# POLYMORPHONUCLEAR GRANULOCYTES AND REACTIVE OXYGEN SPECIES IN THE PATHOMECHANISM OF ACUTE HAEMARTHROSIS

**Ph.D. Thesis**

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# Contents

# Abbreviations

| | |
|---|---|
| **4-HNE** | 4-hydroxy-2 (E)-nonenal |
| **CS** | chondroitin sulphate |
| **DMEM/F-12** | Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 |
| **DMSO** | dimethyl sulfoxide |
| **FCS** | fetal calf serum |
| **FITC** | fluorescein isothiocyanate |
| **GAG** | glycosaminoglycan |
| **GDB** | glucose containing modified Dulbecco's buffer |
| **GS** | glucosamine-sulphate |
| **GSH** | reduced glutathione |
| **HA** | haemarthrosis |
| **LDH** | lactate dehydrogenase |
| **LFA-1** | leukocyte function associated molecule-1 |
| **LTB-4** | leukotrien B-4 |
| **MDA** | malondialdehyde |
| **NSAID** | non-steroid anti-inflammatory drugs |
| **OA** | osteoarthritis |
| **PAF** | platelet activating factor |
| **PBS** | phosphate buffered saline |
| **PMA** | phorbol 12-myristate 13-acetate |
| **PMN** | polymorphonuclear leukocyte |
| **PRF** | phenol red free |
| **RBC** | red blood cell |
| **ROS** | reactive oxygen species |
| **SOD** | superoxide dismutase |
| **SYSADOA** | symptomatic slow acting drugs against osteoarthritis |
| **TUNEL** | terminal deoxynucleotidyl transferase mediated desoxyuridinetriphosphate nick end labelling |
| **WBC** | white blood cell |

# 1. INTRODUCTION

Joint disease affects hundreds of millions of people around the world causing pain and disability. The symptoms and treatment outcome significantly influence the quality of life and social reintegration of the patient, in addition to the considerable financial burden. In this regard activities aim to reveal new findings of pathogenic articular conditions such as the blood-induced joint destruction may have crucial clinical importance.

Despite various clinical observations and experimental investigations the precise pathophysiology of haemarthrosis is still unclear. Especially its acute form has remained illusive even though haemarthrosis more frequently presents as an acute, single episode of intraarticular haemorrhage as a result of sport injuries or other accidents. Moreover, the increasing popularity of rigorous sports and other out-door recreational activities potentially increases the incidence of acute haemarthrosis, an indication of growing risk for the whole population.

Interest in free radicals and reactive oxygen species (ROS) dates back to middle of $20^{th}$ century. Numerous in vitro and in vivo investigations were performed to reveal their role on many fields of biology.

Although the pathogenic role of free radicals and ROS were widely examined in different articular diseases, relatively little evidence was found regarding their harmful effect in haemarthrosis. On the other hand only few studies have investigated the role of polymorphonuclear granulocytes in this respect even though they represent the largest proportion of leukocytes and are an abundant source of ROS during haemarthrosis.

## 1.2. Reactive oxygen species in the pathomechanism of haemarthrosis

From the early 30s, respiratory burst and reactive oxygen species (ROS)–mediated reactions have gradually become a focus for scientific interest. Nowadays it is generally accepted that ROS play important roles not only in the host defence mechanisms but also in ageing and numerous disease states. Thus it is not surprising that new findings from free radical research have shed light on the novel role of both the iron and PMNs in the pathomechanism of degenerative arthropathies, prompting new directions for research in haemarthrosis.

Several studies report that iron is the most likely to stimulate hydroxyl generation and other radical reactions in vivo. Such reactive species are in turn able to attack the plasma membrane, mitochondria and DNA. This leads to interruption of cellular integrity, impairment of cellular energy homeostasis, alterations in the genetic code and finally amounting fatal injury to the cell.

According to *Halliwell and Gutteridge*, when haemoglobin reacts with hydrogen peroxide produces a reactive species, most likely ferril radicals that can degrade DNA and stimulate lipidperoxidation. Moreover reaction of heme protein with excess of hydrogen peroxide can cause heme degradation and release of iron ions from the protein, followed by a Fenton reaction.

On the basis of all above, besides direct matrix damaging potential of iron both RBC-derived ferrous and ferric ion as well as haemoglobin may act as a catalyst for ROS producing reactions in the intraarticular microenvironment. Therefore their contribution in the acute haemarthrosis induced cartilage damage may be considered.

There is proof of non-mitochondrial respiratory burst in all phagocytes – PMNs, monocytes, eosinophils, and lung and peritoneal macrophages - due to activation of the membrane bound NADPH-oxidase. However owing to their high number, PMNs are thought to be influential cellular participant in cartilage damage. Indeed, there is a large body of evidence that following activation PMNs produce large variety of reactive oxygen metabolites, which are able to attack all components of cartilage tissue.

### 1.3 Our hypothesis regarding the pathomechanism of acute haemarthrosis

The mechanisms detailed above can undoubtedly come to play during chronic haemarthrosis as well. However the lack of repeated bleeding episodes and the consequently absence of severe synovitis as well as the brief duration of the unfavourable environment (the 70-80 percent of RBCs are eliminated from the joint within 4-5 days enhance the importance of PMN and ROS-mediated pathways during acute haemarthrosis. According to our hypothesis PMNs are activated by the thrombocyte and endothelial-derived mediators released following the vascular injury. These leukocytes produce enzymes, cytokines and ROS that may induce production of similar factors in the chondrocytes and synovial cells via paracrine as well autocrine pathways. On the other hand, ROS and enzymes directly attack all components of the articular tissues.

In order to provide support for our hypothesis, in vitro and clinical investigations were conducted to further clarify the following:

1. *Extent of activated PMNs and iron mediated chondrocyte damage under in vitro conditions.*

2. *Do the PMNs engender oxidative stress on chondrocytes?*

3. *Are the PMNs activated during clinical presentations of acute heamrthrosis?*

# 2. EXTENT OF ACTIVATED PMNS AND IRON MEDIATED CHONDROCYTE DAMAGE UNDER IN VITRO CONDITIONS
## (First series of experiments)

Regarding acute HA, there is yet relatively little data available on its pathomechanism despite being more common than the chronic form. The key to the pathological damage lies in changes of intra-articular fluid. There are of course significant differences between the constituents of synovial fluid and blood, which is rich in red blood cells (RBC) and polymorphonuclear leukocytes (PMN). Previous studies by *Roosendal et al*. have suggested that RBC and monocytes are responsible for such damage. Furthermore abundant data exists to suggest that PMN-derived reactive oxygen species (ROS) can damage a wide range of cellular components leading to cell death. In addition RBC-derived ferrous ion, a known participant of Fenton reaction, can also lead to the formation of highly reactive hydroxyl radical.

This in vitro study was aimed to investigated interaction between some proposed key participants in the pathogenic processes following intraarticular bleeding. Thus the cytotoxic effects of PMN-derived ROS and increased quantity of ferrous ion on cultured chondrocytes were evaluated. Since DNA is also targeted by ROS, we hence investigated whether short-term chondrocyte exposure to PMN causes histologically detectable DNA lesions.

## *2.1. Materials and methods*

*Animal preparation.* Three healthy Yorkshire pigs (35-40kg) were used as blood donors. Each animal was subjected to an overnight fast and was sedated with ketamine (15mg/kg), induced with pentobarbitone and maintained on 1.5-% halothane inhalation. Following anaesthesia a silicon catheter was inserted into the jugular vein. The drain was led out on the posterior surface of the neck facilitating blood-sampling procedure.

Chondrocyte isolation was carried out on ten healthy Yorkshire pigs, weighting 20-25kg. These animals were sedated with ketamine (15mg/kg) and euthanased with intra cardiac injections of sodium pentobarbital (120mg/kg). All animal procedures were performed in accordance to the International Guiding Principles for Animal Research.

*Isolation and culturing of chondrocytes from pigs.* Whole thickness cartilage was dissected from the articular surfaces of knee joint. The cartilage pieces were chopped and the chondrocytes freed from their extra-cellular matrix by digestion On the basis of the viability, $1x10^6$ living chondrocytes were seeded on each of the 12-well plastic culture plates and incubated in humidified 95% air and 5% $CO_2$ mixture at 37°C Living chondrocytes ($20x10^6$) from the same source, were placed separately on $75cm^2$ plastic culture flasks and cultured as above for histological examinations.

*Isolation of PMN.* EDTA anticoagulated blood was sedimented with dextran and gently hemolised to remove the erythrocytes. The white pellet obtained after repeated centrifugation was suspended in glucose containing modified Dulbecco's buffer and spun against gradient separator medium. Finally, the pellet consisting of about 95% PMN was suspended in cold buffer as above. After cell counting and viability estimation the required amount of cells were used in the experiment.

*Treatment of chondrocytes and measurements of cytotoxicity.* Each well of culture plates were filled with either newly isolated PMN suspension or hydrogen peroxide as well as ferrous-sulphate or ferrous-sulphate with $10^7$PMN/2ml PMN. The antioxidant Trolox was applied together with PMN to evaluate the effect of PMN-derived free radicals. In order to

exclude the non-specific lactate dehydrogenase (LDH) release reference populations of lysed and untreated chondrocytes as well as untretaed PMN and PMN in the presence of 0.1mM ferrous-sulphate were used. Finally, 0.2μg/ml phorbol 12-myristate 13-acetate (PMA) a PMN stimulating agent was added to each well. After 30 minutes of pre-incubation and thereafter the media were sampled every second hour for a total of six hours. The released LDH content of each sample was determined using CytoTox96® Assay kit. The following equation was used for determination of cytotoxicity.

$$\% \text{ Cytotoxicity} = \frac{(OD^{\text{treated chondrocytes}} - OD^{\text{chondrocytes spontaneous}} - OD^{\text{PMN spontaneous (optional)}})}{(OD^{\text{lysed chondrocytes}} - OD^{\text{chondrocytes spontaneous}})} \times 100$$

*Histological examinations.* Chondrocytes, in the presence of PMN (treated groups), and alone (control groups) were co-incubated. At the end of the experimental period cells were washed, fixed with paraformaldehyde and stained with Leishman stain as well as subjected to immunohistochemical (TUNEL) investigations. The samples were analysed semiquantitatively by light microscopy and an image analyser.

*Statistical evaluation.* The experiments were performed on separate populations of chondrocytes, which were randomly allocated to various groups. The mean values of LDH and the standard errors are presented on the charts. One-way ANOVA test was used to define the significance between the mean values. A p-value of less than 0.05 was considered statistically significant.

## 2.2. Results

*Cytotoxic effect of activated PMNs.* The injurious effect of PMA-activated PMN on chondrocytes was tested by the measurement of LDH leakage, which is strongly correlated to the cellular damage. The cytotoxicity began to rise promptly and significantly following the addition of activated PMN. This trend peaked to 48.1±2.3 percent around the fourth hour of the experiment and slightly declined in the following two hours. The cytotoxic effect of hydrogen peroxide developed more slowly and increased continuously up to 46.4± 3.1 percent by the end of the examined period.

*Involvement of free radicals in the PMN-mediated cytotoxicity.* In order to explore the proportion of ROS in the granulocyte cytotoxicity the co-incubation was performed in the presence of Trolox, which is a cell permeable and water-soluble derivative of vitamin E with potent antioxidant property. It was found that Trolox significantly decreased the harmful effect of PMN in a dose dependent manner.

*Role of increased amount of ferrous ion.* The contribution of iron to chondrocyte damage was investigated using ferrous sulphate solutions (0.1-1.0mM final concentration), during the incubation. With ferrous sulphate alone the LDH release was insignificant. Likewise there was no significant difference between the PMN-mediated cytotoxicity measured in the presence or the absence of 0.1mM ferrous sulphate. Repeating the experiment without cells, no significant interference was noted between iron and the test kit or LDH.

*Histological investigations.* Since the chondrocytes were obtained from full thickness cartilage, the population might have contained various kinds of chondrocytes from the tidemark zone up to the superficial layer. Noticeable reduction in chondrocyte number was revealed in the treated groups as compared to the control. Polygonal and basophilic nucleated round shaped cells were found which showed remarkable variability in their size in both slides. The uniformly sized and shaped PMNs with their well known segmented nuclei were practically absent on the co-incubated sections as a consequence of shaking and rinsing procedures.

Lesions of chondrocyte genomic DNA were revealed by TUNEL reaction in PMN treated populations of chondrocytes. On the slides derived from the treated groups the ratio of DAB positive cells significantly exceeded the controls with respective values of $30.696\pm2.72\%$ and $2.03\pm0.27\%$.

## 2.3. Discussion

Based on the approximate cellular ratio occurring under common clinical settings of acute HA, in vitro investigations were performed in order to evaluate the individual and common role of these proposed factors in the pathomechanism of HA. In this study we have shown that activated PMN are remarkably cytotoxic to chondrocytes. This cytotoxicity propagated more rapidly than the effect of hydrogen peroxide, almost equalling that of $10^{-4}$M $H_2O_2$. The additional effects of PMN-derived enzymes and other mediators could explain the difference between the kinetics of these processes. Detrimental role of PMN-derived ROS is confirmed by the fact that the antioxidant (Trolox) was able to significantly suppress the PMN cytotoxicity. On the other hand the partial cytotoxicity in Trolox treated groups underlines the importance of other PMN-derived factors that may have an injurious role.

Despite to several data form the literature on the subject, we did not observe a significant effect of increased ferrous ion concentrations on chondrocyte destruction. Surprisingly, an increased dose of $Fe^{2+}$ also failed to influence PMN-derived cytotoxicity. This outcome correlates with results by *Bates et al.*. It is suggested that the consequent increase in $Fe^{2+}$ has a subordinate pathogenic role in the early, ROS-mediated phase of acute haemarthrosis.

Histological investigations were done on cultured cells to disclose the morphological signs of PMN-mediated chondrocyte injury. In our study, using activated PMN as appropriate source of ROS, the TUNEL postive cell ratio was approximately 30% after the 6 hour long procedure. Thus, our results proved that PMN resulted in DNA lesions in chondrocytes. These findings correlate with the observation that single and short-term exposure to leukocytes and RBCs is able to cause longterm inhibition of proteoglycan synthesis on chondrocytes.

In summary, although the artificial conditions are a limitation of this study, simulating an in vitro model of haemarthrosis was not our intention. Nonetheless we can conclude that activated PMN-derived ROS have significant cytotoxic potential against chondrocytes under in vitro conditions. Due to their potential and their dramatically enhanced presence in the articular cavity following acute HA, PMN are hypothesised to be a major culprit in the underlying pathomechanism of cartilage damage. The histological changes also substantiate our assumption that PMN lead to DNA lesions on chondrocytes. These results also raise the prospect of delayed (apoptotic?) consequences following acute traumatic HA. Although iron is claimed to be a key player in HA, its short-term role in chondrocyte degradation is controversial and its possible importance in the acute HA requires further investigations.

# 3. DO THE PMNS ENGENDER OXIDATIVE STRESS ON CHONDROCYTES ?

## (Second series of experiments)

Intra-articular bleeding leading to articular damage is a well-established clinical entity. Several studies have documented the importance of synovial and blood-borne factors, such as inflammatory mediators and lysosomal enzymes, as contributors of this destructive process.

Although deleterius effects of such agents on cartilage tissue have been proven in numerous studies, specific biochemical markers indicative of the degree of oxidative stress in chondrocytes, evoked by activated PMNs, have previously not been studied in this regard. Oxidative stress is described as a condition where excessive ROS production overwhelms the antioxidant defences. Reduced glutathione (GSH), is an integral element in this antioxidant defence, one that is depleted during oxidative stress. On the other hand superoxide dismutase (SOD), another member of the endogenous antioxidant system, demonstrates compensatory increase in activity following exposure to bursts of superoxide radical. Furthermore it has been proven that the detection of lipid peroxidation via measurements of malondialdehyde (MDA) and 4-hydroxy-2 (E)-nonenal (4-HNE) levels, is a suitable indicator for ROS-mediated membrane damage. Thus monitoring the above parameters is considered appropriate in evaluating oxidative stress.

## *3.1. Materials and methods*

*Animal preparation.* (see at 2.1.)

*Isolation and culturing of chondrocytes from pigs*. (see at 2.1.).

*Isolation of PMNs*. (see at 2.1.)

*Treatment of chondrocytes.*. On the basis of viability, $20 \times 10^6$ healthy chondrocytes were seeded on 75cm$^2$ plastic culture flasks and incubated. Except for the control populations (*Group I.*), the media were either supplemented with a suspension of isolated PMNs (*Group II.*), or freshly prepared hydrogen peroxide solution ($10^{-4}$M final concentration, *Group III.*), as a controlled source of ROS. Finally 8μg phorbol 12-myrsistate 13-acetate (PMA) as PMN stimulator agent was added to each flask. Throughout the experiments, the flasks were incubated at 37°C and continuously agitated on a vibrating table at 60 rpm.

*Measurement of free radical mediated changes.* Following six hours of incubation, the cells were washed removing dead chondrocytes and PMNs. The remaining cells were collected with rubber cell-scraper and homogenized by repetitive freezing/thawing and the use of a plastic pestle. The cell homogenate was sampled for biochemical analysis.

Changes in the endogenous scavenging status were characterized by the measurement of reduced glutathione (GSH) concentration and superoxide dismutase (SOD) activity of the samples using colorimetric assay kits.

The immediate harmful effect of ROS on cell membranes was evaluated by assessing the degree of lipid peroxidation using relevant assay kit. Changes in the levels of malondialdehyde (MDA) and 4-hydroxyalkenals contents of the specimens were assessed.

*Histological examinations.* (See at 3.1).

*Statistical evaluation.* In each experiment the populations of chondrocytes derived from the same animal were randomly divided into different groups (namely control and PMN or H$_2$O$_2$ treated). The mean values of the different parameters and the standard errors are presented. Non-parametric Sign test was used to define the significance between the mean

values of the control and treated groups of chondrocytes. A p-value of less than 0.05 was considered statistically significant.

## 3.2. Results

*Monitoring the endogenous scavengers.* Results show that intracellular levels of GSH (n=8) declined significantly following co-incubation with either $H_2O_2$ (1.36±0.09 µg/mg, p=0,008) or PMNs (1.13±0.14 µg/mg, p=0,008) as compared to controls (1.86±0.12 µg/mg). The difference between the differently treated groups was not significant.

Conversely SOD activity (n=11) in treated cells (PMNs: 24.57±2.34 U/mg, p=0,001; $H_2O_2$: 23.61±1.82 U/mg, p=0,001) showed considerable elevation versus control cells (18.49±1.77 U/mg). Although the effect of hydrogen peroxide was less profound than that of PMNs, the difference again was not statistically significant.

*Measurement of lipid peroxidation.* The data obtained from the simultaneous measurement of MDA and 4-HNE (n=11) indicate significant increase in lipid peroxidation in both groups of treated cells (PMNs: 2.62±0.84 nM/mg, p=0,012; $H_2O_2$: 3.12±1.01 nM/mg, p=0,001) compared to the control group (1.71±0.52 nM/mg). Again the difference between the two treated groups was not significant.

*Histological examinations.* Whole thickness cartilage was used for obtaining chondrocytes, so the populations might have represented all kinds of cells from the superficial layer to the tide mark zone. Polygonal and round shaped cells with their basophilic nuclei were present in both control and co-incubated (treated) slides. Marked reduction in chondrocyte population was seen on slides belonging to treated groups. Due to the shaking and rinsing procedures, similarly sized PMNs with their typical segmented nuclei were practically absent on the sections and therefore could not have interfered with the biochemical analysis of ROS-mediated chondrocyte damage.

## 3.3. Discussion

Recent studies draw attention to the fact that blood itself can harm the cartilage and that its direct effect precedes the consequences of synovial inflammation. Several studies have addressed the damaging role of activated PMNs on cartilage matrix and chondrocytes. In spite such investigations, little is known about the effect of a PMN-evoked oxidative stress on chondrocyte.

Since oxidative stress disrupts the balance of endogenous scavengers and antioxidants, investigating alterations in these systems would appropriately reflect the degree of stress caused by ROS. Reduced glutathione, due to its nucleophilic and reducing properties, plays a central role in the cellular antioxidant defence mechanisms. Evidence for a reduction of this antioxidant provides support for an increased ROS activity during such process. In our study We observed a significant decline in intracellular GSH concentration in treated samples, an indication of the presence of such reactive species.

Superoxide dismutase is an intracellular metalloprotein, which transforms superoxide anion into $H_2O_2$ and oxygen, thus eliminating free radicals. Fridovich and other investigators have reported that increased production of superoxide radical induces SOD activation. Therefore the significant elevation in SOD activity in both our treated groups is considered to be in response to increased quantity of $O_2^-$ radical derived from either activated PMN or the reaction between $H_2O_2$ and ˙OH, which also results in superoxide radicals.

Lipid peroxidation is a well-known mechanism of cellular injury in both plants and animals. On the basis of the immediate deleterious effects of ROS on chondrocyte, we evaluated the process of lipid peroxidation in this model. Significant elevation of MDA/4-

HNE levels in both PMN and $H_2O_2$ treated groups suggest increased ROS-mediated lipid membrane injury. This fact demonstrates not only the presence reactive species but also their consequence, namely oxidative injury to chondrocytes.

In summary our in vitro study has clearly proven the presence of oxidative stress and chondrocyte injury elicited by activated PMNs through demonstrating alterations in specific markers indicative of such a process. Although the artificial conditions are a limitation of this study, simulating an in vitro model of haemarthrosis was not the author's intention. Nonetheless these observations provide evidence that short-term exposure to activated PMNs can lead to oxidative injury of chondrocytes, it is arguable that in clinical situations of haemarthrosis, PMNs and ROS may also contribute to cartilage damage.

# 4. ARE THE PMNS ACTIVATED DURING CLINICAL PRESENTATIONS OF ACUTE HAEMARTHROSIS?

## (Third series of experiments)

In vitro investigations have proven that single or a limited number of bleeding episodes result in cartilage damage, in which cytotoxic oxygen metabolites derived from the interaction between mononuclear cells and red blood cells are supposed to play a role. These results support the importance of the impact of a single haemarthrotic episode. Similarly our previous *in vitro* investigations suggest a key role of activated PMNs in the pathomechanism, yet the question whether leukocytes are activated during acute, clinical haemarthrosis remains unanswered.

This study aimed to assess the leukocyte activation and the production of leukocyte-derived reactive oxygen species (ROS) during acute haemarthrosis. Enhanced expression of various surface antigens is a known characteristic of leukocyte activation, especially polymorphonuclear granulocytes (PMN), which are reported to produce highly toxic ROS. Subsequent to identifying PMN activation and ROS production we intended to gain data regarding the impact of leukocyte activation and its place in the hierarchy of pathogenic events in acute haemarthrosis.

## *4.1. Materials and methods*

*Subjects*. Twenty-two patients admitted to the Department of Traumatology and Hand Surgery (affiliated to the Medical Faculty of University of Pécs) with acute traumatic knee injury were included in this study. There consisted 18 males and 4 female ranging from 18 to 75 (mean 44) years in age. All patients underwent a physical examination and X-ray imaging before their articular effusions were evacuated under sterile conditions as a part of their treatment. The mean duration between the knee injury and joint aspiration was 18 hours with a range of 1 to 60 hours. Corresponding venous blood samples were obtained from the cubital veins from all patients and all samples were placed in ice and immediately sent to the laboratory for fluorescent flow cytometric analysis and chemiluminometry.

*Preparation of leukocytes and flow cytometric analysis*. Blood was treated with EDTA and cells sedimented with dextran and the erythrocytes were removed by hypotonic haemolysis. Appropriate amount of the whole leukocyte fraction was used for immunofluorescence staining with (FITC)-conjugated mouse anti-human CD11a, CD18 and CD97 mAbs. Binding of FITC-labelled antibodies to leukocytes was quantified both as the percentage of cells exhibiting specific FITC fluorescence and the mean channel fluorescence in arbitrary unit that exceeded non-specific background fluorescence.

*Luminol dependent whole blood chemiluminometry*. Respiratory burst activity of leukocytes in corresponding samples of peripheral blood and joint effusion was measured by detection of ROS via modified chemiluminescence method published by *Dandona et al*. Lagtime and ROS producing capacity were calculated on the basis of the curve.

*Statistical evaluation*. Results were expressed as means ± SD. Two-tailed Students paired t-test was used to compare differences between venous blood and joint-fluid leukocyte samples. A p-value of less than 0.05 was considered statistically significant.

## 4.2. Results

*Flow cytometry.*
*Monocytes.* Flow cytometric analysis of leukocyte populations derived from venous blood and joint aspirate failed to reveal any significant differences between the joint and venous samples with regards to the expression of the CD97 on monocytes or the proportion of CD11a, CD18 and CD97 positive cells. There was an enhanced expression of CD18 on these cells but it did not reach significant levels (p=0.094). However a significant decline was found in case of CD11a expression in the articular monocytes compared to peripheral cells (p=0.00167).

*Polymorphonuclear Granulocytes (PMN).* Investigations on the expression of CD18 and CD97 surface antigens showed significantly increased mean density (CD18: p=0.00945; CD97: p=0.00338) of these markers on the joint-derived PMNs comparing to the venous samples. On the other hand there were no marked difference between effusion and peripheral populations either in case of CD11a expression or the proportion of mAb positive PMNs regarding each antigen.

*Lymphocytes.* Paired analysis of peripheral blood and synovial effusion samples demonstrated a significant increase in the number of CD97 positive lymphocytes in the joint derived group (p=0.000149). There was no significant alteration regarding other measured parameters.

*Chemiluminometry*
Comparative assessments of corresponding samples derived from cubital vein and articular cavity revealed a significantly enhanced production of ROS (p= 0,0146) and markedly decreased lag time (p= 0,00272) in case of leukocytes from the latter group**.**

## 4.3. Discussion

Currently it is believed that the inflammatory changes of synovial tissue lead to cartilage damage. However more recent studies have shown that changes in the articular cartilage which precede synovitis may initiate the joint destruction. Moreover such changes occur almost immediately after a brief exposure to blood, suggesting that even a single intraarticular bleeding event may be harmful.

In spite of the numerous studies detailing the importance of different cellular and non-cellular mediators in the pathogenesis of haemarthrosis, the initiating factor remains unclear. The normal synovial fluid contains only few leukocytes, less than 200-300 cells/mm$^3$. This population is dramatically altered during haemarthrosis and there is an obvious increase in the number of leukocytes. These cells have the ability to produce catabolic enyzmes, cytokines and reactive oxygen species following activation by different proinflammatory mediators such as platelet activating factor (PAF), thromboxan-A2 and leukotriens (LTB4). These activators are released from the endothelial cells and from the locally recruited platelets as a consequence of the intra-articular vascular injury.

In this clinical study we aimed to explore the importance of leukocytes as mediator in the inflammatory reactions and the activation of cartilage destruction during acute haemarthrosis. Hence a comparison was made in the expression of alpha-chain of LFA-1 (CD11a), the common beta-chain of β-2 integrins (CD18) and in the leukocyte activation antigen (CD97) between leukocytes derived from traumatic knee effusion and those derived from the peripheral blood.

The significantly decreased expression of CD11a on joint-derived monocytes suggests a subordinate role of LFA-1 during haemarthrosis-associated leukocyte activation. Similar results have been reported by Jones et al., who compared the CD11a and CD11b/CD18

expression on PMNs derived from synovial fluid and systemic circulation in patients with rheumatoid arthritis. The significant increase of CD18 and CD97 expression on PMNs derived from haemarthrosis compared to corresponding venous blood samples provides good evidence of PMN activation. The elevation in the proportion of CD97 positive lymphocytes in the articular cavity as compared with systemic blood suggests that lymphocytes also might have an important role in the early inflammatory reactions in acute haemarthrosis. Since ligand binding to CD97 leads to activation of adenylate cyclase and increase of intracellular cAMP level, it is possible that such process leading to a higher metabolic activity, augmenting the cellular inflammatory response at the binding site. Moreover the increased expression of CD18, as opposed to unchanged density of CD11a on PMNs suggest an upregulation of integrins that is most likely Mac-1 as it found in rheumatoid arthritis.

Production of ROS following activation is characteristic of several immune cells, especially of PMNs. Whole blood chemiluminescence assay has proven to be a rapid, simple and reliable technique for estimation of ROS generating capacity of leukocytes. Our investigations revealed that joint-derived leukocytes required significantly less time to generate ROS, and at higher rate than the corresponding peripheral white blood cells, in other words leukocytes from the articular cavity had been sensitised.

In summary, our findings demonstrate enhanced expression of CD18 and CD97 on PMNs and increased number of CD97 positive lymphocytes in articular effusions of patients with acute traumatic haemarthrosis. On the other hand, leukocytes from the same samples showed significantly higher ROS production with shortened lag time. All these suggest marked leukocyte activation as part of the clinical process. Since the earliest synovial changes are recorded by 4 days following single haemarthrosis, it is likely that leukocyte activation precedes it. The importance of PMNs and lymphocytes in the regulation of inflammatory mechanisms and in cytotoxicity is well recognised. Therefore it is believed that these cells provide the missing link in the chain of events leading to the onset of degenerative events following acute haemarthrosis.

# 5. CONCLUSIONS AND CLINICAL RELEVANCE

The lack of unambiguous data from human investigations proving that single intraarticular bleeding episode is responsible for chronic disabilities, brings to question whether acute haemarthrosis is really dangerous under clinical conditions. Of course we have to beware when drawing direct conclusions from *in vitro* investigations. However it is beyond doubt that blood itself harms the cartilage as verified by several *in vitro* and *in vivo* studies. Our results also support this concept and draw the attention to the PMNs and ROS as important participants in the pathogenic processes. Thus acute haemarthrosis can be viewed as a form of stress, which may have long lasting sub-clinical effect on the joint. However combined with other factors (e.g.: abnormal loading, immobilisation or repeated episodes) their concerted effects can lead to clinically detectable joint injury. Thus acute haemarthrosis may be considered a risk factor for joint damage the elimination of which is essential in order to prevent further articular destruction.

# 6. NOVELTIES

- The first series of experiments highlighted the importance of PMN-derived ROS in predisposing chondrocytes to cytotoxic processes and DNA damage upon exposure to abundant activated PMN. The applied *in vitro* conditions such as the cellular ratio and the concentration of reacting agents reflected the clinical settings in a certain degree, thus our results underline the importance of PMNs and ROS in chondrocyte damage as is probably the case in acute haemarthrosis.

- In the second series of experiments we demonstrated for the first time that activated PMNs are able to exert oxidative injury on chondrocytes in vitro. Our results confirm previous suggestions that PMN-derived ROS contribute to cartilage degradation in acute haemarthrosis. Moreover these results support the importance of ROS scavenging and PMN-inhibiting drugs as therapeutic alternatives in the treatment of this condition.

- The clinical trial provided clear evidence for leukocyte activation during acute haemarthrosis, a fact that had remained disclosed so far. Our previous *in vitro* observations regarding the deleterious role of PMNs have also been confirmed by this trial.

- Also novel to this work was the review of therapeutic alternatives and its proposed revision regarding especially the routinely applied plaster immobilisation in case of acute

# 7. ACKNOWLEDGEMENT

# 8. LIST OF PUBLICATIONS

*Manuscripts*

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