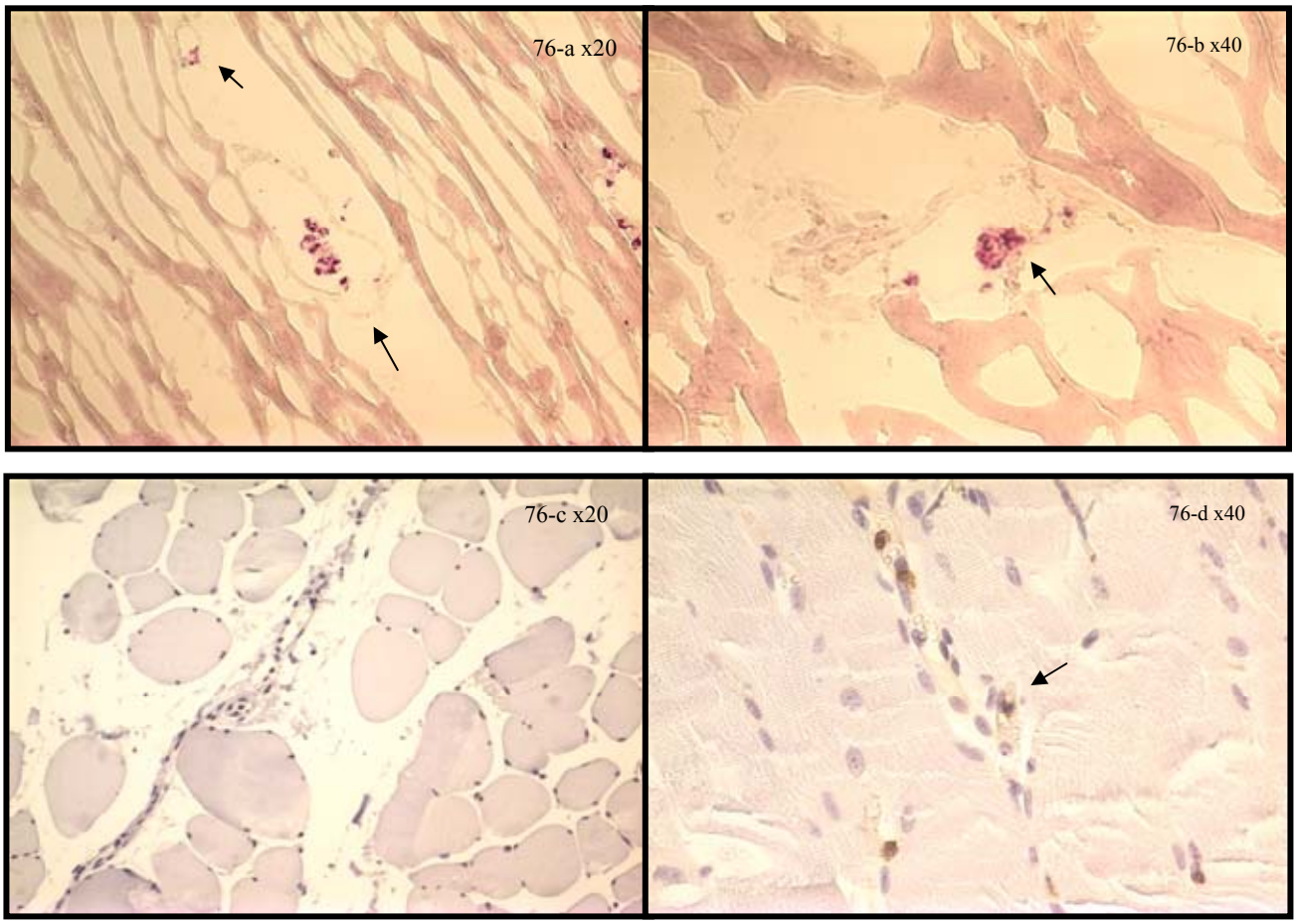


Fig. 76: Biopsies from to the BSI group show intravascular presence of neutrophils (76-a, arrow), adhering to the endothelial wall in some cases as demonstrated with granulocyte esterase staining e.g. on the picture 76-b (arrow). This group shows slight muscular and connective tissue infiltration. Most positive CD45 immunoreactions are found in perivascular or vascular spaces (76-d, arrow).

3.4.3.3: Fresenius Short Ischemia

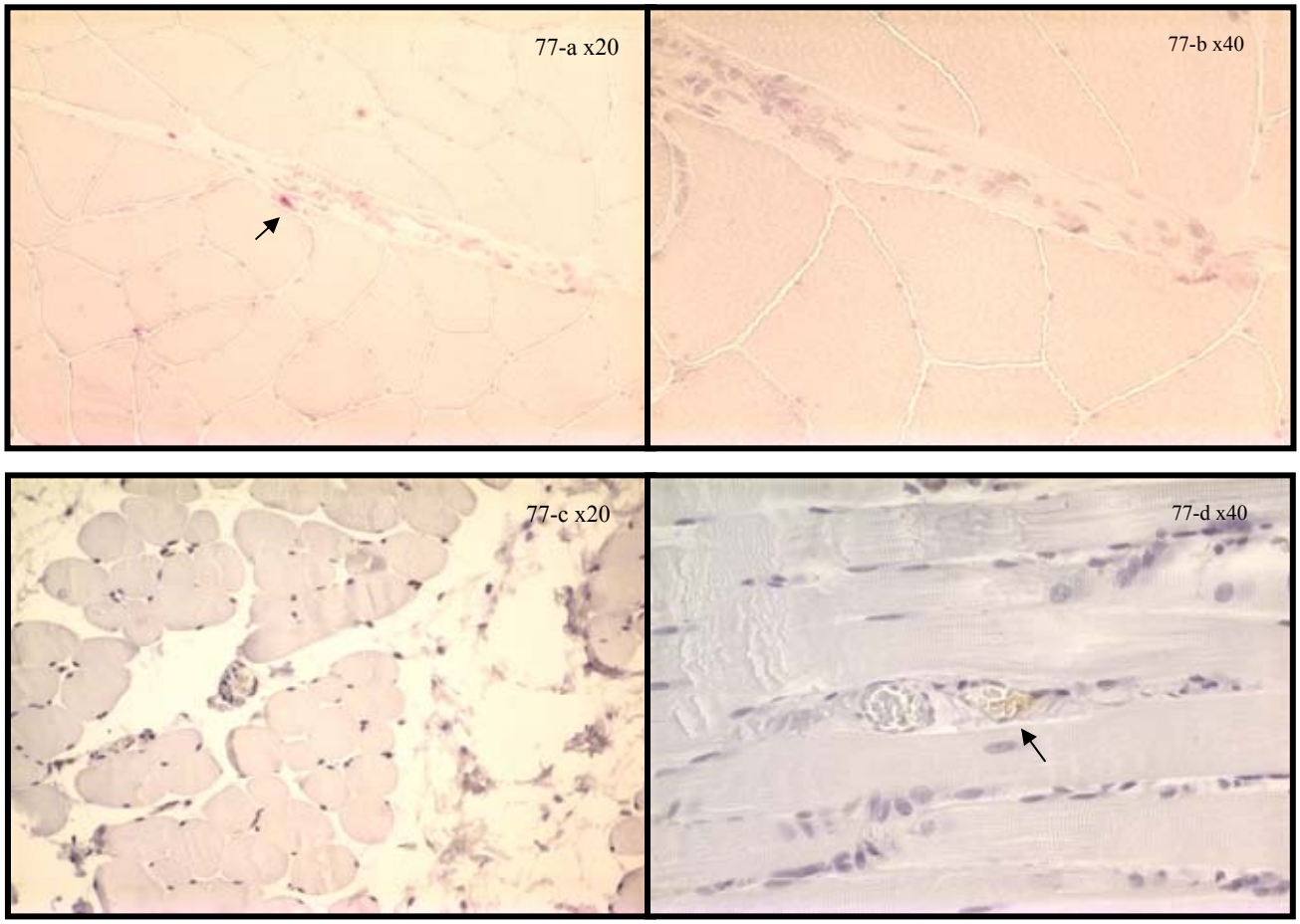


Fig. 77: FSI group shows slight presence of neutrophils in muscular and connective tissue (77-a, 77-b). Presence of these WBC could be demonstrated in perivascular connective tissue in a very low quantity (Fig. 77-a, arrow). Focusing on vascular areas, presence of PMN was much lower than in the control and the other study groups. CD45-like immunoreaction was observed in few vascular spaces and could scarcely be detected in muscular tissue (77-c, 77-d, arrow).

3.4.3.4: Merieux Short Ischemia

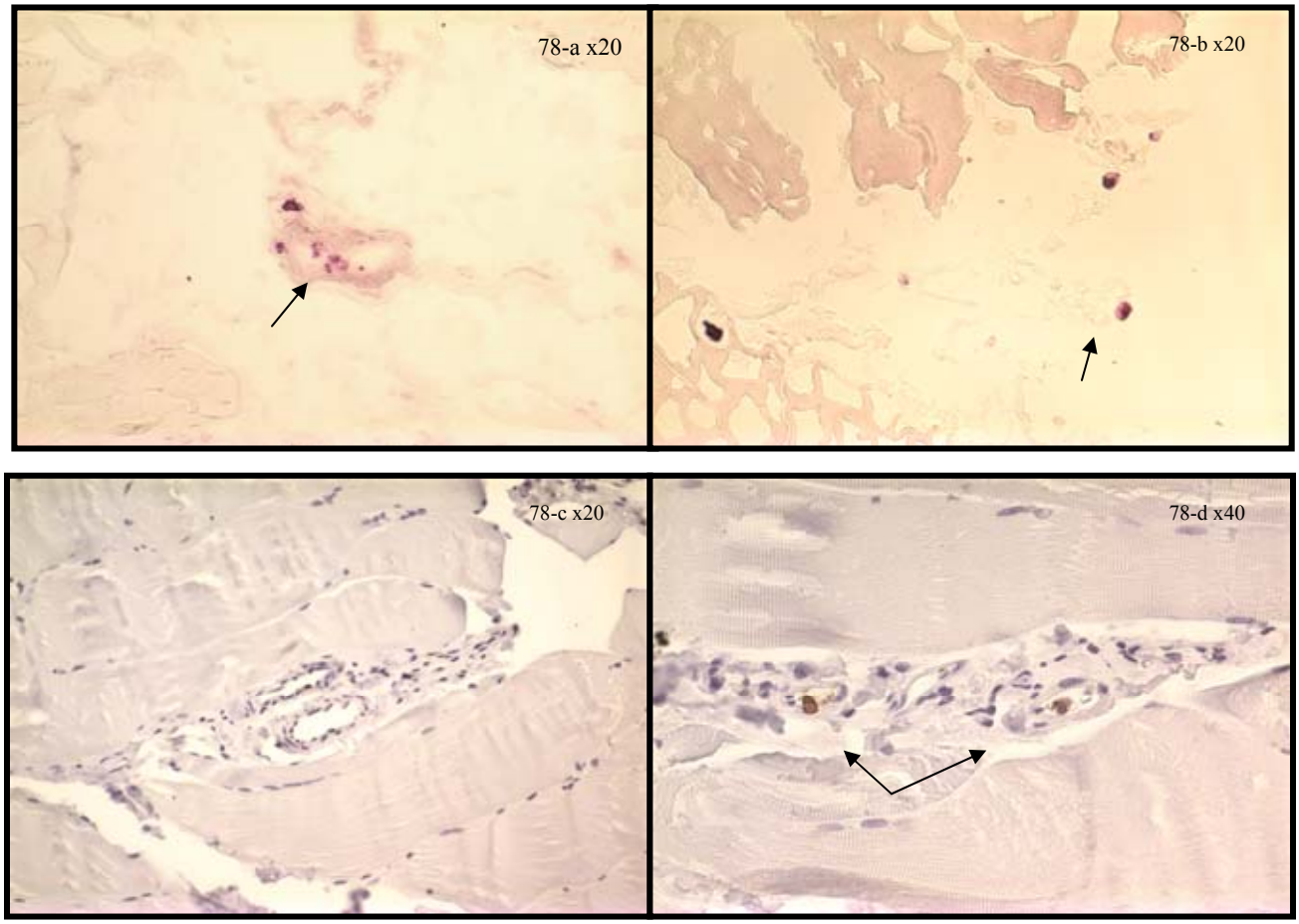


Fig. 78: Granulocyte esterase reaction for the MSI demonstrated presence of few PMN, mostly localized in connective or perivascular areas (78-b, arrow). Some neutrophil aggregates were to be seen attached to the vascular wall, as seen on the left hand side picture (78-a, arrow). Positive cells for CD45 were located in the vascular spaces, as shown on the right lower picture (78-d, arrows). Muscular and connective tissue showed slight other no infiltrates of CD45+ cells.

3.4.3.5: Control Long Ischemia

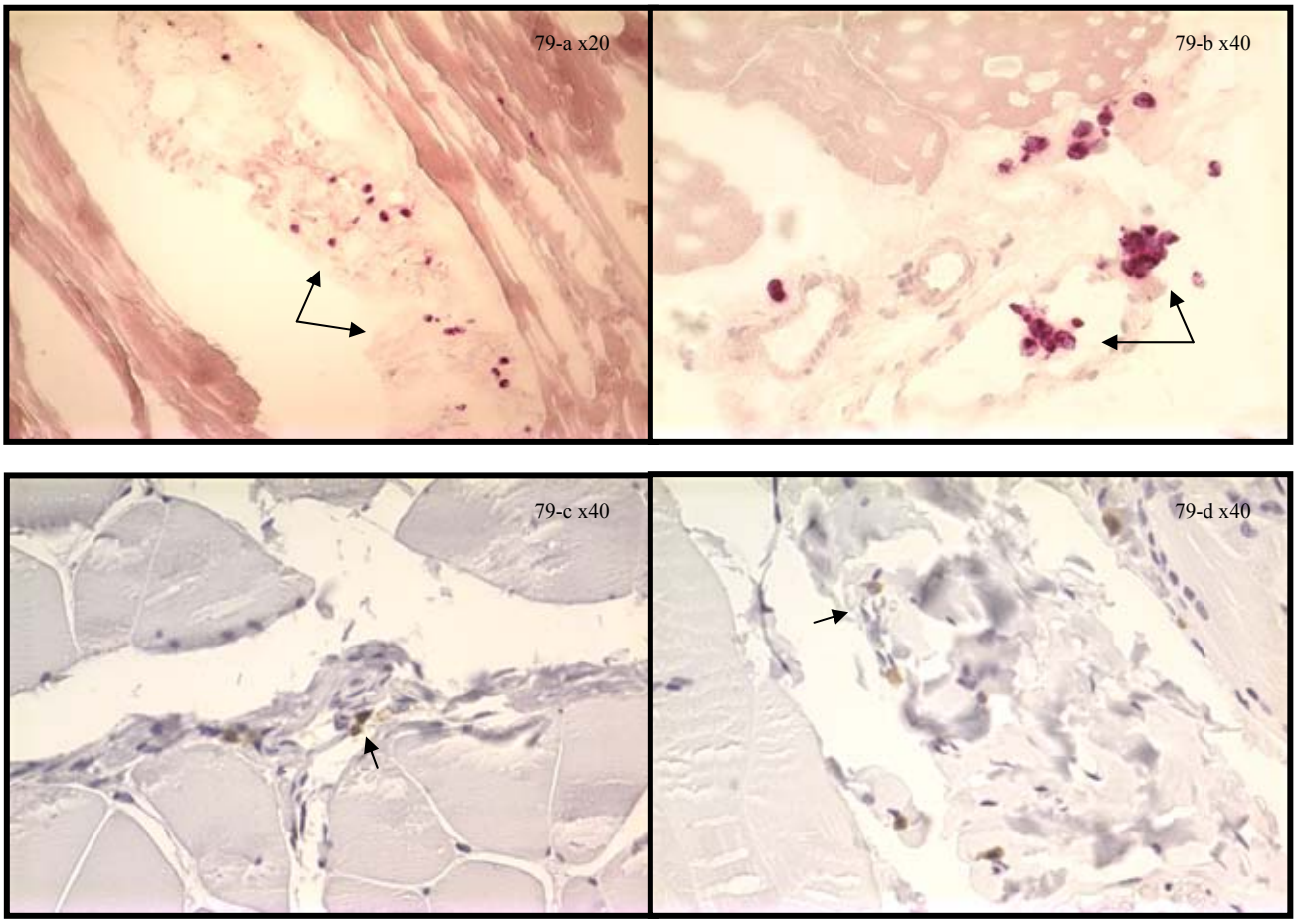


Fig. 79: Biopsies of the CLI group show diffuse muscular, connective and perivascular neutrophil infiltration, as demonstrated by means of granulocyte esterase staining. In the left hand side picture (Fig. 79-a), many positive cells can be observed in these structures. Aggregates of PMN were found attached to the endothelial wall and in the perivascular connective tissue (Fig. 79-b, arrow). Positive staining for CD45 was detected in all of the structures studied, most of the positive cells being located in perivascular tissue (Fig. 79-c, arrow). However, presence of CD45+ cells in connective tissue was also detected (79-d, arrow) in higher amounts than in the study groups.

3.4.3.6: Biotest Long Ischemia

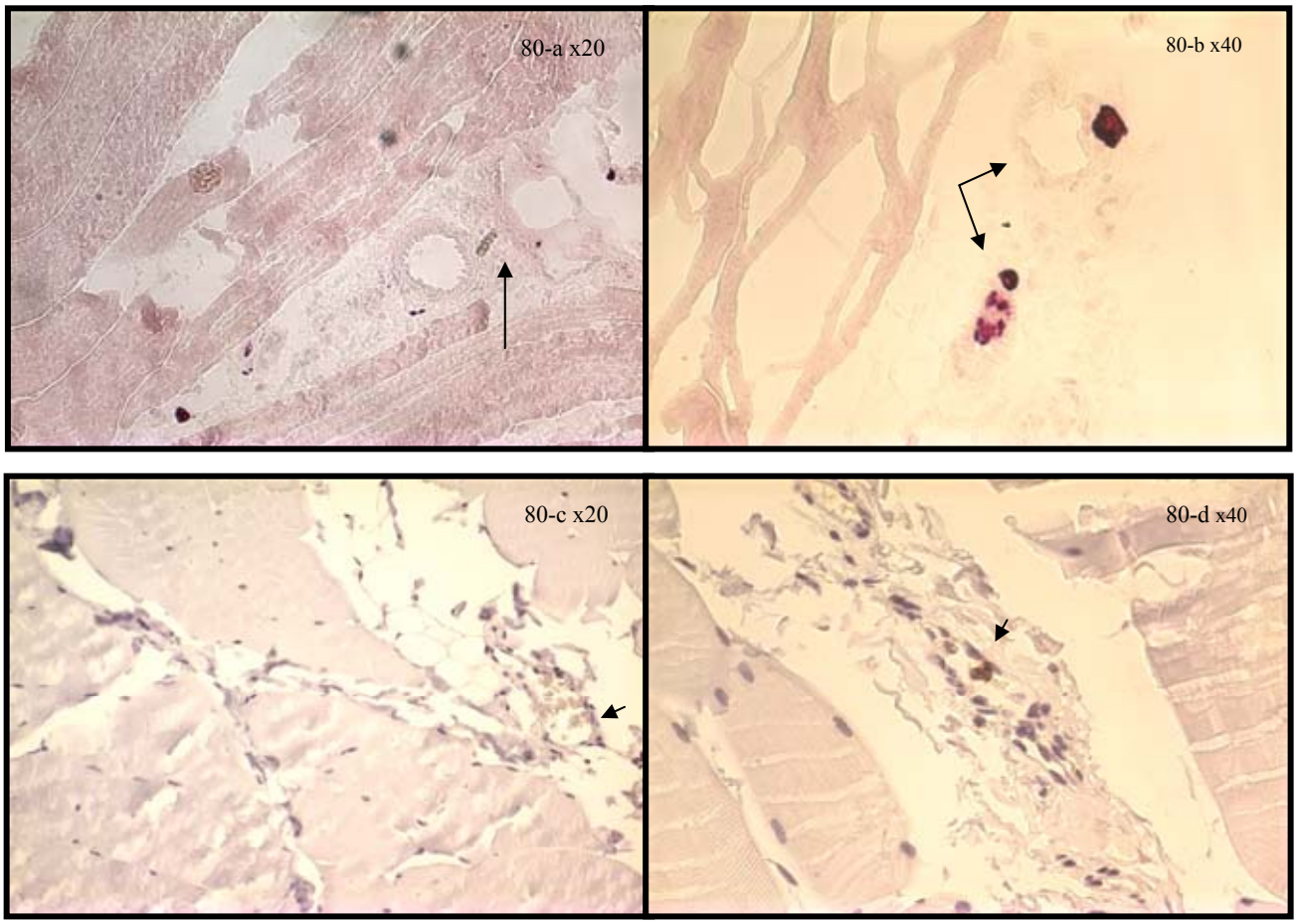


Fig. 80: Positive reaction for granulocyte esterase was found in the BLI mostly in form of aggregates attached to the endothelial wall or in the perivascular connective tissue (Fig. 80-b, arrows). However, not all the vessels presented such formations, as shown on the left hand side picture (Fig. 80-a, arrow). CD45-like immunoreaction was mostly detected in intravascular or adherent WBC aggregates (Fig. 80-d, arrow), showing slight muscle affectation (Fig. 80-c).

3.4.3.7: Fresenius Long Ischemia

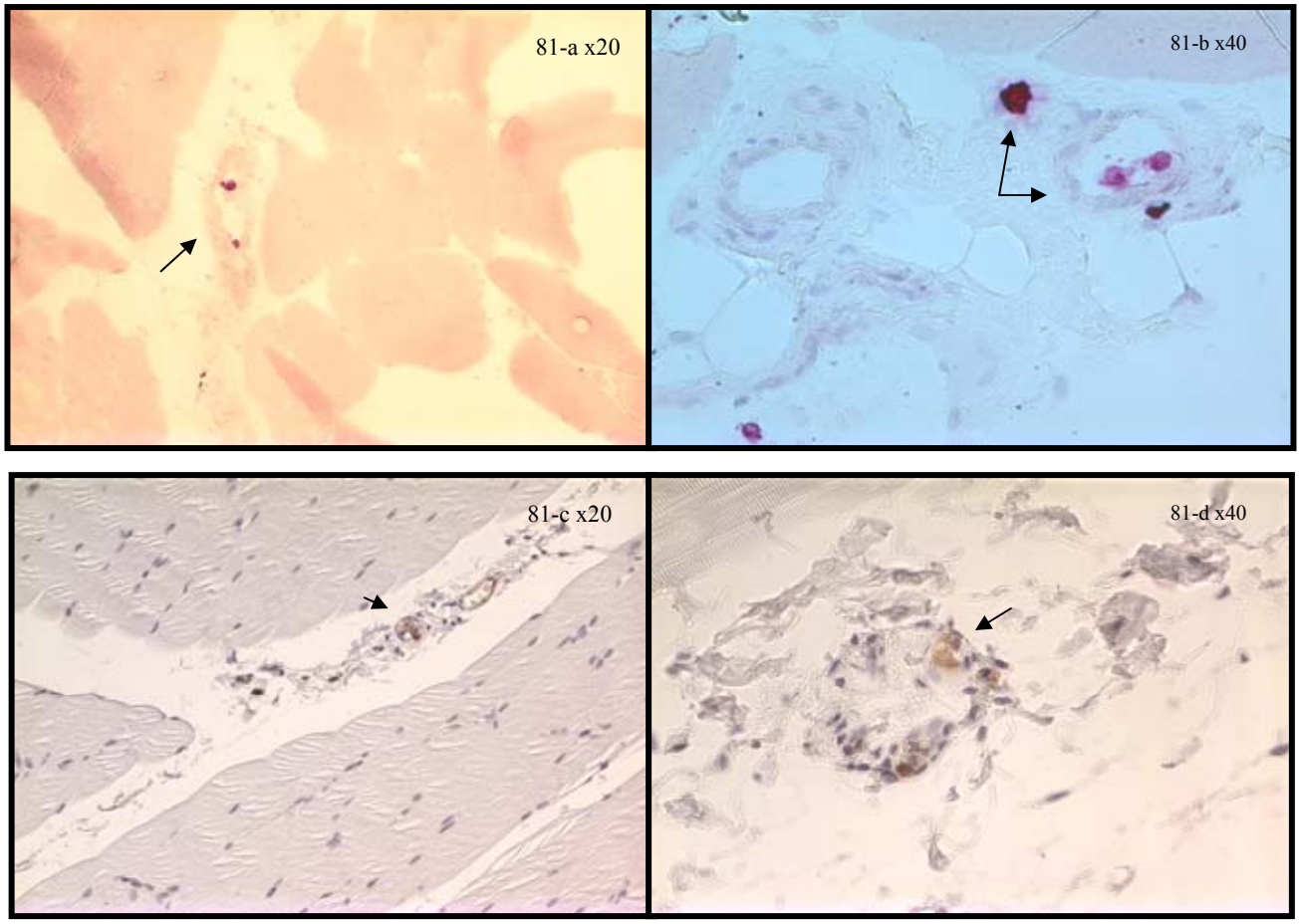


Fig. 81: Positive staining for granulocyte esterase could be observed in the FLI group in the vascular spaces (81-a, 81-b, arrows). However, the incidence of aggregates attached to the endothelial wall was not as high as in the other study groups. Positive CD45 immunoreaction was observed in the same locations (81-c, 81-d, arrows), showing slight positive or no reaction at all the muscular structures.

3.4.3.8: Merieux Long Ischemia

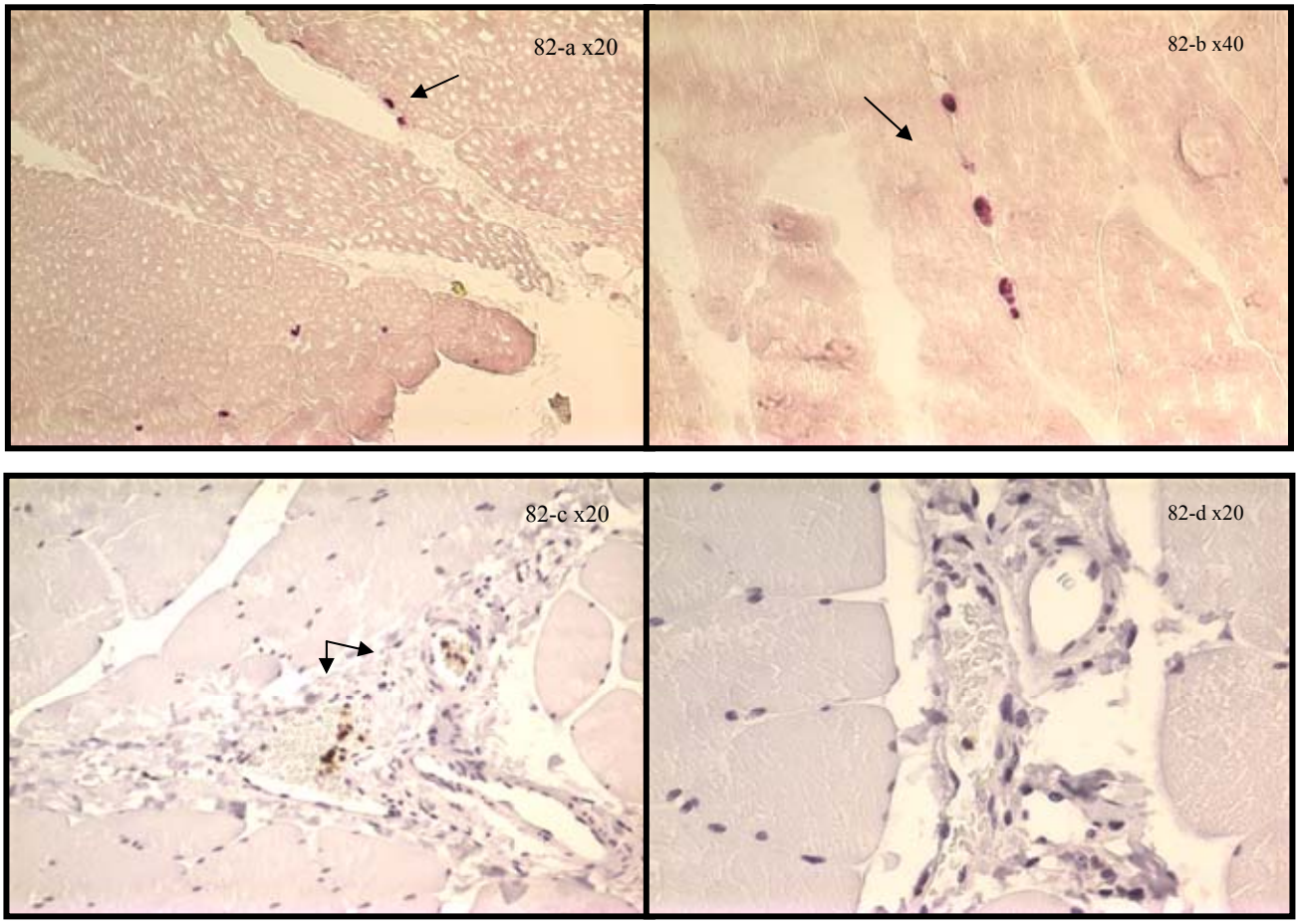


Fig. 82: The biopsies belonging to the MLI groups showed differences when compared to the same drug short ischemia group. More PMN positive staining was found, especially in vascular and perivascular spaces (Fig. 82-b, arrow). Positive esterase staining was also found in muscular tissue (Fig. 82-a, arrow), although in low quantity. Positive immunoreaction for CD45 was found in perivascular and vascular spaces (Fig. 82-c, arrows), as well as in connective tissue. However, this fact was not generalized, as most of the vessels showed slight or no infiltration (Fig. 82-d).

3.4.4: Fibrin, platelets and EC: Weiger`s technique, Masson's Thrichrome and CD31-Immunoreaction

The following microphotographs focus on the vascular structures. The left upper image corresponds to Masson's Thrichromic. The upper right picture corresponds to Weiger`s hematoxiline (specific for fibrin). The lower pictures show immunoreaction to CD31 (PECAM).

3.4.4.1: Control Short Ischemia

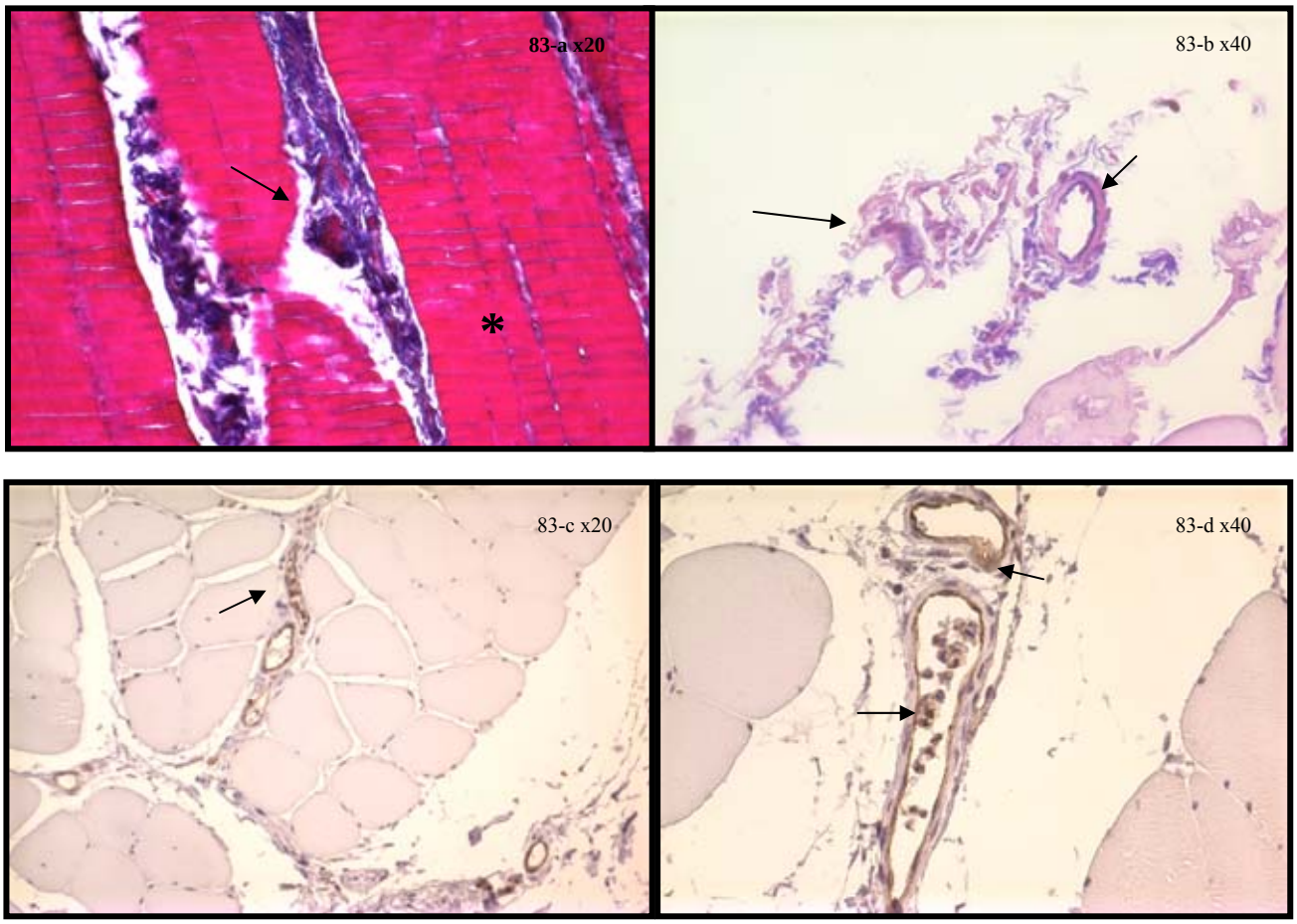


*Fig. 83: The upper images from the CSI group show vascular occupation and perivascular loss of structure (83-a, 83-b, arrows). Masson's reaction shows a damaged muscle with extended areas of haemorrhage (83-a, *). CD31 immunoreaction shows positive staining on endothelia with activated thrombocyte aggregates in the vascular lumen and adherence to the endothelial wall (83-c, 83-d, arrows).*

3.4.4.2: Biotest Short Ischemia

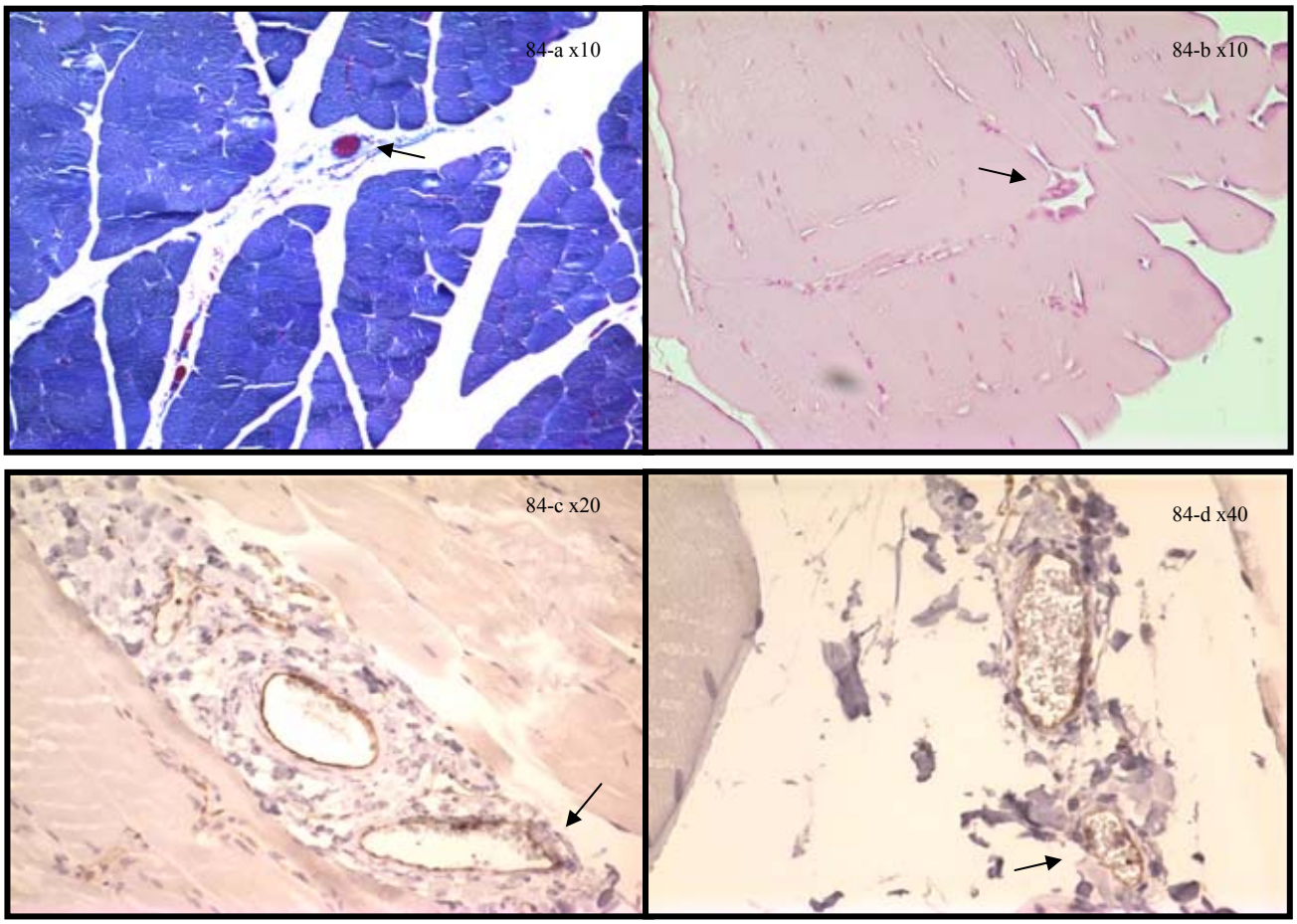


Fig. 84: BSI group shows presence of fibrinoid aggregates in the small vessels, as can be seen in the two upper pictures (84-a, 84-b). However, the muscle is well structured and no signs of ischemia or necrosis are observed. In the lower pictures, CD31-like positive immunoreaction is detected in endothelia (84-c, arrow) and in small cellular aggregates adherent to the endothelial wall of small vessels (84-d, arrow).

3.4.4.3: Fresenius Short Ischemia

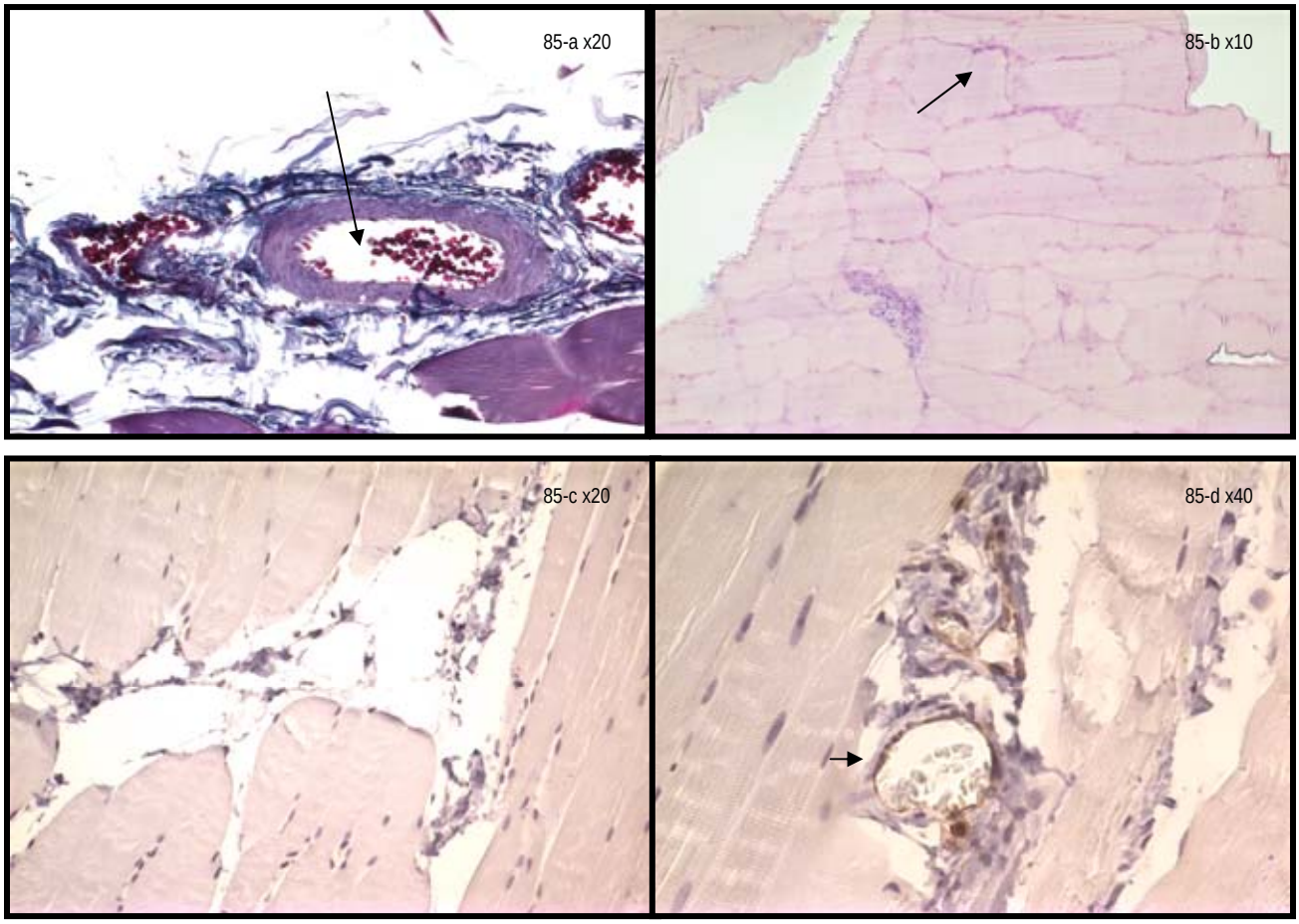


Fig. 85: The vessels belonging to the FSI group are well conserved. None or few fibrinoid aggregates are seen in the vascular spaces with Masson's Thrichrome technique (Fig. 85-a, arrow). However, presence of fibrin between the muscle fibers was detected in low quantity (Fig. 85-b, arrow). Positive immunoreaction for CD31 was weak (Fig. 85-c) and limited to endothelia (Fig. 85-d, arrow).Few positive intravascular or perivascular cell aggregates including activated thrombocytes were observed.

3.4.4.4: Merieux Short Ischemia

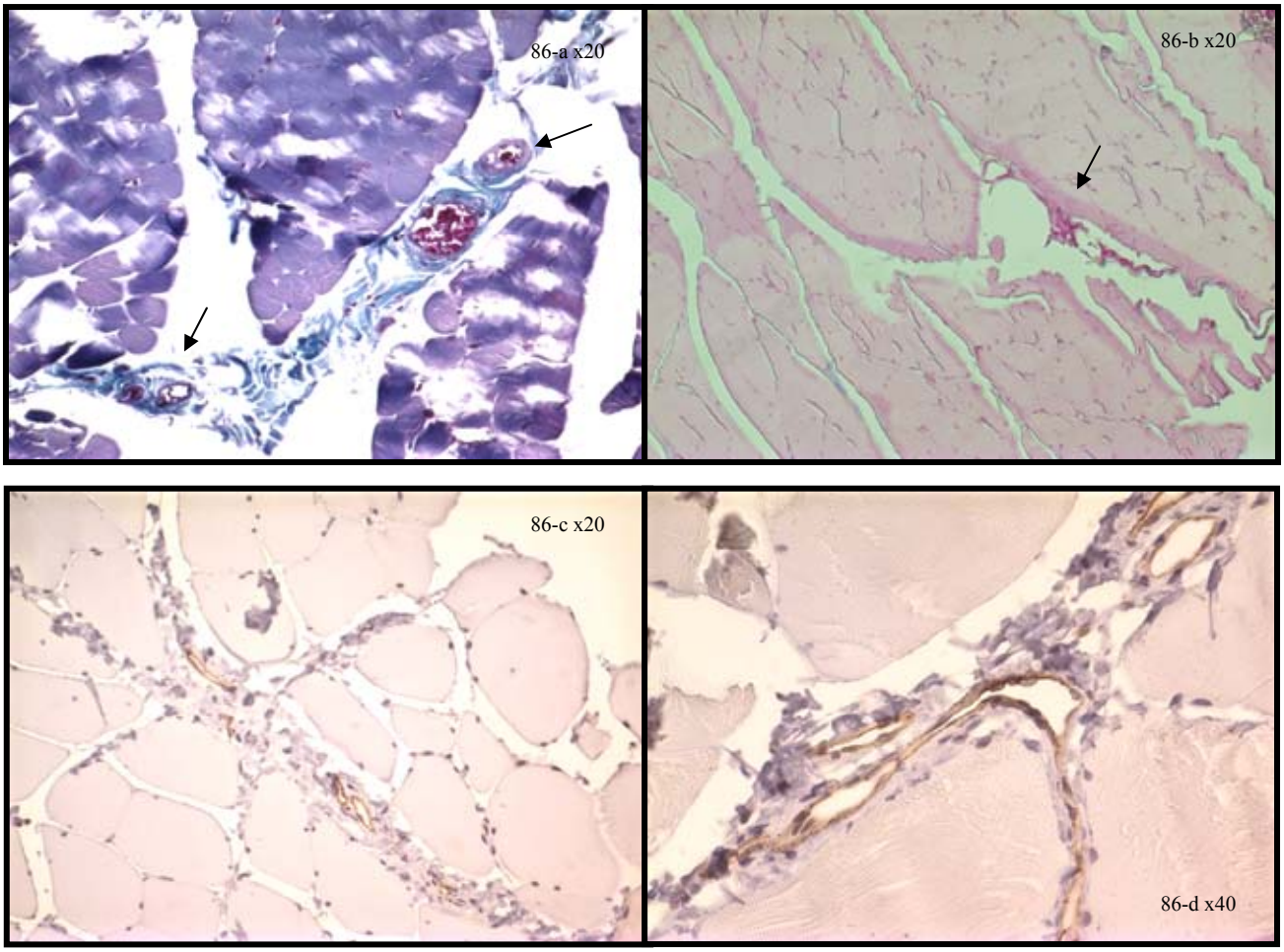


Fig. 86: MSI group demonstrated low presence of fibrinoid aggregates in perimisial and intramuscular vascular spaces. Most of the positive reactions were found in the perimisial or endomisial connective tissue vessels (Fig. 86-a, arrow). Positive reaction for the Weiger`s technique was also observed in the connective tissue adjacent to the muscle fibers (Fig. 86-b, arrow). CD31-like immunoreaction was detected in vascular endothelia (Figs. 86-c, 86-d), showing no presence of positive reactions in the vascular lumina or thrombocyte aggregates.

3.4.4.5: Control Long Ischemia

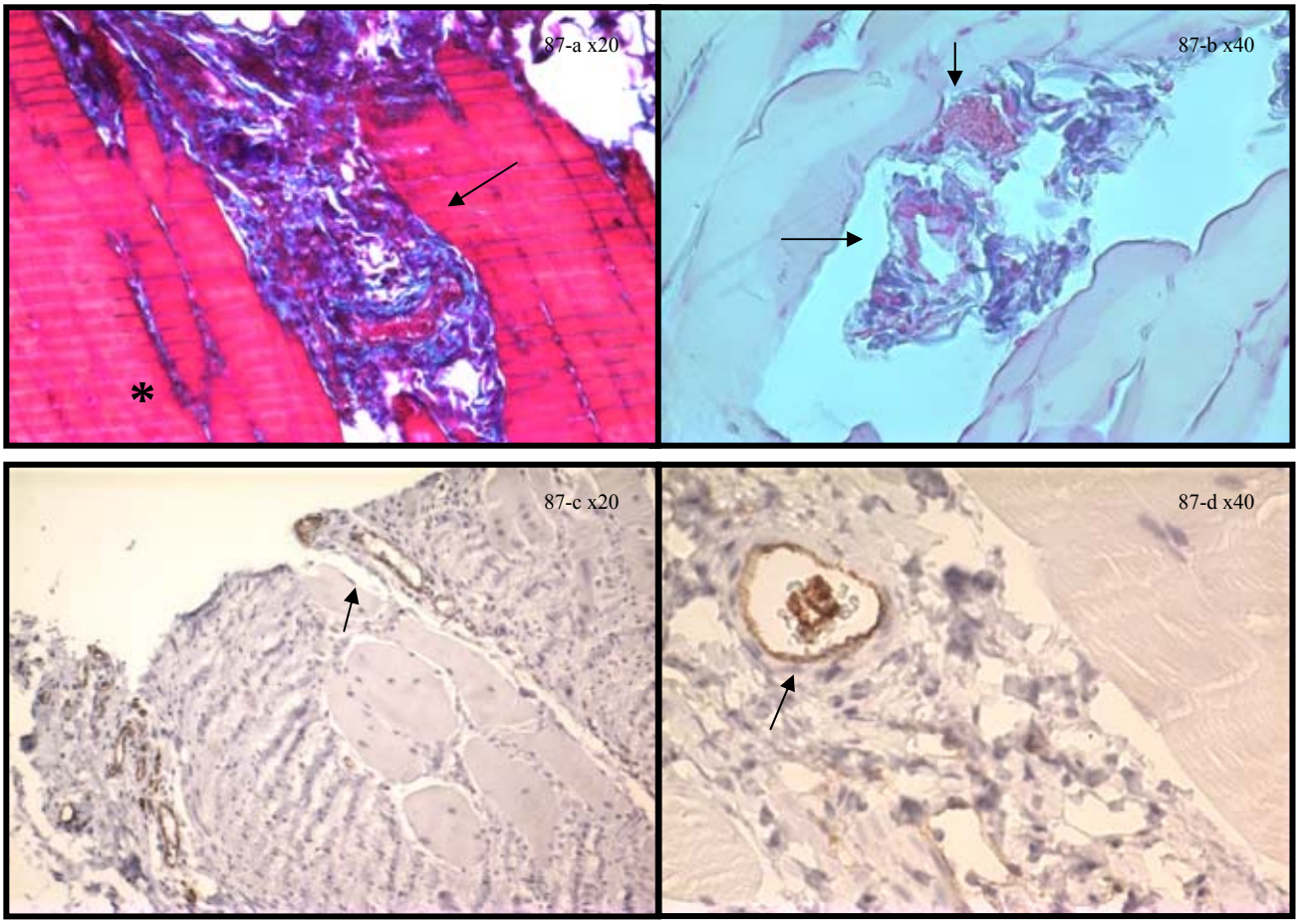


*Fig. 87: CLI group biopsies' analysis with conventional techniques showed diffuse muscle damage and haemorrhage, presence of huge fibrinoid aggregates in the vascular spaces and general muscle damage (Figs. 87-a, 87-b, arrows, *). Positive immunoreaction for CD31 was observed both in endothelia and in adherent cells (Fig. 87-c, arrow). Intravascular aggregates showing positive immunoreaction could also be seen (Fig. 87-d, arrow)*

3.4.4.6: Biotest Long Ischemia

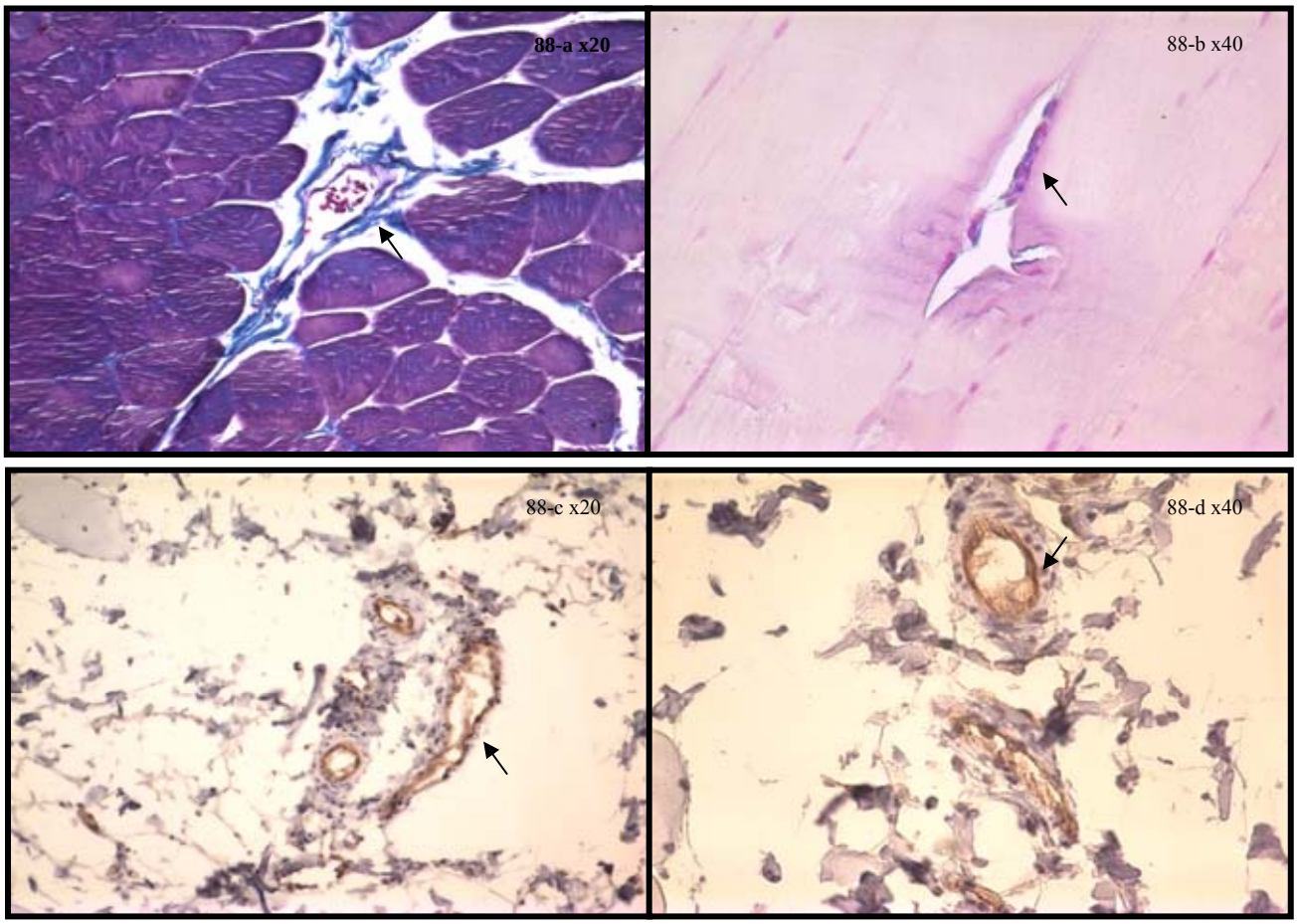


Fig. 88: Presence of fibrinoid structures and positive reaction for fibrin were demonstrated in vascular spaces of the BLI group. However, these findings were not present in all the vessels. On the upper left hand side, a small accumulation of cells in the vascular lumen can be observed (Fig. 88-a, arrow). On the right hand side, a fibrin aggregate with cells included is shown (Fig. 88-b, arrow). CD31 immunostaining showed the same phenomena as the non-specific techniques. In both pictures the presence of CD31-positive aggregates and cells can be observed (Figs. 88-c, 88-d, arrows).

3.4.4.7: Fresenius Long Ischemia

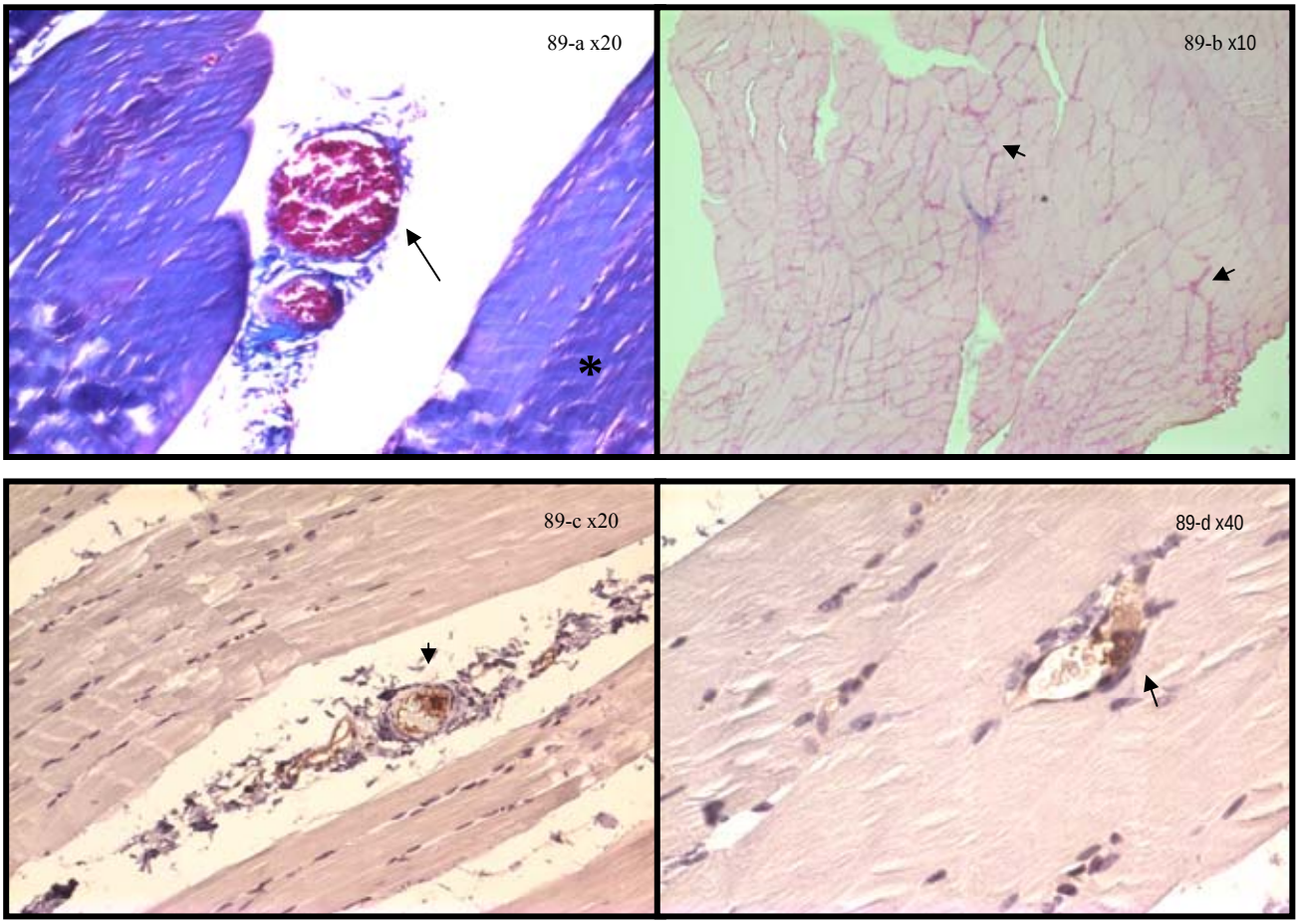


Fig. 89: FLI group shows low presence of fibrinoid aggregates in the small vessels, the bigger vessels being permeable, as can be seen in the left upper picture (Fig. 89-a, arrow). Furthermore, the muscle is well structured and little presence of fibrin is observed (Fig. 89-b, arrow). In the lower pictures, CD31-like positive immunoreaction is detected in endothelia as well as in cellular aggregates adherent to the endothelial wall of small vessels (89-c, 89-d, arrows).

3.4.4.8: Merieux Long Ischemia

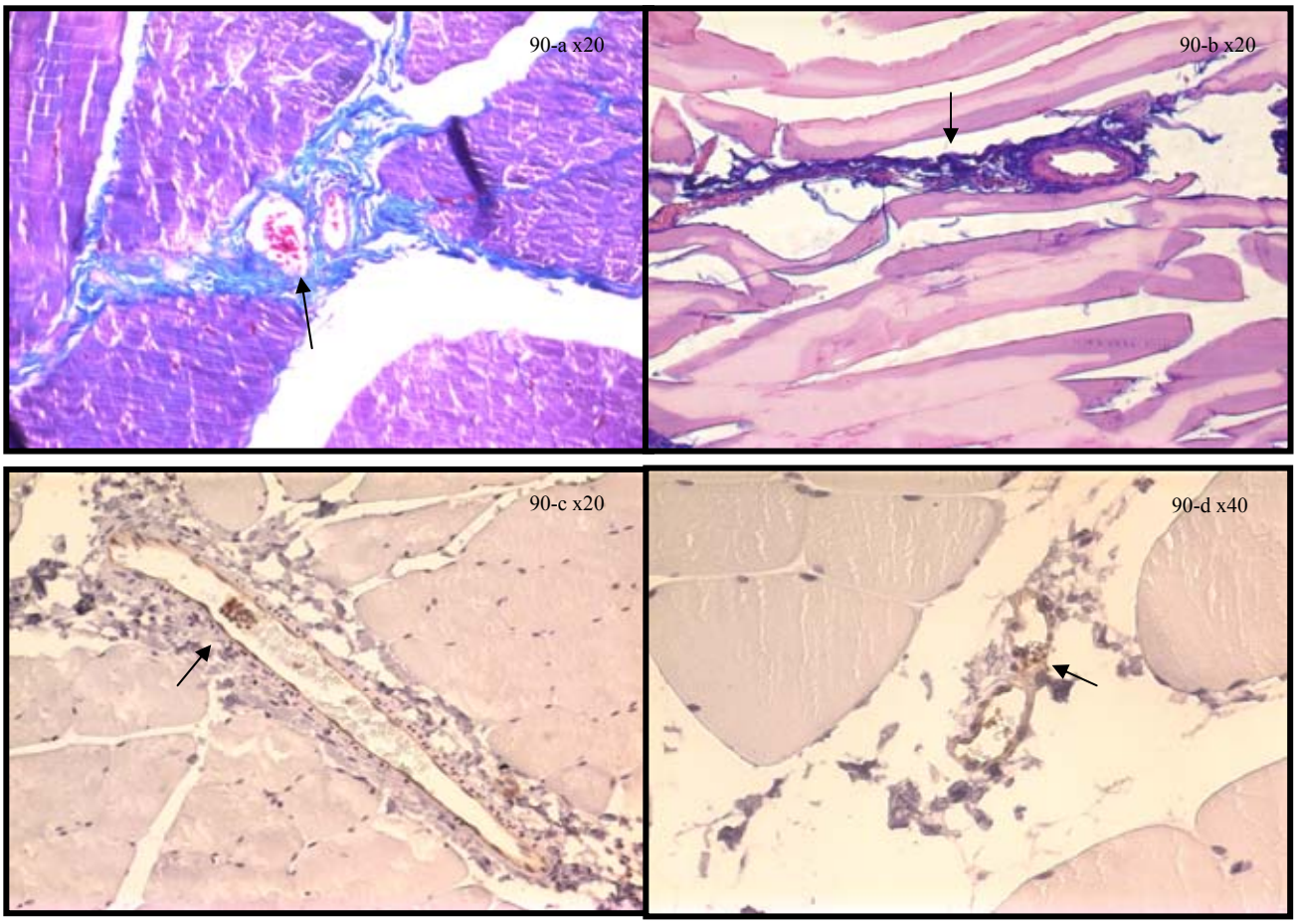


Fig. 90: Masson's Thrichrome shows fibrinoid or cellular aggregates present in the vascular lumina of the Merieux long ischemia group (Fig. 90-a, arrow). Fibrinoid structures are observed in the perivascular space (Fig. 90-b, arrow). This fact is confirmed by the CD31 immunostaining, as shown in the lower pictures (90-c, 90-d arrows) were cellular aggregates show positivity for CD31, thus demonstrating the existence of thrombocytes on these structures.

3.4.5: IL-4 Immunoreaction

3.4.5.1: Control Group

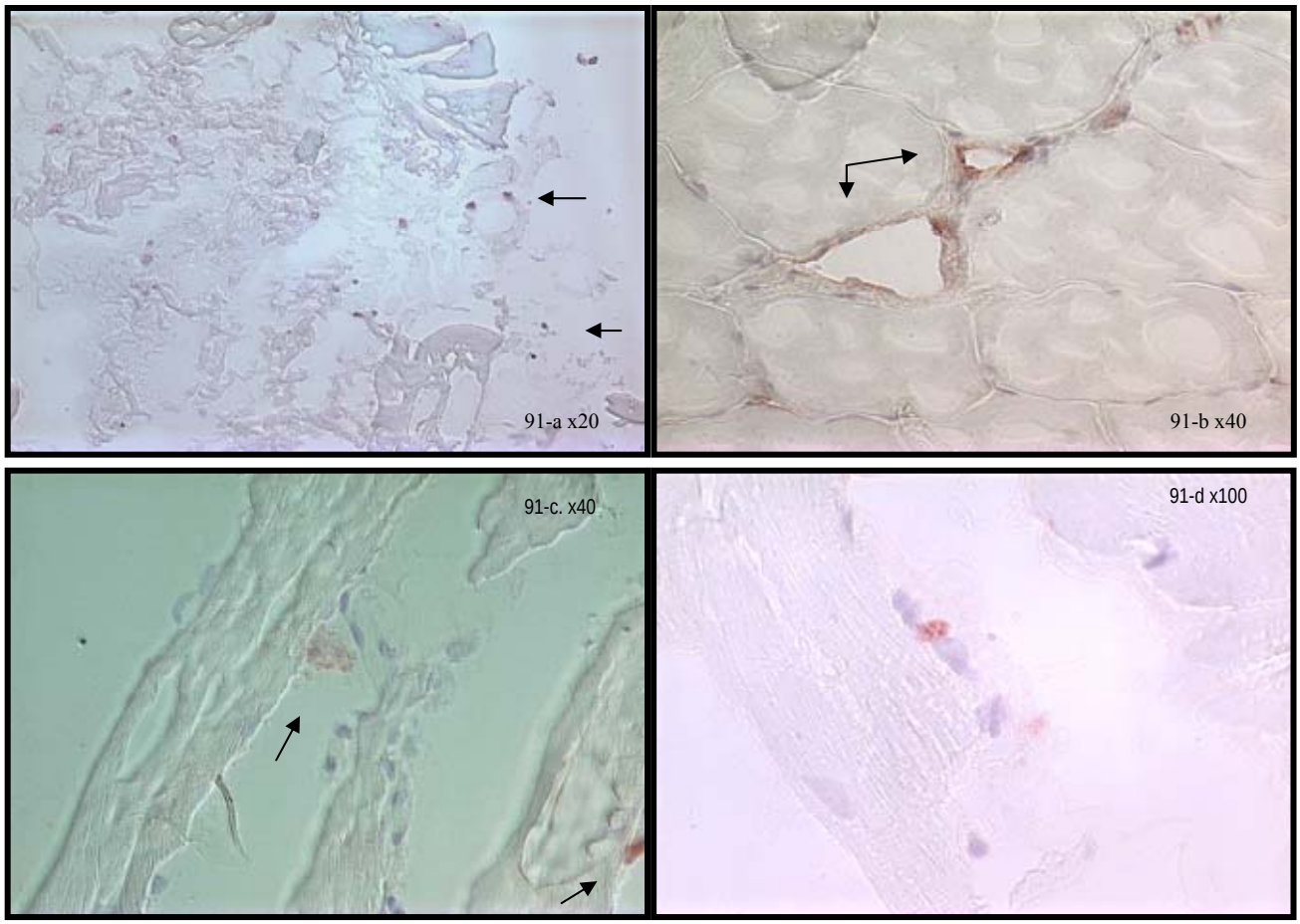


Fig. 91: Positive immunoreaction for the monoclonal antibody anti-IL-4 was found in the control groups in connective, vascular and muscular structures (Figs. 91-a,-b,-c,-d). In the upper left picture, stained cells in all these structures are shown (Fig. 91-a, arrows). Endothelial expression of IL-4 is shown in the right upper picture (Fig. 91-b, arrows). The cellular expression of IL-4, surrounding a WBC (Fig. 91-c, arrow) or between two WBC in the figure 91-d can be observed in the lower pictures at a higher magnification.

3.4.5.2. Study groups and negative control of the technique

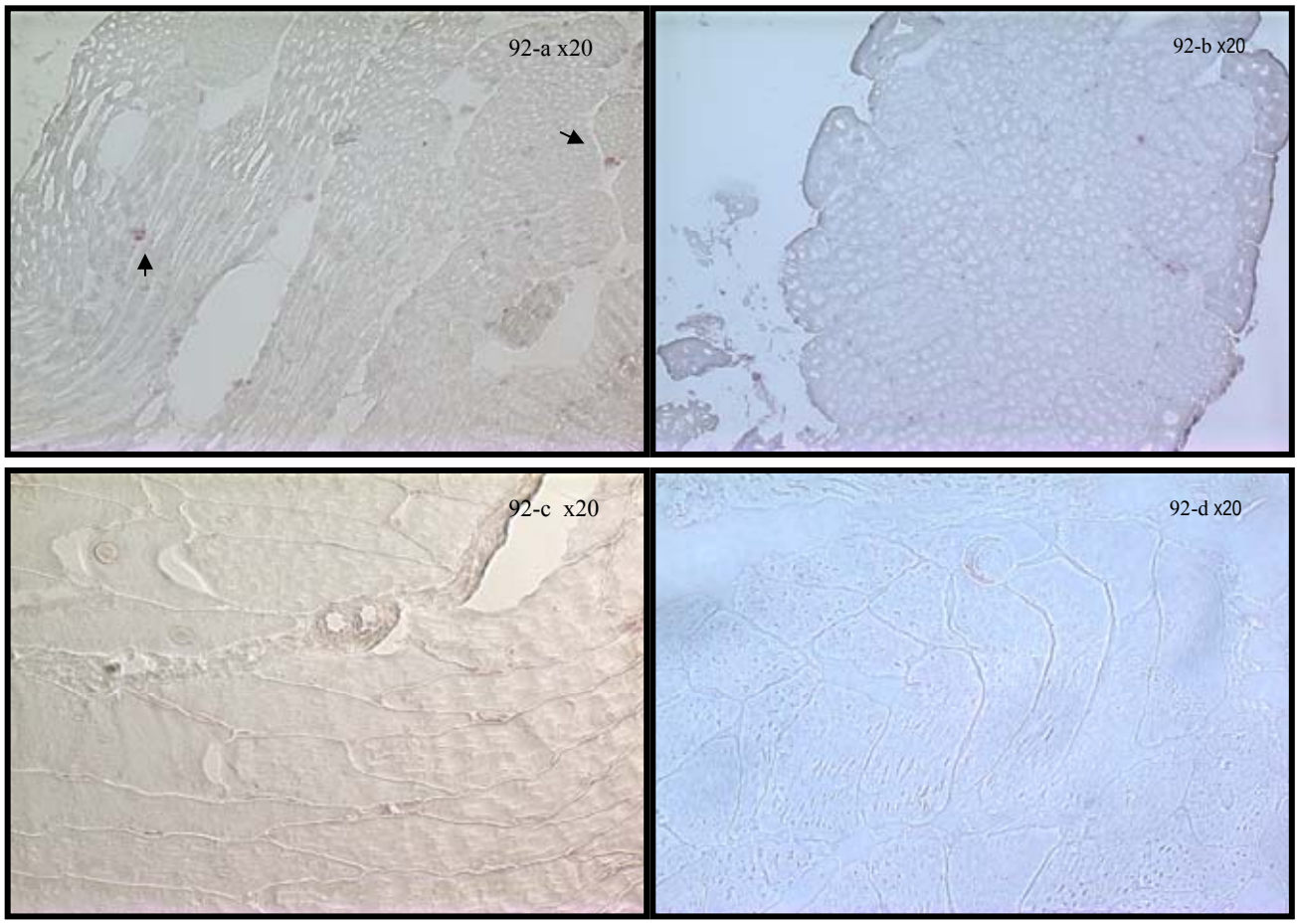


Fig. 92: No or low immunoreaction was observed in the study groups. We can observe in the pictures that the muscle and connective tissues are free from positive staining for IL-4 (92-a,-b,-c). However, as can be seen in the left upper picture, there are cells showing immunoreaction for IL-4 in muscular tissue in the Biotest-ATG group (Fig. 92-a, arrows). The right bottom picture is a negative control of the technique, performed by substituting the primary antibody for PBS (Fig. 92-d).

4. DISCUSSION

4.1. Discussion of material and methods

4.1.1 Ischemia-Reperfusion Injury and Transplantation

Organ, tissue and cell transplantation has achieved impressive results in the last three decades as a treatment modality for patients with end-stage organ diseases (148). Since the first successful heart transplantation (11) in Cape-Town took place, transplantation was thought to be the easiest solution to improve the life quality of many patients as well as the key to life's prolongation. However, it was not easy to achieve the actual status in this field. First, this achievement can be attributed to the development of advanced technical skills and technological means. Advances in surgical technique have improved the recovery time of the patients (60, 186, 202), decreased the mortality in those surgical procedures (106), treated congenital end-stage diseases at low ages (138, 189), diminished the use of long-term medical devices such as dialysis thus improving life quality. Second, the development of immunosuppressive agents has considerably reduced the number of organ rejections and subsequent transplantation failure (177) as well as controlled the incidence of graft vs. host disease (179). This development is related to research in transplantation immunobiology, which has much improved our understanding of the alloimmune response and the complex mechanisms undergoing cell and cytokine interaction (148). However, other problems are still to be solved and among them is ischemia/reperfusion injury.

In organ transplantation, IRI is a multifactorial process that leads to organ damage and primary graft dysfunction (82). Cessation of blood flow deprives the cell and subsequently the grafted organ of energy metabolism and appropriate activity. Different mechanisms are implied in IRI such as macrophage and leukocyte interactions, microcirculatory changes, release of oxygen-derived free radicals, nitric oxide and calcium metabolism, endothelial cell molecules and

inflammatory mediators. There is little question about IRI importance upon transplantation processes and multiple studies to investigate all these molecular and cellular events associated to the deleterious effects of IRI are being carried out.

4.1.2 Polyclonal antibodies

Multiple strategies have been designed to avoid or lessen the cellular and tissue derangements produced after reperfusion. Use of immunosuppressive agents such as Tacrolimus (182, 212), Rapamycin (159), Cyclosporine (107, 132, 159), anti-CD11 monoclonal antibodies (155) and OKT3 (178) have been suggested. Antibodies blocking other molecules present in IRI have been employed, e.g. anti-endothelin receptor (147), adenosine (121), different antibodies anti-adhesion molecules, complement and activating factors (123, 136), anti- oxygen-derived free radicals or arachidonic acid inhibitor (16, 170), calcium-channels blocker (48), allopurinol and superoxid dismutase (68), etc. Physical methods have been suggested as possible strategies to block IRI, among them leukocyte depletion pre-reperfusion (105, 175), changes of perfusion times and temperature (143), or different preservation and storage solutions (84, 110, 172). Organ or tissue preconditioning with different methods has also been performed by means of heat (119), gas (167) or drugs (208). Gene therapy against certain activation molecules and endothelial compounds of the reperfusion process has been developed without definitive results (54, 81, 154). As the number of immunosuppressive agents increases, the election of the correct agents for a precise therapy becomes more difficult. This is one of the reasons why the mechanism of these different drugs and their effect on the immune cells must be accurately studied and described. Polyclonal ATGs are antibody preparations obtained by immunization of horses or rabbits with human lymphocytes from definite cell lines (8) or suspended human thymocytes (193, 198) and with a wide clinical application. The mechanism by which polyclonal

antilymphocyte preparations suppress immune responses is not fully understood, but T-cell depletion and immune modulation on a molecular level are included (166, 211). ATGs contain a variety of antibodies recognizing key receptors on T-cells, being able to inactivate or kill them, thus reversing the rejection process.

ATGs have been and, in some cases, still are used in the clinical practice in a broad spectrum of therapeutical activities in different fields. One of the most important is transplantation. Polyclonal ATGs have been employed as induction therapy (28, 75, 137, 207), concomitant immunosupression (29, 76, 79), treatment of graft vs. host disease (34, 76, 161), acute rejection therapy (18, 26, 152). They have been employed in aplastic anaemia (199, 201), treatment of autoimmune diseases, such as lupus (130) or rheumatoid arthritis (192) and skin diseases, e.g. cutaneous lymphoma (43) or contact allergy (174). These drugs cause, however, a wide range of adverse reactions, which sometimes superpose the possible benefit of their administration. The most common are cardiopulmonary reactions (108), fever (94), elevation of TNF-α in plasma (39), concomitant infection with CMV (98, 180, 204), hypersensitivity to rabbit or horse proteins (158) or serum sickness with cutaneous manifestations (36).

To our knowledge, this is the first time that the effect of pATGs upon IRI has been investigated. Our choice was based on the wide range of cellular and molecular effects explained in the introduction of this work which could have an influence on the establishment and development of IRI and its associated features. It is also, to our knowledge, the first time that the three existing polyclonal antibodies on the German market, Fresenius-ATG © (Fresenius, Bad Homburg, Germany), Tecelac© (Biotest Pharma GmbH, Dreieich, Germany) and Thymoglobuline©(Imtix-Sangstat California, USA)], are compared with each other.

4.1.3 The animal model

Transplantation and xenotransplantation experiments can be performed either by experimental organ or tissue transplantation or by using artificial perfusion systems where donor organs can be perfused with allogeneic blood, xenogeneic blood or blood components (59, 142). Ex vivo perfusion of an organ or a tissue with a perfusion system is valuable in establishing the relative value of the factors involved in the rejection process. Several models have been designed to perfuse isolated organs with allogeneic or xenogeneic blood ex-vivo to investigate not only inflammation (140) or rejection phenomena (206), but also interactions between different drugs and vascular endothelia (25) or tissue response to definite antigens (90). Pascher *et al.* (145) defined a suitable model for perfusing isolated organs simulating the normal physiological conditions. In these systems the organ is isolated and connected to an artificial circulatory circuit to perfuse it at physiologic pressure, normothermia and oxygenation conditions (50, 145). Furthermore, these models allow obtaining tissue specimens for histopathological studies, measurement of functional parameters, the test of different drugs as potential therapeutic agents for IRI or acute rejection (59). Perfusion of isolated limbs is considered an ex-vivo perfusion model, as no systemic interactions are present after the access to general blood circulation is avoided.

Our model was designed to study the interactions between antithymocyte globulins (ATG) and cells or tissues during ischemia reperfusion injury, to assess the potential effect of these drugs as therapeutical agents in IRI. These polyclonal antibodies are directed against human cells; therefore the experiments had to be performed using human blood. However, this experimental set-up cannot be applied to humans, both due to ethical and legal reasons. To test these polyclonal antibodies under in vivo condition, a model of perfusion in non-human primates was designed, although there are legal restrictions on performing research with primates. The ethical

issues are much more complicated than in research with other animal species due to the behavioural similarities of men and these primates and the pressure of certain anti-animal-research groups. Our choice of non-human primates is related to the similarities of the non-human primate endothelia with human endothelia (80, 210) and thus the possibility of obtaining comparable and clinically valid cell interactions when these vessels were perfused with human blood of the corresponding blood group. Although our model can be criticized for employing two different species, immunological concordance, morphological vascular similarities and the impossibility of other in vivo experiments to test the effect of ATGs in IRI may still allow us to extract valid conclusions from our results.

Perfusion of isolated limbs is extensively used in the literature as a valid model to test different drugs without affecting the systemic circulation (45, 50, 196). Our model is designed to evaluate the results obtained in the capillary superficial net of muscle vessels and to extrapolate it to the microcirculation of sinusoidal organs. That is because, due to its functional similarities (10, 209), superficial circulation of the extremities, especially upper muscle and dermis, reproduces the capillary circulation of most of the solid organs. Our technical set-up has also been extensively discussed, demonstrating no cell activation or damage as well as no cellular interactions during the process of reperfusion (144).

4.1.4 Evaluation of the results

To evaluate the results obtained, different methods and techniques were applied. The number of blood cells and other blood parameters in PB were counted to monitor the state of the animal during the surgical procedure and the reperfusion process. The number of WBC was studied to assess the cytotoxicity of the three different ATGs applied to PB. The number of RBC and

platelets were also measured to differentiate a possible toxic effect of the employed drugs. These methods have been extensively described in the literature to evaluate the immunological and macrohaemodynamic situation of patients during (3, 49) and after transplantation (164, 191), or animals during research procedures (42).

Peripheral blood smears are of current use in some clinical procedures, such as haematological and oncologic diagnostic (9). Blood smears were also performed to examine the state of the various cell subpopulations in peripheral blood and to test the toxicity of certain drugs or procedures. We have used this cytological tool for various reasons. First of all it was our objective to correlate the morphological findings with the cell numbers obtained in the PB counting. A second reason was to investigate possible erythrocyte malformations due to the cytotoxic effect of ATGs. Another reason was to assess the cellular variation of the different WBC subpopulations in answer to IRI and the three different drugs employed and to compare them.

Cyto-immunological monitoring is a non invasive tool used in the clinic and in experimental research allowing one to differentiate the various subpopulations of lymphocytes and their activated forms (63). This tool has a special importance because it permits one to distinguish between different inflammatory events. With CIM we studied the behaviour of the different subpopulations of WBC isolated from peripheral blood. It allowed monitoring the activation of WBC and the relationship between the different cell subpopulations during the reperfusion injury and with the three different immunosuppressive agents employed. CIM has been used in experimental procedures to investigate the extent of acute rejection, graft damage or infection and other inflammatory processes (64, 92, 176, 188).

Routine histological techniques such as staining with haematoxylin-eosin or Masson's Thrichrome are usually performed in experimental and clinical procedures to evaluate the extent of histological lesions, the intensity of tissue inflammation, tissue damage or leukocyte

infiltration (46, 117, 197). They can also be employed to detect fibrosis in the vascular bed or presence of fibrinoid aggregates (6, 13, 93). We used haematoxylin-eosin, Masson's thrichromic and Weiger's haematoxylin staining to investigate the general state of the reperfused muscle, the extent of leukocyte infiltration and muscle damage as well as fibrosis, necrosis or thrombosis phenomena.

Leukocyte infiltration, inflammatory mediators and neutrophil-mediated tissue injury are important factors in the development and latter consequences of IRI. These cellular factors were studied by means of immunohistochemical methods. All of them play an important role during IRI as CD45 (LCA) shows positive reactions to WBC and granulocyte esterase is also expressed on neutrophils. CD31 (PECAM) is an adhesion molecule leading leukocytes to adhesion through endothelial and platelet activation. Stainings with CD45, CD31 and granulocyte esterase were performed in our experiments to evaluate the presence of WBC in different tissues, activation of platelets leading to leukocyte adhesion and neutrophil-mediated cytotoxicity. These techniques have often been employed not only in the study of IRI but also in other inflammatory events (47, 61, 67, 139).

IL-4 is a central cytokine involved in the development of inflammation and in the regulation of the cooperating T-lymphocyte response (205). T helper lymphocytes play an important role in IRI as they are able to activate other WBC subpopulations such as NK cells or macrophages (57). We investigated the effect of ATGs on the expression and release of IL-4 by immunohistochemical means and correlated these results with tissue damage and further cell activation.

The evaluation of the histological and immunohistochemical staining was scored by two independent observers. Minor difference on the scoring was resolved by agreement. Semi-quantitative analysis was performed and the results classified according to previously established criteria that graded the muscle damage, leukocyte infiltration, vascular damage and the

presence/absence of IL-4. These evaluation methods are accepted in the literature (38, 95) as a reliable tool for studying tissue damage and morphological changes in rejection and IRI.

The statistical evaluation of the results was performed by means of different statistical techniques. Descriptive statistics were presented by median $\pm$ standard deviation, a method commonly used in the literature (184). Analytical study of the groups by means of ANOVA is recommended to compare different groups and permitted us to investigate the effect of taking different variables as dependent or independent. Scheffe´s correction is one of the methods employed to correct the global results of ANOVA when comparing groups one-by-one. Our choice to use nonparametric function estimation with stochastic data or smoothing-spline as a method to design a multivariate model for each group is due to the versatile behaviour of these models and their fair correlation with the analytical study of the groups.

Analysis of several study groups with dependent and independent variables as well as correction of the results and construction of multivariate models are well-established methods for scientifical statistical analysis (32, 184).

4.2 Discussion of the results

4.2.1 Influence of ATG on peripheral blood counts

The absolute number of circulating WBC during the reperfusion was reduced in the ATG groups when compared to the control group. Our results are in agreement with other clinical and experimental studies. This reduction may be related to the lymphocytotoxicity of these polyclonal antibodies (156) and at the same time to the higher induction of apoptosis of peripheral lymphocytes through CD95 ligand (52, 53). When comparing the groups one-by-one, only the Merieux-ATG group showed a lower number of peripheral WBC in short ischemia,

suggesting a reduction of the lymphocytotoxic activity parallel to the increase of the ischemia time. No data supporting or negating this fact have been found in the literature.

With respect to RBC, the ATG groups present higher values of circulating erythrocytes in comparison to the control group. This reduction of circulating RBC may be due to the presence of haemorrhage and muscle damage in the control groups during the reperfusion and also to the increased number of thrombi or fibrinoid aggregates present in the vessels and the capillary net of the control groups compared to the treated groups.

These facts, higher incidence of haemorrhage, muscle damage and higher presence of fibrin, thrombi and fibrinoid aggregates in the control groups may, in the same way, be related to the increased values of RBC, haemoglobin and haematocrit presented by the ATG groups in comparison to the control groups. In fact, although the haematocrit was set in 30% to enable a better circulation through the perfusion system, all blood related parameters suffered a parallel decrease in the control groups, significantly more severe than the decrease of the ATG groups.

The number of circulating thrombocytes had a particular behaviour that may be separately discussed. The number of thrombocytes in the control groups was already higher at the beginning of the reperfusion when compared to the ATG groups. This fact suggests a relative toxicity of ATGs to platelets, a conclusoin supported by the property of ATGs to recognize antigens expressed in non-lymphoid cells (platelets, endothelium, etc.) as shown by Bonnefoy-Berard *et al* (21). During the following reperfusion process, the values of platelets in the Fresenius-ATG groups decreased parallel to the control groups while the number of circulating thrombocytes in the Merieux-ATG and Biotest-ATG groups decreased significantly. This finding may be related to the different origin of the three ATGs: both Merieux and Biotest ATGs deriving from human thymocytes, Fresenius from a cultured Jurkat cell line. However, no previous investigation to support these theories was found in the literature. A further

investigation to test in vitro toxic properties of ATGs against blood components is recommended.

4.2.2 Influence of ATG on peripheral blood smears

Blood smears were performed to evaluate the behaviour of the different WBC subpopulations in whole blood both for the control and the ATG groups as well as to test the influence of ATGs on the different types of WBC after a period of ischemia and later reperfusion.

A significant decrease of the number of lymphocytes in the ATG groups in comparison to the control groups both in SI and LI times is observed. This finding shows a good correlation between the morpho-cytological study and the counts obtained from PB. Diminution of lymphocytes was constant for all the ATG groups, but showing significant differences between them, probably related to lymphocytotoxicity and apoptosis induction of ATGs for this WBC subpopulation. There is, however, a significant difference in the Merieux-ATG group according to the "time of ischemia" variable. In the PB counts, the number of WBC in the LI group was higher than in the SI group, while in the blood smears the percentage of lymphocytes was higher in the SI group. In our opinion, the relative increase of WBC in peripheral blood of the long ischemia group is related to the increase of neutrophils, having no direct relation with the percentage of lymphocyte in the preparation.

The percentage of neutrophils in PB counted in whole blood smears showed a significant difference between control and study groups. The percentage of neutrophils is significantly higher in the ATG groups than in the control groups although the number of circulating WBC is lower. This may be explained by the lymphocyte depleting activity of ATGs. The number of circulating WBC is fundamentally reduced due to the lymphocyte depletion. Therefore there is a

reduction of the percentage of lymphocytes and consequently a higher percentage of neutrophils in the extensions.

The same occurs when studying the percentage of monocytes, although in this case, the percentage of these cells does not change significantly during the reperfusion.

In the microphotographs presented, the presence of mononuclear cells is much higher in the control groups than in the ATG groups both in SI and LI times. In the latter, one finds more polymorphonuclear cells. That is consistent with the results shown and the relative increase of PMN in the ATG groups due to the lymphocytotoxic properties of these drugs. Occasional presence of damaged erythrocytes was documented although it had no relation to the drug employed or the time of ischemia.

4.2.3 Influence of ATGs on WBC subpopulations

Cyto-immunomonitoring has been used to study inflammatory events in the field of transplantation, as explained before. With the help of this technique we studied the influence of the ATGs upon the behaviour of the different WBC subpopulations. These data are also shown in percentage, meaning that the values of the different subgroups of cells are relative to the total of WBC in the cytological preparation. CIM results are consistent with the percentages of WBC shown in whole blood and blood smears. The percentage of lymphocytes in CIM was significantly lower in the ATG groups than in the control groups. This is explained by the direct lymphotoxicity of ATGs and the increased rate of apoptosis in this cell subpopulation. No differences were found related to ischemia time in this part of the study.

Neutrophil and monocyte percentages also presented a parallel behaviour to the whole blood smears and were higher in the ATG groups than in the control groups. However, the Biotest-ATG group presented lower values of neutrophils when compared to the Fresenius-ATG group

in the SI time and lower values than the other groups in the percentage of monocytes also in the SI time. This may be explained by the presence of eosinophils and LGLs in some of the samples of the Biotest-ATG group which are not shown although counted, perhaps modifying the percentage of neutrophils and monocytes.

The microphotographs revealed a higher presence of PMN in the ATG groups. The nuclear modifications of these cells suggest regeneration of this cell subpopulation after cellular damage. The nuclei of the PMN are hypersegmented and these cells show in some ATG groups' membranous damage. A higher presence of mononuclear cells is observed in the control groups during the reperfusion.

4.2.4 Influence of ATGs on tissue, vascular damage and leukocyte infiltration

All groups treated with one of the three different ATGs presented less muscle damage than the control groups. The differences were statistically significant. As could be observed in the microphotographs shown, the tissue samples of the control groups presented a high rate of fibre necrosis, wide areas of haemorrhage and areas of diffuse infiltration throughout the muscular and connective tissue. Biochemical features such as release of OFR or proinflammatory cytokines and cellular features such as neutrophils and endothelial cell activation are mainly responsible for this muscle damage and loss of structure (96). Focusing on the cellular features, the presence of infiltrating neutrophils and other WBC such as lymphocytes or macrophages in tissue was due to an increase in the release of proinflammatory mediators and adhesion molecules provoked by the activation of endothelial cells after reperfusion (35). This cellular activity may be decreased by ATGs, as no comparable muscle damage was observed on the ATG groups.

The muscle damage observed has a tight relationship to the extent of the leukocyte infiltration in muscular, vascular, connective perivascular and perimisial tissue. Decrease of leukocyte

infiltration was observed by immunohistochemical staining with CD45 and granulocyte esterase in all the ATG groups in comparison to the control groups. Differences between the ATG groups were restricted to the LI time. After LI, the Fresenius-ATG group showed less muscle and perivascular tissue infiltration in one-by-one comparison with the other two ATG groups. These differences were not found in connective or vascular infiltration according to the study criteria. Merieux-ATG groups showed more general infiltration after LI than after SI, consistent with the previously explained results for PB counts and blood smears. These differences may be related to the different origin of the three ATGs.

WBC presence in tissue after reperfusion is directly related to increased neutrophil adhesion and activation of immune and endothelial cells (165). Although the exact nature of interactions between endothelial and cellular adhesion molecules has not been clearly defined, a decrease of WBCs present in muscle and muscular vessels as well as a reduction in the number of infiltrating neutrophils in the same area suggest that ATG may play a role in blocking the process of transendothelial leukocyte migration. This anti-inflammatory action might be due to the lymphocyte depletion and subsequent decrease of the activation of endothelial cells after reperfusion or might be related to a direct effect on adhesion molecules and proinflammatory mediators. Both hypotheses are partially supported by our data, as less endothelial or vascular damage is strongly associated to a diminution of the circulating WBC observed in PB counts, smears and CIM and to a decrease of the extent of tissue infiltration. However, decrease of CD-31 and IL-4 expression also partially supports the direct blocking of adhesion molecules and proinflammatory cytokines. In any case, further experiments must be performed to define the exact mechanism of action of ATGs in preventing leukocyte transendothelial migration and subsequent muscle damage after reperfusion.

Vascular obliteration related to fibrin or fibrinoid aggregate formation as well as to endothelial and thrombocyte activation was measured by means of histological (Masson's Thrichrome,

Weiger`s Haematoxylin) and immunohistochemical (CD31) techniques. Control groups showed more presence of fibrin and positive reaction for CD31 than the ATG groups. Activated thrombocytes adhered to the endothelial cells or were included in vascular aggregates. They were observed in most of the biopsies studied both in SI and LI. ATG groups, however, showed a lower presence of positive reaction for CD31 and fibrin staining as compared to the control groups. The intragroup differences observed for the ATG groups may be related to the different origin of the three ATGs.

Decrease of the positive reaction for CD31 (PECAM) can explain the diminution of the transendothelial leukocyte migration, as CD31 is one of the most important adhesion molecules implied in this process (35, 58). In fact, many publications assert that decrease of the expression of PECAM attenuates reperfusion injury (30, 47) and ameliorates the outcome of the reperfused tissue (89). This fact supports the hypothesis that ATGs have a protective effect upon IRI by direct blocking adhesion molecules or by diminution of the production of these molecules as a consequence of leukocyte depletion.

Cellular inflammation and infiltrating WBC in tissue also lead to muscle damage. Demonstration of IL-4 in our model is related to a higher WBC presence and greater muscle damage. This fact may be related to a participation of IL-4 in the proinflammatory cascade accompanying IRI as an activator of Th2 helper lymphocytes. Therefore, inhibition of IL-4 release might result in a decrease of the cooperation between lymphocytes, decrease of other inflammation molecules such as TNF-α or IL-2 and subsequently less endothelial activation and vascular damage (44, 162). ATG groups show in our model significantly less presence of IL-4 than the control groups. These results are consistent with the hypothesis that IL-4 release may have a proinflammatory role in muscle ischemia reperfusion injury.

5- SUMMARY AND CONCLUSIONS

Background:

Ischemia-reperfusion injury (IRI) is a non-specific, antigen independent event, which significantly influences the outcome of transplanted organs. Anti-thymocyte globulins (ATGs) are used to prevent acute rejection after transplantation, to induce immunosuppression and to overcome graft vs. host disease or haematological disorders. ATGs induce apoptosis and complement-mediated cell death in peripheral T-lymphocytes and have the potential to inhibit leukocyte adhesion by directly binding to adhesion molecules. We analysed, by means of cytology, histology and Immunohistochemistry, the microvasculature and the different blood cell-subpopulations upon ischemia/reperfusion after induction of immunosupression by three different ATGs.

Material and Methods:

Extremities of cynomolgus monkeys were flushed with Ringer's lactate solution at 4 C° via either the femoral or the brachial artery. After 60 minutes of ischemia the limbs were reperfused with human blood of the corresponding blood group (BG 0). ATGs were added to the blood, diluted to a haematocrit of 30 % with Krebs-Henseleit-buffer, 20 min prior to the reperfusion. Perfusion was carried out in a re-circulatory perfusion system. The limbs (n=60) were assigned to four groups: Biotest-ATG group (n=20), Fresenius-ATG group (n=12), Merieux-ATG (n=11) and control group (without ATG; n=17). The perfused muscle was investigated using intravital microscopy to determine the influence of ATGs on the microcirculation. Monitoring of haematological parameters was performed and counts of RBC, WBC, platelets, haematocrit and haemoglobin were realised. Cytology and Cyto-immunological-monitoring (CIM) were performed in blood samples taken at different time points (0,1,5,10,15,30,45 and 60 min) after

onset of reperfusion. Biopsies from muscular tissue were taken after the experiments. Histological and immunohistochemical techniques were used to investigate the vascular damage and the distribution of WBC in vascular, perivascular and muscular tissue, applying semi-quantitative analysis to evaluate the results.

Results and conclusions:

The following conclusions may be drawn from the results:

- Monitoring of the haematological parameters showed a decrease in the number of WBC in the treated groups compared to the control groups. The amount of RBC, haemoglobin and the haematocrit was significantly higher in the treated groups than in the control groups.
- ATG-Biotest and Merieux-ATG groups showed a decrease in the number of circulating platelets in comparison to Fresenius-ATG and control groups.
- Cytological and CIM studies demonstrated significant differences in lymphocytotoxicity and depletion of peripheral lymphocytes in the ATG groups in comparison to the control groups.
- Histological and immunohistochemical analyses showed a decrease in vascular and perivascular infiltration as well as muscle inflammatory reactions after ATGs treatment.
- Expression of IL-4 was reduced in the ATG groups when compared to the control group.
- ATGs influence IRI, having a positive effect on the outcome of reperfused tissues

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6- ZUSAMMENFASSUNG

Hintergrund:

Der Ischämie-Reperfusionsschaden ist ein unspezifischer, Antigen unabhängiger pathophysiologischer Prozess, welcher bedeutenden Einfluß auf das Überleben transplantierter Organe hat. Antithymozyten-Globuline (ATGs) werden als Immunsuppressiva in der Therapie akuter Abstoßungsepisoden und zur Unterdrückung der Graft vs Host Disease sowie hämatologischer Funktionsstörungen eingesetzt. ATGs führen zu Apoptose und Komplement vermitteltem Zelltod, wobei die direkte Bindung an Adhäsionsmoleküle die Leukozyten-Adhäsion hemmt.

Wir haben mittels Zytologie, Histologie und Immunhistochemie den Einfluß dreier verschiedener ATGs auf die Mikrozirkulation sowie die unterschiedlichen Zellsubpopulationen nach Ischämie/Reperfusion untersucht.

Material und Methoden:

Arterie und Vene der Extremitäten von Affen (M. fascicularis) wurden isoliert, mit 4 C° kalter Ringer-Laktatlösung gespült und nach einer Ischämiezeit von einer bzw. zwei Stunden über die femorale bzw. brachiale Arterie mit Blutgruppen-kompatiblen Humanblut reperfundiert.

Dem mit Krebs-Henseleit-Puffer auf einen Hämatokrit von 30% verdünntem Blut wurden ATGs 20 Minuten vor der Reperfusion zugefügt. Die Perfusion wurde mit Hilfe eines Perfusionssystems rezirkulierend durchgeführt. Die Extremitäten (n=60) wurden entsprechend dem Versuchsansatz vier verschiedenen Gruppen zugeteilt: Biotest-ATG Gruppe (n=16), Fresenius-ATG Gruppe (n=16), Merieux-ATG Gruppe (n=12) und eine Kontroll-Gruppe (ohne ATG; n=16). Während der Perfusion wurde die Mikrozirkulation der perfundierten Muskulatur mittels Intravital-Mikroskopie untersucht. Neben der Bestimmung hämatologischer Parameter,

wurden die Anzahl der Rot Blutzellen (RBZ)**,** weiß Blutzellen (WBK), Thrombozyten sowie die Hämatokrit- und Hämoglobinspiegel im Perfusat zu verschiedenen Zeitpunkten bestimmt. Zytologische Untersuchungen und zyto-immunologisches Monitoring (CIM) wurde in Blutproben, welche zu unterschiedlichen Zeitpunkten (0,1,5,10,15,30,45,60 Min.) abgenommen wurden, durchgeführt.

Nach den Versuchen wurden Biopsien von Muskelgewebe entnommen. Histologische und immunhistologische Techniken wurden angewandt, um den Einfluß der ATGs auf die Integrität des Gewebes und die Infiltration der weißen Blutzellen (WBZ) im vaskulären, perivaskulären und muskulären Gewebe semi-quantitativ zu analysieren.

Ergebnisse und Folgerungen:

- Die hämatologische Untersuchung ergab eine signifikante Reduktion der zirkulierenden WBZ in den behandelten Gruppen im Vergleich zu der Kontrolle. Die Anzahl der RBZ, sowie der Hämatokrit und der Hämoglobinspiegel waren im Vergleich zur Kontrolle signifikant erhöht.
- Die Anzahl zirkulierender Thrombozyten in den ATG-Biotest und Merieux-ATG Gruppen war im Vergleich zu Fresenius ATG Gruppe und Kontrolle signifikant reduziert.
- Die zytologische Untersuchung sowie das CIM zeigten signifikante Unterschiede hinsichtlich der Lymphzytotoxizität und der Depletion peripherer Lymphozyten in den ATG Gruppen im Vergleich zur Kontrolle.
- Die histologische und immunhistochemische Analyse ergab eine reduzierte vaskuläre und perivaskuläre Infiltration, sowie eine Verminderung der Inflammation des muskulären Gewebes nach Behandlung mit ATG.
- Die Expression von IL-4 war in den ATG Gruppen signifikant niedriger als in der Kontrolle.

7- APPENDIX: Tables

The values of all the parameters studied distributed into groups are presented in this appendix.

Values are shown as median ± standard deviation.

App 1.1.- Blood parameters

App 1.1.1.-WBC

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	6,45	1,1705	6,45	0,8255	5	1,7742	9	2,7809
0	3,92	0,7488	4,4	0,8280	3,3	1,3427	6,8	1,9602
1	2,95	0,6124	3,3	0,7348	2,8	0,9571	5,9	3,9016
5	2,9	0,7064	2,6	0,6129	2,4	1,2149	5,3	1,5093
10	2,65	0,7160	2,3	0,5033	2,1	1,1870	3,55	1,9091
15	2,55	0,9109	2,3	0,5219	2,1	0,9945	3,65	2,4748
30	2,7	0,8354	1,9	0,4353	1,9	1,1840	1,5	1,9157
45	2,2	1,0714	1,6	0,5468	1,6	1,2436	2,5	1,6802
60	2,25	1,0342	2	0,7805	1,55	0,0707	2,6	1,2503

Tab. 1: WBC-Short Ischemia: Number of WBC in PB. Values for every time point are expressed in number of cells x $10^3/\mu L$

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	6,45	1,1846	6,1	0,7996	6,75	1,7075	9	2,8598
0	4,15	0,5343	4,2	0,4615	4	1,1672	6,3	2,7624
1	2,65	1,1519	3,2	0,5856	4,15	1,8266	5,1	2,21698
5	2,7	0,9008	2,7	0,3193	3,45	1,4854	3,65	2,568
10	2,65	0,7740	2,6	0,5727	3,2	1,3744	5,1	2,4724
15	2,85	0,5873	2,3	0,3633	3,1	1,4974	4,05	1,2120
30	2,6	0,6664	2,2	0,2607	2,55	1,0801	3,8	1,1344
45	2,2	0,2810	2,1	0,1923	1,9	0,9609	2,8	0,70946
60	2,25	0,7176	1,88	0,2178	2,3	0,5033	3,45	1,4849

Tab. 2: WBC-Long Ischemia: Number of WBC in PB. Values for every time point are expressed in number of cells x $10^3/\mu L$

App. 1.1.2.- RBC

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	5,095	0,3628	4,93	0,5676	4,79	0,4527	4,87	0,2885
0	3,53	0,1943	3,4	0,2559	3,43	0,1109	3,47	0,4029
1	3,375	0,3295	3,4	0,7274	3,52	0,4872	3,45	1,1664
5	3,525	0,4471	3,35	0,6986	3,52	0,3440	3,33	0,7249
10	3,615	0,5395	3,42	0,7135	3,5	0,202	3,34	0
15	3,705	0,5149	3,33	0,8312	3,49	0,2135	3,38	0,0707
30	3,875	0,5549	3,55	0,6945	3,44	0,5029	3,4	1,3741
45	3,85	0,5889	3,92	0,5908	4,24	0,7442	3,3	0,4147
60	4,26	0,8616	3,98	0,7166	3,285	0,6293	3,31	0,5200

Tab. 3: RBC-Short Ischemia: Number of RBC in PB. Values for every time point are expressed in number of cells x $10^6/\mu L$

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	4,705	0,4915	4,93	0,6565	5,185	0,455	4,315	0,4936
0	3,355	0,2312	3,4	0,2306	3,475	0,2242	3,39	0,4445
1	3,255	0,4305	3,49	0,2847	3,42	0,27702	3,46	0,7469
5	3,315	0,5207	3,54	0,5558	3,36	0,2808	3,18	1,4345
10	3,465	0,3605	3,59	0,2338	3,5	0,2542	2,9	1,0128
15	3,495	0,2633	3,55	0,2977	3,615	0,3092	3,26	0,5899
30	3,795	0,5576	3,91	0,4403	3,81	0,4011	3,38	0,8441
45	4,165	0,5572	4,06	0,6333	4,19	0,5416	3,45	0,5676
60	4,21	0,6321	4,02	0,7984	4,32	0,8083	3,12	0,791

Tab. 4: RBC-Long Ischemia. Number of RBC in PB. Values for every time point are expressed in number of cells x $10^6/\mu L$

App. 1.1.3. Platelets

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	220	63,13	210	59,688	192	44,679	274	43,482
0	162,5	49,00	151	64,86	138	38,379	188	29,593
1	119	52,770	146	19,093	128	31,100	140,5	33,826
5	97,5	48,113	149	33,585	82	47,975	155	26,368
10	108,5	52,151	145	36,404	118	34,095	157,5	0,7071
15	88	52,415	137	26,333	108	28,342	154	8,4852
30	86	50,974	132	64,605	93	24,513	140	52,252
45	59	41,764	122	68,670	80	35,98	133	74,332
60	64	29,518	117	93,811	115	38,37	119	12,342

Tab. 5: Thrombocytes-Short Ischemia: Number of platelets in PB. Values for every time point are expressed in number of cells x $10^3/\mu L$

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	212	67,744	210	71,578	242	20,832	270,5	45,919
0	160,5	52,512	151	75,457	154,5	51,990	176,5	41,126
1	106	38,536	152	17,908	155	34,635	137,5	22,911
5	91,5	36,492	148	41,644	156	30,309	134	71,092
10	105,5	56,505	148	40,228	132	28,099	202	43,675
15	69	58,585	140	52,276	127	23,614	136	22,278
30	79	44,468	128	40,350	108,5	24,102	158	54,995
45	73,5	44,159	128	39,268	98,5	30,490	147	54,921
60	66,5	54,020	120	33,484	57	30,347	129,5	19,091

Tab. 6: Thrombocytes-Long Ischemia: Number of thrombocytes in PB. Values for every time point are expressed in number of cells x $10^3/\mu L$

App. 1.1.4.Haematocrit

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	45,05	3,5139	45,5	5,0753	43,6	5,1250	38,6	2,6758
0	30,8	1,4931	29,6	1,5126	31,5	1,1757	30,6	3,2957
1	30,3	2,0465	29,6	6,9158	30,05	4,7053	30,8	10,140
5	30,8	3,3028	30,3	6,4523	30,9	3,5888	29,8	6,3749
10	31,2	4,2536	31,3	6,6725	31,3	1,9475	29,9	0,1414
15	32,55	4,0083	30,5	7,5627	31,9	2,0958	30,25	0,6363
30	34	3,8818	32,3	6,0717	30,3	4,3665	30,1	11,869
45	33,8	4,2149	35,3	6,2353	34,55	6,061	29,7	2,783
60	36,9	6,3443	35	5,9733	29,5	3,9597	29,9	4,050

Tab. 7: HCT-Short Ischemia: haematocrit in % before and after dilution.

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	41,55	4,1379	43,5	5,4550	48,8	4,1931	38,75	3,4372
0	30,4	1,5099	29,6	1,4024	30,95	1,6713	30,35	3,1784
1	29,2	2,9794	30,9	2,6949	31,05	3,3491	27,05	6,1190
5	29,15	3,8354	31	4,5025	31,35	1,7858	28,6	13,389
10	30,35	2,3998	31,9	2,1349	32,2	1,7757	25,6	8,8017
15	31,75	1,9449	31,1	3,0792	32,35	1,8779	29,15	5,4178
30	35	4,1262	34,3	4,7072	33,55	3,2176	30,3	7,4503
45	37,15	4,2589	36,8	7,4031	36,9	3,8974	29,2	5,2204
60	38,5	5,5568	35,3	8,4690	38	6,3437	27,7	7,0710

Tab. 8: HCT-Long Ischemia: haematocrit in % before and after dilution

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App. 1.1.5. Haemoglobin

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	15,05	1,2035	14,6	1,8087	14,1	2,023	13,9	0,8173
0	10,6	0,8530	9,9	0,7184	10,3	0,7114	10,3	1,4060
1	10,7	0,9057	10,3	1,9146	10,8	1,4138	10,6	3,9041
5	10,95	1,0340	10,3	1,8680	10	1,3246	9,9	2,2576
10	10,65	1,4945	10,8	1,9171	10,4	0,7967	10,35	0,6363
15	11,15	1,3680	10,8	2,2825	11,1	1,0014	10,55	0,9192
30	11,75	1,1400	11,3	1,9659	10,9	1,5565	10,1	4,2335
45	11,7	1,1150	11,8	1,6118	11,7	2,1238	10,3	1,1590
60	12,6	1,5793	12	2,1562	9,7	0,8485	10,5	1,3276

Tab. 9: Hb-Short Ischemia: Amount of haemoglobin during the reperfusion after a SI period.

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	13,6	1,4232	14,6	1,9603	16,15	1,5649	12,8	1,1923
0	10,45	0,7946	9,9	0,5449	10,6	0,7274	10	1,0264
1	10,4	0,9818	10,1	0,7791	9,85	0,668	9,5	2,1173
5	10,2	1,4030	10,4	1,2136	10,1	0,5	9,7	4,3431
10	10,65	1,0192	10,8	0,7968	10,3	0,5477	9,3	2,9517
15	11,2	1,0077	10,8	0,8700	10,45	0,6849	10,2	1,2419
30	11,80	1,2668	11,5	1,1631	10,75	1,2027	9,9	1,9537
45	12,8	1,6317	11,6	2,0005	11,5	1,285	9,9	0,7767
60	13,3	2,1229	11,8	2,5449	12,1	2,0663	9,35	1,9091

Tab. 10: Hb-Long Ischemia: Amount of haemoglobin during the reperfusion after a LI period.

App. 1.2.-Smears

App. 1.2.1. Lymphocytes

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	30	13,69	28	12,12	54	14,76	39,5	20,14
0	44	12,33	50	21,94	34	14,57	38	36,90
1	40	8,51	28	16,02	42	14,24	50,5	41,71
5	31	12,64	38	15,10	46	6,75	69	26,50
10	44	19,49	29	25,37	48	9,94	58,5	53,03
15	35	6,80	43	21,11	40	14,95	58,5	44,54
30	31	7,14	30	18,41	40	9,21	94	32,33
45	34	5,62	30	16,10	35	9,55	59	46,66
60	29	7,27	32	20,17	24	5,50	88	26,15

Tab. 11: Lymphocytes-Short Ischemia: Percentage of lymphocytes in PB smears after a period of SI (median and standard deviation are expressed in %).

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	32	13,64	24	8,67	35,5	11,57	39	16,25
0	44	12,57	50	19,54	48	16,35	70	23,57
1	50	22,58	36	21,33	25	18,09	32	20,42
5	44	16,72	28	17,30	38	5,61	59	23,44
10	30	18,00	28	16,04	32,5	11,09	63	26,65
15	32	6,75	28	20,94	35,5	14,47	60,5	17,83
30	37,00	9,17	30	19,21	29	11,75	69	17,38
45	32	15,46	25	22,77	25,5	27,53	50	45,25
60	26	17,31	24	23,55	21	9	52,5	36,06

Tab. 12: Lymphocytes-Long Ischemia: Percentage of lymphocytes in PB smears after a period of LI (median and standard deviation are expressed in %).

App.1.2.2 Neutrophils

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	58	13,33	61	8,99	44	12,73	55,5	18,73
0	47	13,88	44	19,48	49	13,43	49	36,59
1	55	10,18	45	14,07	51	14,85	45	41,01
5	60	11,46	46	18,67	48	6,21	36,5	26,16
10	49	15,57	53	23,71	50	10,46	37	49,49
15	60	6,22	55	22,13	51	16,65	38,5	43,13
30	63	5,99	59	16,58	58	5,47	29,5	38,89
45	58	5,77	58	14,97	60	5,12	37,5	44,54
60	62	10,46	48	18,21	69	4,04	4	24,82

Tab. 13: Neutrophils-Short Ischemia: Percentage of neutrophils of the different study groups during the reperfusion after a SI period (median and standard deviation are expressed in %).

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	57	13,02	65	7,39	56,5	8,84	55	14,36
0	47	13,26	44	17,60	43	11,16	26	20,55
1	44	22,56	54	21,89	73,5	20,07	66	26,85
5	54	16,87	58	29,35	53	10,37	40,5	22,41
10	58	16,95	55	20,48	58,5	14,72	35	29,56
15	58	14,43	50	24,26	60,5	13,04	33,5	21,07
30	56	9,77	40	25,40	62	16,58	16	20,29
45	60	17,17	32	22,42	62	22,41	41,5	50,20
60	66	18,87	40	25,54	70	11,01	37,5	43,13

Tab. 14: Neutrophils-Long Ischemia: Percentage of neutrophils of the different study groups during the reperfusion after a LI period (median and standard deviation are expressed in %).

App. 1.2.3. Monocytes.

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	8	4,02	9	3,77	6	4,19	5	2,58
0	8	2,34	8	4,15	6	1,97	8	2,88
1	6	3,73	4	1,60	3	2,96	4,5	0,70
5	5	3,25	5	3,03	3	3,35	4	5,29
10	7	3,90	3	4,18	6	5,61	4,5	3,53
15	5	2,94	5	2,26	1,5	6,28	3	1,41
30	4	3,14	7	2,54	2	3,20	5	1
45	5	4,54	6	2,50	2	4,38	3,5	2,12
60	5	3,71	6	3,09	4	2,51	7	3,21

Tab. 15: Monocytes; Short Ischemia: Percentage of monocytes during the reperfusion after a SI time (median and standard deviation are expressed in %).

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	8	4,02	10	3,43	6	4,19	6	2
0	7	2,85	8	4,43	7,5	2,62	8	4,50
1	4	3,54	4	3,19	4	3,30	3	4,35
5	4	3,45	5	2,30	7	1,5	4	4,52
10	8	3,04	3	2,34	6,5	2,08	4	3,05
15	6	3,31	3	3,27	4,5	1,41	7	3,46
30	5	1,51	4	1,94	7,5	6,70	10	4,04
45	6	2,22	5	2,54	5	5,59	7,5	3,53
60	6	3,76	6	3,56	8	3,51	8,5	6,36

Tab. 16: Monocytes-Long Ischemia: Percentage of monocytes during the reperfusion after a LI time (median and standard deviation are expressed in %).

App. 1.3.-CIM

App. 1.3.1. Lymphocytes

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	73	14,19	69	4,25	56,5	13,21	32	0
0	64	20,83	62	21,28	62	14,18	50	16,97
1	39	17,33	65	14,86	36	26,49	64	7,07
5	30	6,04	39	13,16	42	13,59	85	28,21
10	32	13,77	29	12,53	36	17,68	22	0
15	23	9,80	30	10,62	34	19,79	11	0
30	26	6,28	28	13,56	33,5	9,89	57	55,15
45	27,5	7,32	42	23,22	27	14,24	29	0
60	22	6,01	36,5	16,09	45	17,03	62	48,08

Tab. 17: Lymphocytes-Short Ischemia: Percentage of lymphocytes with CIM after a SI period. (median and standard deviation are expressed in %).

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	72	7,99	69	5,01	63	8,42	62	0
0	54	24,59	66	25,80	66	10,50	63	1,41
1	41	10,86	39	19,25	41	21,31	29	0
5	39,5	11,78	41	12,32	35,5	10,42	78	22,62
10	41	14,95	36	17,22	37	7,54	68,5	34,64
15	32,5	15,58	33	13,88	29	10,78	61	32,52
30	28,00	11,43	34	13,44	33	13,50	58,5	48,79
45	38,5	15,31	41,5	17,68	23,5	5,73	49	46,66
	24	14,82	25	12,50	24	14,50	16	0

Tab. 18: Lymphocytes-Long Ischemia: Percentage of lymphocytes with CIM after a LI period. (median and standard deviation are expressed in %).

1.3.2. Neutrophils

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	0	16,01	0	0	1,5	3,50	60	0
0	8	23,72	11	27,68	21,5	21,23	26,5	31,81
1	49	18,34	27	22,26	60,5	28,12	24	0
5	64	7,69	50	19,67	56	23,51	26	36,76
10	62	12,52	62	17,73	59	19,62	69	0
15	68	10,12	60	14,78	65	20,49	84	0
30	69	8,44	62	16,31	63	10,03	35,5	50,20
45	63,5	10,47	44	20,13	69,5	13,83	66	0
60	74	5,40	59,5	16,49	53	33,04	33	46,66

Tab. 19: Neutrophils-Short Ischemia: % of neutrophils after a period of SI. (median and standard deviation are expressed in %).

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	0	9,77	0	0	0	4,5	21	24,04
0	22	28,96	11	32,76	15	13,01	45	12,72
1	46	11,97	42	25,71	33	19,73	28	0
5	52,5	10,69	52	15,75	56	32,53	22,5	23,33
10	55	11,64	54	15,05	59	6,94	30	32,52
15	62	13,27	55	15,11	57	11,06	42	41,01
30	63,5	14,75	54	12,70	62	8,62	42	49,49
45	57	17,70	42,5	16,89	67,5	3,51	46	45,25
60	68	15,70	59	16,56	74	19,54		

Tab. 21: Neutrophils-Long Ischemia: % of neutrophils after a period of LI. (median and standard deviation are expressed in %).

App.1.3.3. Monocytes

	BSI		FSI		MSI		CSI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	23	10,03	29	4,68	42,5	13,61	8	0
0	18	13,26	20	15,22	12	10,27	23,5	14,84
1	7	3,74	9	8,38	4,5	3,61	14	0
5	5	4,816	9	6,50	3	5,472	10	7,07
10	3	4,70	5	6,06	1,5	4,35	7	0
15	4	4,03	4	7,38	2,5	1,36	5	0
30	4	5,23	6	3,46	1	0,81	6	2,82
45	5	4,05	5	5,43	1	1,5	4	0
60	3	1,83	4	2,33	2	19,92	4	0

Tab. 21: Monocytes -Short Ischemia: % of monocytes (CIM) after a SI period (median and standard deviation are expressed in %).

	BLI		FLI		MLI		CLI	
Time	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**	**Median**	**Sta.De.**
-30	28	12,98	28	5,06	34	10,66	0	0
0	15	10,77	18	13,74	27	12,66	17	24,04
1	9	8,39	18	10,22	7,5	2,16	8,5	12,02
5	3	3,02	7	5,49	3	4,19	5	7,071
10	6,5	2,87	6	2,44	6	4,12	8,5	12,02
15	4,5	2,67	7	3,36	2	4,61	4	5,65
30	4	2,60	3	5,41	3	1	2,5	3,53
45	4	3,50	6	4,85	4	5,47	2,5	3,53
60	3	4,50	1	8,38	7	5,50	3	0

Tab. 22: Monocytes-Long Ischemia: % of monocytes (CIM) after a LI period (median and standard deviation are expressed in %).

App. 1.4: Histological results

	CSI		FSI		BSI		MSI		CLI		FLI		BLI		MLI	
	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D	mean	St. D
Muscle Damage	2,6	0,51	0,9	0,73	1,1	0,73	1,2	0,63	2,8	0,42	1	0,66	1,6	0,69	1,4	0,51
Connect. Infiltration	2,5	0,52	1,1	0,31	1,2	0,42	1,3	0,48	2,3	0,67	1,3	0,48	1,4	0,51	1,3	0,48
Vascular Infiltration	2,3	0,48	1,1	0,31	1,3	0,48	1,4	0,51	2,4	0,69	1,5	0,52	1,5	0,52	1,3	0,48
Perivasc. Infiltration	2,3	0,67	1,2	0,42	1,3	0,48	1,3	0,48	2,3	0,67	1,2	0,42	1,4	0,51	1,5	0,52
Muscular Infiltration	2,4	0,69	0,5	0,52	0,4	0,51	0,4	0,51	2,7	0,48	0,4	0,51	0,6	0,51	0,9	0,79
Presence Fibrin	1,9	0,56	0,3	0,48	1,2	0,63	0,8	0,63	2,2	0,63	0,8	0,63	1,3	0,67	1,1	0,56

Tab. 23: Values obtained after semi-quantitative analysis of the histological and immunohistochemical sections.

Results expressed as mean ± standard deviation

8. LITERATURE

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