Malaysian Journal of Medicine and Health Sciences Vol. 6 (2) June 2010: 11-17

The Effect of Human Mesenchymal Stem Cell on Neutrophil Oxidative Burst

[1]R Ramasamy*, [1,2]K Krishna, [1]M Maqbool, [1]S Vellasamy, [1]VH Sarmadi, [1]M Abdullah & [1]S Vidyadaran

[1]Immunology Laboratory, Department of Pathology, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

[2]Faculty of Health and Life Sciences, Management and Science University, Shah Alam, Malaysia

ABSTRACT

Objective: Mesenchymal stem cells (MSC) are multipotent, non-haematopoietic stem cells that are capable of differentiating into different varieties of mature cell types such as osteoblasts, chondrocytes, adipocytes, and myoblasts. There is abundant evidence showing that MSC not only affect the differentiation of haematopoietic progenitors, but also the function of mature cells like lymphocytes and neutrophils. However the effect of MSC on neutrophil function and its responses is not well studied. Therefore, this study was conducted to assess the effect of MSC on neutrophil nitric oxide production. **Method:** Neutrophils from heparanised venous blood were isolated using Ficoll-Hypaque density gradient centrifugation followed by Dextran sedimentation and red blood cell (RBC) lysis. Isolated neutrophils were on average of 97% purity as determined by morphologic analysis. MSC were generated from human bone marrow and characterised by immunophenotyping (monoclonal antibodies CD105, CD73 and CD34) using a flowcytometer. In order to test the effects of MSC on neutrophil function, isolated neutrophils were co-cultured in the presence or absence of MSC at different ratios for 24 and 48 hours. The amount of nitric oxide released was used as an indication of oxidative burst and measured using the Griess assay. **Result:** The results indicate that MSC neither elevate the NO level when cocultured with resting neutrophils nor alone. However MSC profoundly inhibit the secretion of nitric oxide in PMA stimulated neutrophils after 24hr of incubation. **Conclusion:** MSC exert an immunomodulatory effect on neutrophil by suppressing neutrophil oxidative burst *in vitro*.

Keywords: Mesenchymal stem cell (MSC), Neutrophils, nitric oxide, oxidative burst.

INTRODUCTION

The bone marrow (BM) gives rise to mainly two populations of stem cells; haematopoietic stem cells which form blood cells and mesenchymal stem cells (MSC) to support the haematopoietic system. MSC have the capacity to differentiate into mesenchymal cell lineages such as osteocytes, adipocytes, chondrocytes[1] and thus play a crucial role in supporting haematopoiesis by providing the necessary cytokines and cell-mediated signals for haematopoietic stem cells to self-renew or differentiate.[2, 3] Neutrophils are mature white blood cells derived from haematopoietic stem cells through differentiation into common myeloid progenitor's lineages. Beside their site of production, the bone marrow (BM) also serves as a place where large amounts of mature non-proliferating neutrophils are retained in the storage pool of BM sinusoids.[4] In the bone marrow niche, MSC protects neutrophils of the storage pool from apoptosis, preserving their effector functions and preventing excessive or inappropriate activation of the oxidative burst a function in microbial killing.[4] Although there is accumulating data indicating a substantial role of MSC in physiology of mature lymphocytes,[5, 6] yet their effect on neutrophil function is not fully studied.

Neutrophils, also known as polymorphonuclear leucocytes (PMN) are the major cell type that constitutes innate immune system. They comprise approximately 50-70% of leucocytes and predominate in eliminating pathogens that induce acute inflammation.[7] Elimination of pathogens by neutrophils involves a series of physiological sequences that comprise chemotaxis, phagocytosis and microbial killing. The success of pathogen elimination by neutrophils depends on oxidative burst a major process that mediates microbial killing through formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The ROS production is initiated by NADPH oxidase which yields various oxygen reactive species such as hydrogen peroxide, superoxide anion, oxygen cations, nitric oxide and other free radicals.[8] RNS results from the catalyst of L-arginine by nitric oxide synthase (NOS) thus produce nitric oxide reactive species. ROS and RNS are essential for neutrophil defence system and the failure of their production causes

*Corresponding Author: *r.rajesh@medic.upm.edu.my*

severe bacterial infection whilst overproduction triggers vascular damage in chronic diseases such as hypertension and arthrosclerosis.[9]

Few studies have been carried out to verify the effect of MSC on mature neutrophils. According to Raffaghello *et al*, MSC at low concentrations inhibits the *in vitro* apoptosis of resting and interleukin-8 (IL-8) activated neutrophils and formyl-Methionyl-Leucyl-Phenylalanine (f-MLP) induced respiratory burst. This study further revealed that, MSC protects neutrophils from apoptosis by constitutive release of interleukin-6 (IL-6), which is signalled through a signal transducer and activator of transcription 3 (STAT-3) dependent mechanism.[4] Furthermore, the phagocytosis, chemotaxis and adhesion molecules expression by neutrophils were not affected by MSC.

Our study specifically tested the effect of MSC on neutrophil nitric oxide production upon stimulation with lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA). Both LPS and PMA have a stimulatory effect on PMN and induce nitric oxide secretion. However, when MSC and PMN were co-cultured and stimulated with LPS or PMA, the nitric oxide level reduced significantly. This study demonstrates that human MSC potently inhibit respiratory burst of activated neutrophils.

METHODS

Generation of mesenchymal stem cells

Human bone marrow samples were purchased from Stem cell Technologies, USA in accordance with local ethics requirement. The bone marrow aspirate was diluted and subjected to Ficoll density gradient centrifugation at 2000 rpm for 20 minutes at 10^0C. Mononuclear cells were collected from interface of density gradient centrifugation and performed viability count using tryphan blue dye exclusion. Mononuclear cells were seeded in 25 cm^2 culture flask in 10% preselected fetal calf serum supplemented Dulbecco's Modified Eagle Medium (DMEM) media and incubated at 37^0C in a humidified 5% CO_2 incubator. The culture was incubated for three days and non-adherent cells were removed by replacing the culture media. When the adherent cells achieved 80% of confluency, cells were detached by using 0.5% trypsin solution (GIBCO, USA) at 37^0C for 5 minutes. The detached cells were sub-cultured and ready for characterisation and further assays.

Immunophenotyping of MSC

MSC were detached by trypsinisation, washed and suspended in 1X phosphate buffer solution (PBS). The cells were stained with flourochrome conjugated anti-human monoclonal antibodies CD105-phycoerythrin (PE), CD73-PE, and CD34-fluoresceinisothiocyanate (FITC) for 30 minutes at 2-8^0C. After the incubation, MSC were washed with 1X PBS and analysed using flow cytometer (FACSCalibur, Beckton Dickinson, USA). The data were analysed using CellQuest analysis software.

Neutrophil isolation

Neutrophils were isolated from human peripheral blood after the informed consent from donors in accordance with UPM, Faculty of Medicine and Health Sciences Ethics Committee requirement. The following methods were performed to optimise the neutrophil isolation. Twenty (20) ml venous blood was collected and diluted in 1X Hank's balanced salt solution (HBSS) medium at 1:1 ratio. Ten millilitre (10ml) diluted blood was then layered over 5ml Ficoll-Hypaque and centrifuged at 1500 rpm for 40 minutes at 10^0C. Plasma and the mononuclear cell layer were discarded. The red cell pellet which contains the PMN and red blood cell (RBC) was suspended in 5ml HBSS. Red cell suspension was layered on 3% Dextran and left at room temperature for 45-60 minutes to further sediment the RBC. Supernatant from dextran sedimentation was collected and the remnant RBC was lysed using RBC lysing solution to obtain pure PMN population.

Morphological analysis

Leishmen staining was done to confirm neutrophil morphology. Few drops of neutrophils preparation was spread over the glass slide and covered with Leishman solution for 2-3 minutes. Subsequently the smear was immersed with phosphate buffer solution for 15 minutes. The slide was rinsed off with tap water, dried and examined under light microscope at 20 and 40X magnifications.

Griess Assay

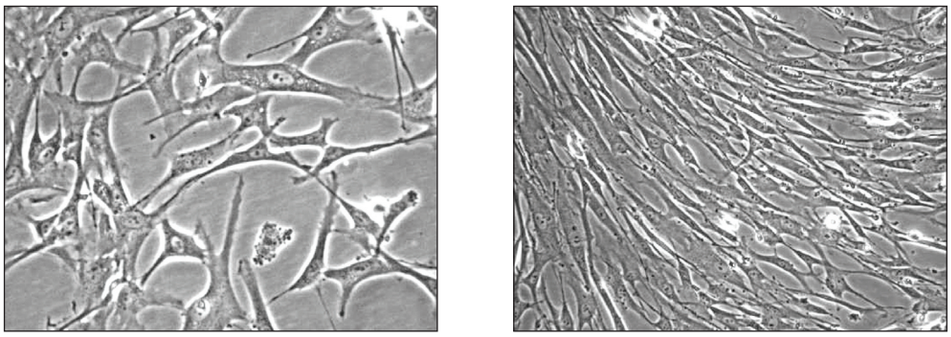
Prior to assay, MSC were seeded into a 96-well plate and incubated overnight to allow adherence of MSC. After the pre-plating, the supernatant was removed as the MSC had adhered. 1.0 x105 of neutrophils were added into the wells and stimulated with of 5µg/ml LPS or PMA, incubated for 24 hours. At end of incubation, 50uL of supernatant was

taken from the wells and mixed with 50uL Griess reagent. The mixture incubated at room temperature for 10 minutes and read by an ELISA reader.

RESULTS

Generation of MSC

MSC were generated from bone marrow aspirates. The bone marrow cells formed colonies at day 7 with morphology resembling fibroblast cells (Figure1A). The colonies expanded rapidly in exponential phase and achieved confluenced state with 14 days of culture (Figure 1B).



(a) (b)

Figure 1. Generation of MSC. One million ($1x10^6$)/cm^2 cells of bone marrow mononuclear cells were cultured in a T25 flask for 7 days. (a) MSC form colonies at day 7 with the appearance of fibroblastic cells. (b) MSC at 80% confluency at day 14

Immunophenotyping of MSC

Bone marrow adherent cells were subjected to immunophenotyping after passage 3 using flow cytometer. Cells appeared, uniformly as large and highly granulated (Figure 2A) and they were positive for CD105 (Figure 2D) and CD73 (Figure 2C). For the haematopoietic stem cell marker CD34, MSC stained negative (Figure 2B).

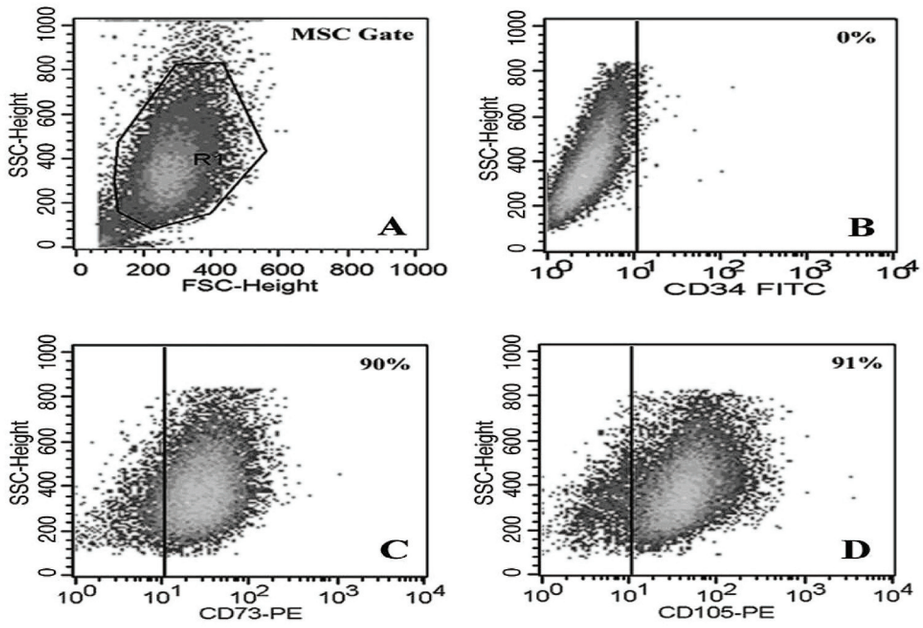
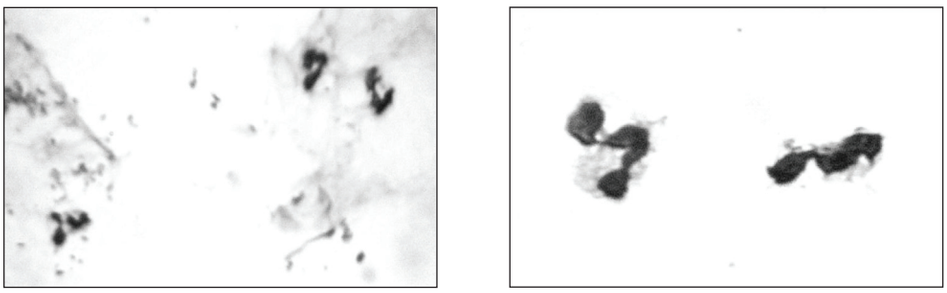


Figure 2. Immunophenotyping of MSC. (A) Gating of the cell population to exclude dead cells and debris. (B) MSC with negative staining for CD34-FITC. (C) MSC showing positive staining for CD73-PE. (D) MSC showing positive staining for CD105-PE

Morphology of isolated neutrophils

Assessment of neutrophil morphology was performed using Leishman staining as a confirmatory test for neutrophil isolation and purity. The dye component of Leishman solution stained the nucleus lobules with dark blue and the cytoplasm stained light pink (Figure 3). Leishman staining indicates that more than 90% of cells isolated were 3-5 multi lobulated, ascertaining their purity.



(a) (b)

Figure 3. Morphological examination of neutrophils. Neutrophils stained with Leishman staining to clearly distinguish nucleus and cytoplasm. (a) Neutrophils with lobulated nucleus at 20x magnification. (b) Magnification of cells at 40x

MSC inhibits the nitric oxide (NO) production of neutrophils

Griess assay was conducted to assess nitric oxide production of neutrophils. Neutrophils were co-cultured with or without MSC at different ratios of neutrophil:MSC (1:100) for 24 hours supplemented with or without 5µg/ml LPS and PMA. The amount of NO released from the cell was derived from the standard curve (Figure 4). The results revealed that PMA served as potent stimulator of neutrophils compared to LPS. Although MSC were allogenic to respondent neutrophil, MSC did not stimulate the resting PMN. In the presence of MSC and PMA, NO production of neutrophils was dramatically reduced more than three folds.

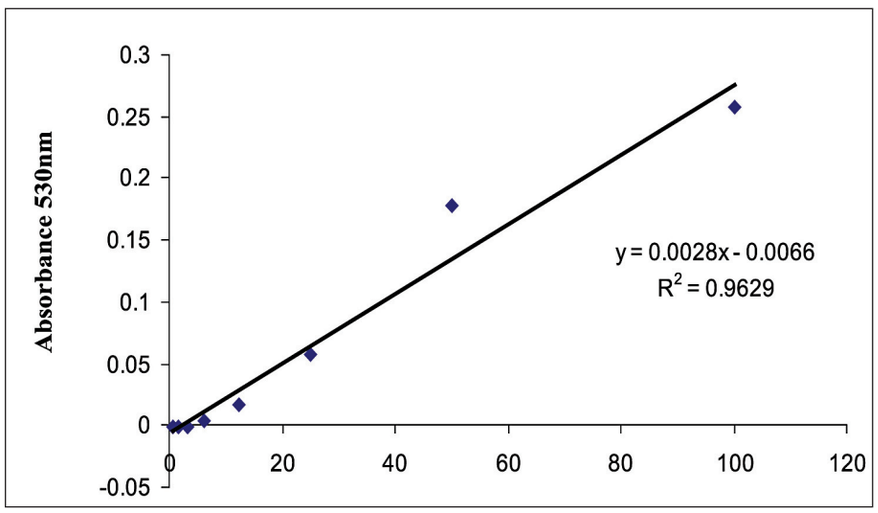
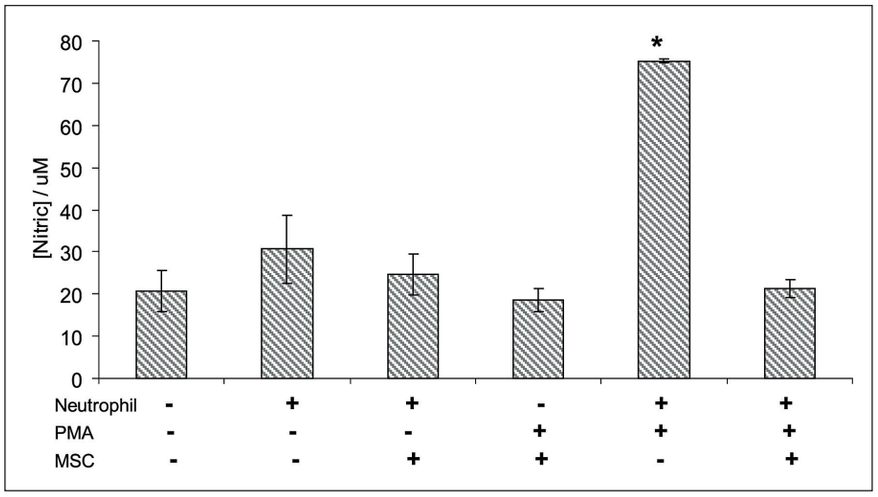


Figure 4. Standard curve of nitric oxide. Standard curve generated for Griess reaction using sodium nitrite at absorbance 530nm and recorded using ELISA reader. Y-axis represents optical density (OD) readings and X-axis represents sodium nitrite concentration



[U4]

Figure 5. MSC inhibits nitric oxide secretion by neutrophils. Neutrophils were cultured in the presence or absence of PMA and/or MSC for 24 hours. At end of incubation, supernatant from cultures was retrieved and their nitric oxide levels determined by Griess assay

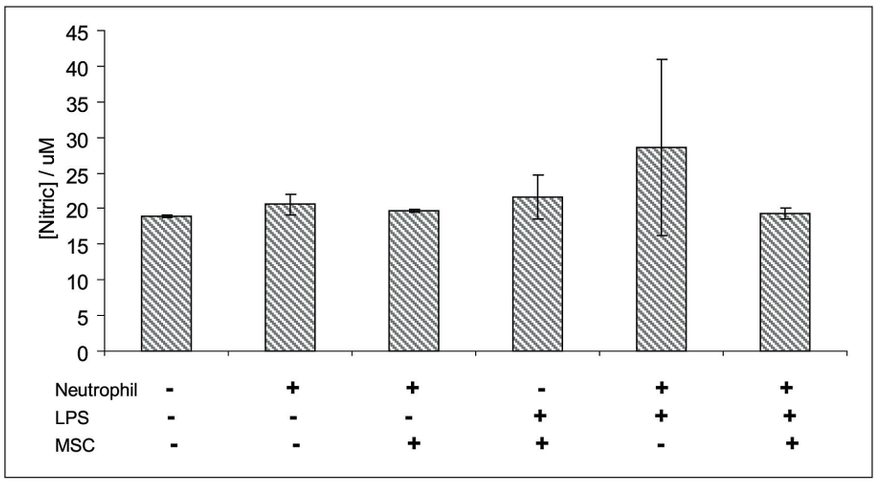


Figure 6. MSC inhibit the nitric oxide secretion by neutrophil. Neutrophil was cultured in the presence or absence of LPS and/or MSC for 24 hours. At end of incubation, supernatant from cultures was retrieved and their nitric oxide levels determined by Griess assay

DISCUSSION

Neutrophil activation occurs due to inflammation induced by immune response. Upon activation, neutrophils release ROS and RNS to kill the invading pathogens.[10] The controlled releases of ROS and RNS in phagolysosome upon degranulation of activated neutrophils serve as major mechanism to kill the pathogens. However, the overproduction of reactive species or the impairment of antioxidant defence mechanisms may results in detrimental effects as they can cause degradation of lipids, proteins and DNA.[11, 12] Recent evidences consolidating the fact that an overproduction of nitric oxide by neutrophil at synovial fluid also proved to be one of the causes for autoimmune diseases such as rheumatoid arthritis.[8]

In the present study, we have addressed the immunomodulatory effect of MSC on neutrophil effector function. We demonstrated that human MSC potently inhibited RNS production of both PMA and LPS activated neutrophil *in vitro*. Neutrophils responded well with PMA stimulation whereby the magnitude of PMA stimulation is much higher than LPS stimulation. Similar results was reported by others and this discrepancy is due participation of different signalling mechanisms in activating respiratory burst machinery.[13] In line with this, studies on human neutrophils have shown that PMA stimulates the cell through the protein kinase C signalling pathway (PKC) directly[14] meanwhile LPS utilises LPS-binding protein to facilitate the binding of LPS to CD14 on human monocytes.[15]

Beside neutrophils, MSC also produce NO in the culture, in fact some studies support the notion that MSC inhibit lymphocyte immune function by secreting NO.[16] However in this study, the stimulators PMA and LPS did not elevate the NO level in MSC cultures. When MSC were cultured with PMA and neutrophils, the production of NO is significantly reduced compared to PMN and PMA positive control. This suggests that neutrophil oxidative burst is completely suppressed by MSC. Rafaghello *et al*, reported that in the bone marrow niche, MSC protects neutrophils of the storage pool from apoptosis, preserving their effector functions and preventing excessive or inappropriate activation of the oxidative burst.[4] This study provides additional evidence that MSC protects neutrophil from apoptosis because of suppression of oxidative burst.

The immunomodulatory effect of MSC is not only restricted to neutrophil but also to other immune cells. Previous *in vitro* and *in vivo* studies have highlighted the immunoregulatory activities of MSC, which include inhibition of T cell, B cell and NK cell proliferation and effector functions,[17, 18, 19] as well as of dendritic cells maturation, activation, and antigen presentation.[20] When neutrophil is activated it will produce reactive species and this is an essential step required to kill pathogens. This is exemplified by chronic granulomatous disease, a genetic disorder characterised by defective production of superoxide metabolites,[21] in which neutrophils display impaired intracellular killing of ingested micro organisms. On the other hand, when activation of the respiratory burst is excessive or inappropriate, ROS participate in severe host tissue injury and involved in various pathological conditions, including ischemia-reperfusion injury, chronic obstructive pulmonary diseases, acute respiratory distress syndrome, atherosclerosis, malignancy and rheumatoid arthritis.[22] In such pathological conditions MSC can be proposed to be used as a therapy. However one should consider that, dis-regulated oxidative burst by neutrophils also could be way of potent persistent infection, therefore usage of MSC in future clinical application should be explored in well designed clinical studies. In conclusion, MSC mediated anti-oxidative burst activity could be a novel mechanism whereby the inflammatory potential of activated neutrophils is harnessed by inhibition of reactive oxygen species production.

ACKNOWLEDGEMENT

This study was funded by Research Universiti Grant Scheme (RUGS), Project No: 04/01/07/0106RU. S Vellasamy and VH Sarmadi are supported by Graduate Research Fund (GRF).

REFERENCES

[1] Pittenger MF, Mackay AM, Beck SC, *et al.* Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.

[2] Bonnet D. Hematopoietic stem cells. Birth Defects Res C Embryo Today 2003; 69: 219-229.

[3] Dazzi F, Ramasamy R, Glennie S, *et al.* The role of mesenchymal stem cells in haemopoiesis. Blood Rev 2006; 20: 161-171.

[4] Raffaghello L, Bianchi G, Bertolotto M, et al. Human Mesenchymal Stem Cells Inhibit Neutrophil Apoptosis: A Model for Neutrophil Preservation in the Bone Marrow Niche. Stem Cells 2008; 26: 151-162.

[5] Glennie S, Soeiro I, Dyson PJ, *et al.* Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 2005; 105: 2821-2827.

[6] Krampera M, Glennie S, Dyson J, *et al.* Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003; 101: 3722-3729.

[7] Greenberg S, Grinstein S. Phagocytosis and innate immunity. Current Opinion in Immunology 2002; 14: 136-145.

[8] Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 1998; 92: 3007-3017.

[9] Elbim C, Lizard G. Flow cytometric investigation of neutrophil oxidative burst and apoptosis in physiological and pathological situations. Cytometry A, 2009.

[10] Freitas M, Porto G, Lima JL, *et al.* Optimization of experimental settings for the analysis of human neutrophils oxidative burst *in vitro*. Talanta 2009; 78: 1476-1483.

[11] Azad N, Rojanasakul Y, Vallyathan V. Inflammation and Lung Cancer: Roles of Reactive Oxygen/Nitrogen Species. Journal of Toxicology and Environmental Health, Part B 2008; 11: 1-15.

[12] Ricciardolo FLM, Di Stefano A, Sabatini F, *et al.* Reactive nitrogen species in the respiratory tract. European Journal of Pharmacology 2006; 533: 240-252.

[13] Tung J-P, Fraser J, Wood P, *et al.* Respiratory burst function of ovine neutrophils. BMC Immunology 2009; 10: 25.

[14] Sheppard FR, Kelher MR, Moore EE, *et al.* Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. J Leukoc Biol 2005; 78: 1025-1042.

[15] Amura CR, Kamei T, Ito N, *et al.* Differential Regulation of Lipopolysaccharide (LPS) Activation Pathways in Mouse Macrophages by LPS-Binding Proteins. J Immunol 1998; 161: 2552-2560.

[16] Sato K, Ozaki K, Oh I, *et al.* Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood 2007; 109: 228-234.

[17] Rasmusson I, Ringden O, Sundberg B, *et al.* Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation 2003; 76: 1208-1213.

[18] Rasmusson I, Uhlin M, Le Blanc K, *et al.* Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. J Leukoc Biol 2007: jlb.0307140.

[19] Ramasamy R, Tong CK, Seow HF, *et al.* The immunosuppressive effects of human bone marrow-derived mesenchymal stem cells target T cell proliferation but not its effector function. Cellular Immunology 2008; 251: 131-136.

[20] Ramasamy R, Fazekasova H, Lam EW, *et al.* Mesenchymal Stem Cells Inhibit Dendritic Cell Differentiation and Function by Preventing Entry Into the Cell Cycle. Transplantation 2007; 83: 71-76.

[21] Dinauer MC OS. Chronic granulomatous disease. Annu Rev med 1992; 43: 117-124.

[22] Gao J, Arbman G, Rearden A, *et al.* Stromal staining for PINCH is an independent prognostic indicator in colorectal cancer. Neoplasia 2004; 6: 796-801.