Original Article

Flow Cytometric Analysis of Inflammatory Cells in Experimental Acute Pancreatitis

Shohreh Fakhari[1], Kamaal Abdulmohammadi[2], Yaser Panahi[2], Mehri Ghorashi[2], Mohammad Ali Rezaie[2], Ali Jalili[1,2*]

[1] Liver & Digestive Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran.
[2] Department of Immunology & Hematology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.

Abstract

Background: Accumulating evidence indicates that inflammatory cells migrate into the pancreas tissue and play an important role in the pathogenesis of acute pancreatitis (AP). The aim of this study was to establish a flow cytometric method to enumerate these infiltrating cells in the pancreas of an experimental AP. **Materials and Methods:** Twelve hours after inducing of AP, mice pancreatic tissues were cut into small fragments and single cells were prepared by mechanical dissociation. The isolated cells were stained with either anti-mouse CD45-PerCP or isotype antibody and analyzed by flow cytometry. Using side scatter (SSC)/CD45 gating we were able to identify inflammatory cells from non-inflammatory cells. **Results:** The mean percentage of leukocytes was 5.9 ± 1.6 in the control group whereas, it was 26.7 ± 8.1 in the AP. Moreover, we found that the percentage of lymphocytes, monocytes and granulocytes were 1.1 ± 0.2, $0.9\pm.04$ and 2.9 ± 1.8 of total pancreatic cells, respectively, in the control mice. In contrast to lymphocytes, the percentage of monocytes and granulocytes were significantly increased in the AP group and it was 3 ± 1.3 and 18.2 ± 3.2 for monocytes and granulocytes, respectively. **Conclusion:** Quantitative flow cytometric analysis is feasible and provides a reliable and rapid assay to determine the number and percentage of inflammatory cells in experimental AP.

Keywords: Acute pancreatitis, flow cytometry, inflammatory cells

*Corresponding Author: Ali Jalili, PhD; Associate Professor, Department of Immunology & Hematology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanadaj, Iran. Tel: (+98) 871-6131290; E-mail: Ali130@gmail.com

Introduction

Acute pancreatitis (AP), an autodigestive disease with various severities and several etiologies, initiates with premature activation of digestive enzymes. Most of the patients express mild-self limited symptoms and do not need any treatment, but up to 25% of patients show a severe attack and unfortunately between 30-50% of these patients will die (1, 2). Intra-acinar activation of digestive enzymes

such as trypsinogen is believed to be the first triggering event of AP leading to interstitial edema, vacuolization and inflammation (2). Accumulating evidence indicates that AP is an inflammatory disease in which many immunological events and cascades play many crucial roles in both pathology and course of the disease (3, 4).

Previous studies have shown that activated leukocytes play an important role in both local and distance damages. Migration of inflammatory cells

into tissues is highly regulated and many cytokines such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1β), IL-8 and monocyte chemotactic protein-1 (MCP-1), have been reported as key activators for these cells (3, 5, 6). Infiltration and subsequent activation of inflammatory cells in the pancreas can lead to release a variety of proteolytic enzymes and oxygen radicals resulting in cellular damage of endothelial and pancreatic parenchymal cells (7-10). Furthermore, it has been suggested that organ dysfunction and fetal pancreatitis are due to excessive leukocytes in the pancreas of patients with AP(11). Supporting of this notion, previous reports employing animal models have clearly shown that leukocyte depletion reduces severity of pancreatitis and lung injury(12, 13). Morphological studies have also demonstrated that the number of inflammatory cells have significantly elevated in the pancreas of patients with AP and experimental AP animals (14, 15). However, the quantification and enumeration of these cells have not yet been analyzed.

Myeloperoxidase (MPO) assay has been previously employed for quantifying infiltrated neutrophils in the injured spinal cord and AP of murine models (16, 17). However, the relatively low sensitivity of MPO assay may limit its application to be used for samples with small size. Moreover, it just quantifies neutrophils, but not other inflammatory cells such as lymphocytes and monocytes. Flow cytometry analysis has been recently shown to be a feasible approach for enumerating infiltrating neutrophils in inflamed tissues including cerebral ischemia (18) and experimental autoimmune encephalomyelitis (19). Noteworthy, Tjoa T et al. have recently induced spinal cord contusion injury in mice and reported that flow cytometry is a suitable approach for detecting of inflammatory cells in injured murine spinal cord. Furthermore, the flow cytometry analysis showed that the number of infiltrated neutrophils is significantly higher in severe spinal cord injury than moderate one (20). The aim of the current study was to develop and optimize a method for quantifying and enumerating inflammatory cells in the pancreas. We have found that flow cytometry is a reliable and feasible approach for quantifying of leukocytes in the pancreas of both normal and AP mice.

Methods

Induction of AP

Healthy 8 week-old C57 mice weighting 25-30 g were used for the experiments. Mice were purchased from Iran Pasture Institute (Tehran, Iran) and let them acclimate to the environment for at least 72h after arrival in the laboratory. Experimental AP was induced as previously reported (17). Briefly, Mice were randomly allocated into two groups (n=8 for each group). The first group was treated hourly (×8) with saline intraperitoneally (IP) and served as the control group. The second group was treated hourly (×8) with cerulein (50μg/kg, suspended in saline solution, IP) and served as the experimental AP group. Twelve hours later, mice were killed and blood samples were obtained by direct intracardiac puncture. Pancreas was removed immediately, half of it was used for flow cytometry analysis and the rest were fixed in formaldehyde for histopathological examination.

Histopathological and biochemical examinations

Paraffin-embedded pancreas samples were sectioned (5mM) and stained with hematoxylineosin and then were analyzed as previously reported (8, 21). Amylase was detected in serum of both sham and AP in a diagnostic Lab (Behsat Hospital, Sanadaj, Iran).

Isolation of pancreatic cells

Pancreatic cells were isolated from pancreas tissue as previously reported (22). Briefly, pancreas gland was cutted into small fragments and suspended in FACS buffer (Dulbecco's phosphate-buffer saline containing 10% fetal calf serum and 5mM EDTA). Tissue fragments were more grinded by pipetting with 1ml sampler for 30s. The remaining large fragments were allowed to sediment for 20s, the supernatant collected and reserved. Then, the sedimented large fragments were triturated again and the all of collected supernatants from each pancreas were pooled, centrifuged (250g, 4ºC, 25min). The resulting cell pellets were then resuspended in FACS buffer for immediate staining for flow cytometry.

Flow cytometry analysis

Isolated pancreatic cells from each mouse were washed three times with FACS buffer, resuspended in 200μL of FACS buffer and then distributed equally into two 1.5mL microtubes. In order to stain the cells

for CD45 antigen, 10μl rat anti-mouse CD45-PerCP (eBiosciences, San Diego, CA) was added to one tube and incubated for 45 min at 4ºC in dark place. To determine cut-off values and true positive staining, 10μl isotype control antibody conjugated with PerCP (eBioscience) was added to the second tube and incubated for 45 min at 4ºC in dark place. Immunostanined cells were washed three times, resuspended in FACS buffer and subjected to flow cytometry using FACS Calibur (Beckman Dickinson, San Jose, CA) within an hour. FCAS data were analyzed by FCS Express software (De Novo Software, Los Angeles, CA).

Statistical analysis

Results are expressed as Mean±standard error of mean (SEM) and Error Bar charts. Statistical analysis was performed with the used of Independent Samples T Test. Data analysis was carried out using SPSS software Version 20 and significant levels were considered at P<0.05.

Results

Histological and biochemical examinations

First, in order to show that AP is induced in mice, we determined amylase activity in both the control and AP groups and found that amylase activity is significantly elevated in the AP group (Figure 1A). In addition, our histopathological examinations showed that the control group displayed a normal pancreas histology in both endocrine and exocrine. In contrast, the AP group showed an edematous pancreatitis with infiltration of inflammatory cells (Figure 1B).
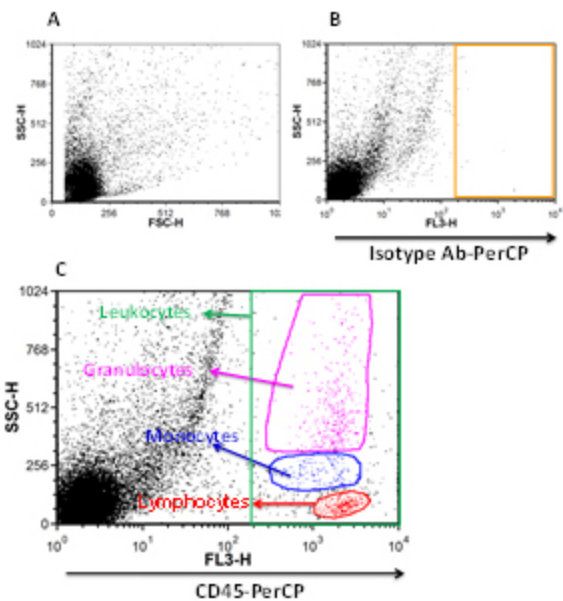
Flow cytometry analysis

Next, we enumerated the number of infiltrating inflammatory cells in pancreas of both the control and AP groups. In general, we observed that isolated pancreatic cells from the normal and AP mice consisted of a wide variety of cells as evidenced by side scatter (FSC) and forward scatter (SSC) (Figure 2A, 3A). Isotype control antibody was used to set the threshold of positive fluorescence (Figure 2B, 3B). In order to clarify various inflammatory cells we stained the isolated cells for CD45 and gated all leukocytes as these cells are strongly positive for CD45 antigen, but possess different SSC. The CD45 negative cells
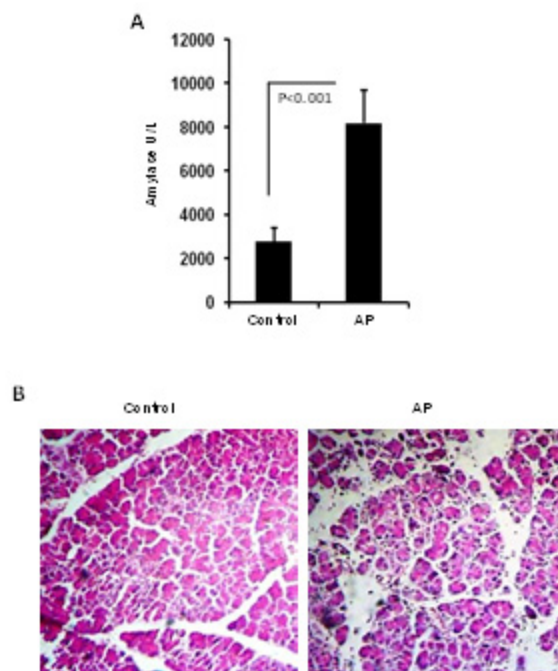
Fig. 1. Biochemical and histopathological examination of AP. AP was induced in mice as stated in material and methods. (A) Shows that amylase activity is significantly higher in the AP than the control group. (B) A representative H & E staining of pancreas tissue from both the control and AP groups. * P values <0.05.

which are non-inflammatory cells including pancreatic cells were excluded and did not count. As shown in figure 2C, all three inflammatory cells including granulocytes, monocytes and lymphocytes were present in the normal pancreas. Similarly, the isolated cells from mice with AP consisted of a numerous number of granulocytes, monocytes and lymphocytes. However, the number of these cells was significantly higher in the AP group than control mice (Figure 3C). Finally, we enumerated and quantified the number of CD45 positive cells (leukocytes) in both groups and obtained the percentage of each leukocyte population by dividing the number of these cells to the total of isolated pancreatic cells (CD45+ and CD45-). We observed that leukocytes consisted of 5.9%±1.6 percentage of total of isolated pancreatic cells in the control group, whereas these cells made up 26%±8.1 in the AP (Figure 4A). Moreover, when we quantified the percentage of each inflammatory lineage, we found that the percentage of granulocytes
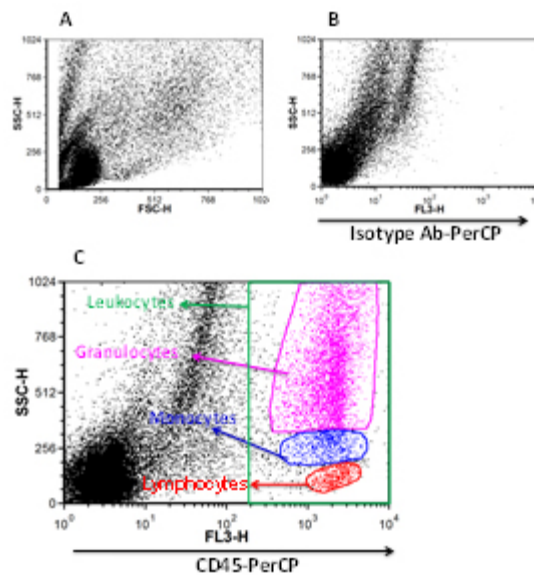
Fig. 2. Flow cytometry analysis of inflammatory cells in pancreas of the control mice. (A) Representative forward and side scatter plot for isolated pancreatic cells in the control group. (B) A threshold of fluorescence intensity was set using isotype antibody. (C) CD45/SSC gating was used to identify various leukocytes populations in the control mice. Representative plots from 6-8 mice are shown.

is 2.9%±1.8 in the control group, but the percentage of these cells were 18.2%±3.2 in the mice with AP (Figure 4B). Moreover, our data show that the percentage of monocytes is 0.9%±0.4 in the control mice whereas; it was 3%±1.3 in the AP group (Figure 4C). In contrast, we observed that lymphocytes only consist of 1.1%±0.3 percentage of pancreatic cells and did not significantly change in the AP mice (Figure 4D), indicating that lymphocytes may not play a crucial role in pathobiology of AP.

Discussion

To our knowledge this is the first report demonstrating the use of flow cytometry to quantify and enumerate infiltrating inflammatory cells in the pancreatic tissue. Herein, we reported that flow cytometry is a reliable and reproducible method for quantifying leukocytes in panaceas. Our flow cytometry data clearly show that the numbers of myeloid cells including granulocytes and monocytes, but not lymphocytes, are remarkably enhanced in the

Fig. 3. Flow cytometry analysis of inflammatory cells in pancreas of mice with AP. (A) Representative forward and side scatter plot for isolated cell in the AP group. (B) A threshold of fluorescence intensity was set using isotype antibody. (C) CD45/SSC gating was used to identify various leukocytes populations in the AP group. Representative plots from 6-8 mice are shown.

pancreas tissue of experimental AP compared to the control mice.

There is a considerable interest in better understating the role of inflammatory cells particularly neutrophils in injured tissues (23). Neutrophils have been shown to be the first leukocytes which arrive in the inflamed organs. These cells phagocytose cell debris and release various inflammatory mediators such as proteases and reactive oxygen species. In the case of AP, activated neutrophils release matrix methaloproteinase-9 (MMP-9) which in turn lead to activation of trypsinogen and subsequently further pathological inflammation and tissue damage in AP (9). MPO assay and immunohistochemically staining of neutrophils are two classical methods for determination of neutrophils in injured tissues. MPO is an enzyme existing in the granules of neutrophils
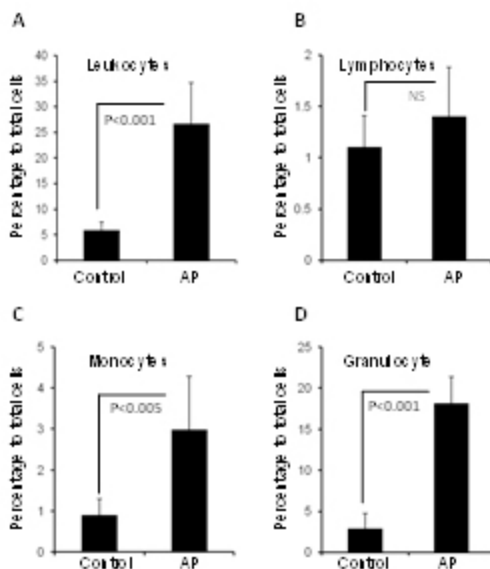
Fig. 4. Quantifying the percentage of all inflammatory cells by flow cytometry. Inflammatory cells were determined as stated in figures 2 and 3 and the percentage of each cell population was analyzed by dividing the number of each population to total of isolated pancreatic cells. (A) Shows the percentage of leukocytes in the control and AP groups. The percentage of granulocytes, monocytes and lymphocytes are shown in graphs B, C and D, respectively. Pooled data from 6-8 mice per group are shown. Values are the mean percentage of each population ± SD. *P<0.05; ns: non-significant.

and is detectable by a colorimetric assay called MPO assay (24). This test has been used to detect infiltrated neutrophils in various injured organs such as pancreas(10) and murine spinal cord injury (20). On the other hand, accumulating evidence implies that because of relatively low sensitivity of MPO assay, an alternatively more sensitive method is necessary (20). In the current study, we employed flow cytometry for quantifying infiltrating leukocytes in pancreas of control mice and experimental AP. To do this, leukocytes were identified by CD45/SSC gating and the percentage of leukocytes and each population of inflammatory cells were determined. As shown in figure 2A and 3A, a similar gating protocol was used for identification and qualifying of these cells in the both control and AP groups. Granulocytes, monocytes

and lymphocytes were shown to have distinctive gates in the control mice. However, these cells particularly monocytes and granulocytes displayed a few overlapping in the experimental AP group. As we used similar gating protocols in all the mice, these overlapping cells were neglectable and did not have any significant effect on the percentage of each leukocyte population.

Our flow cytometry data showed that granulocytes consisted only 2.9 percentage of pancreatic cells in the control group, whereas these cells possessed more than 18.2 percentage of pancreatic cells in the AP group, indicating that granulocytes are the greatest inflammatory cells in the terms of number which migrate into the pancreas during AP. Supporting of these findings, data from a previous study showed that administration of anti-neutrophilic serum significantly reduced the extent of necrosis and inflammatory infiltrates (25). Moreover, our histopathological examinations demonstrated an elevated number of neutrophils in AP compared to normal group. However, histopathological findings are not quantitative and are much less sensitive than flow cytometric analysis. Taking together, flow cytometry is a feasible and reliable method for quantifying granulocytes in pancreas. However, we have to consider that this method may not have any application for acute pancreatitis in human. So far, supporting diet is the only treatment of AP and finding novel therapeutic approaches are absolutely necessary. Thus, flow cytometry would be a suitable method for enumerating inflammatory cells in animal models of AP before and after treatment with new pharmaceutical drugs.

Accumulating evidence from in vitro and in vivo studies suggests a vital role for monocyte/macorpages in regulating the injury responses in various tissues (26). In animal models Ly-6Chi monocytes are released from bone marrow in response to distance organ injury and are believed to play a curial role in initial responses to tissue injury. In contrast, Ly-6Clo monocytes have been suggested to be involved in tissue repair (27). Herein, our flow cytometry data showed that infiltrate monocytes consisted of only 0.9 percentage of all pancreatic cells. However, the numbers of these cells were significantly increased (3.33 times) and reached to 3

percentage of pancreatic cells in the AP mice. Our observation is in agreement with a report showing that the number of Ly-C6hi monocytes are remarkably enhanced in the AP compared to control group(22). However, enumerating the subpopulation of Ly-6Chi and Ly-6Clo monocytes in the normal and AP mice by flow cytometry remains to be elucidated.

Finally, when we quantified the number of infiltrated lymphocytes in the pancreas of both the normal and AP mice, we found that the percentage of lymphocytes in both groups are almost similar, indicating that these cells might not play an important role in pathobiology of AP. our data are in the line with a previous report showing that injury in renal ischemia-reperfusion is not dependent on both B and T-lymphocytes (28).However, we did not examine the lymphocyte sub-populations in our work and we have to consider that there might be a difference between CD4+ and CD8+ T cells in pancreas tissue which needs to be further clarified. On the other hand, recent studies have demonstrated that not only the number of peripheral blood lymphocytes are decreased in patients with AP but also the CD4+/CD8+ ratio is lower in patients with severe than mild AP (29, 30).

In conclusion, we have implemented flow cytometry as a new technical approach to quantify inflammatory cells in pancreas and found that the percentage of these cells was remarkably enhanced in experimental AP. Thus, flow cytometry as a feasible and reproducible method could be a suitable approach for studying inflammatory diseases including pancreatitis.

Acknowledgment

Conflicts of Interest

The authors declare that they have no competing financial interest.

References

1. Sah RP, Garg P, Saluja AK. Pathogenic mechanisms of acute pancreatitis. Curr Opin Gastroenterol. 2012;28(5):507-15.

2. Bhatia M, Wong FL, Cao Y, Lau HY, Huang J, Puneet P, et al. Pathophysiology of acute pancreatitis. Pancreatology. 2005;5(2-3):132-44.

3. Dambrauskas Z, Giese N, Gulbinas A, Giese T, Berberat PO, Pundzius J, et al. Different profiles of cytokine expression during mild and severe acute pancreatitis. World journal of gastroenterology : WJG. 2010;16(15):1845-53.

4. Mayerle J, Dummer A, Sendler M, Malla SR, van den Brandt C, Teller S, et al. Differential roles of inflammatory cells in pancreatitis. J Gastroenterol Hepatol. 2012;2:47-51.

5. Panek J, Kusnierz-Cabala B, Dolecki M, Pietron J. Serum proinflammatory cytokine levels and white blood cell differential count in patients with different degrees of severity of acute alcoholic pancreatitis. Pol Przegl Chir. 2012;84(5):230-7.

6. Ohmoto K, Yamamoto S. Serum interleukin-6 and interleukin-10 in patients with acute pancreatitis: clinical implications. Hepatogastroenterology. 2005;52(64):990-4.

7. Ramudo L, De Dios I, Yubero S, Vicente S, Manso MA. ICAM-1 and CD11b/CD18 expression during acute pancreatitis induced by bile-pancreatic duct obstruction: effect of N-acetylcysteine. Exp Biol Med. 2007;232(6):737-43.

8. Abdulla A, Awla D, Thorlacius H, Regner S. Role of neutrophils in the activation of trypsinogen in severe acute pancreatitis. J Leukoc Biol. 2011;90(5):975-82.

9. Awla D, Abdulla A, Syk I, Jeppsson B, Regner S, Thorlacius H. Neutrophil-derived matrix metalloproteinase-9 is a potent activator of trypsinogen in acinar cells in acute pancreatitis. J Leukoc Biol. 2012;91(5):711-9.

10. Chooklin S, Pereyaslov A, Bihalskyy I. Pathogenic role of myeloperoxidase in acute pancreatitis. Hepatobiliary Pancreat Dis Int. 2009;8(6):627-31.

11. Rinderknecht H. Fatal pancreatitis, a consequence of excessive leukocyte stimulation? Int J Pancreatol. 1988;3(2-3):105-12.

12. Barie PS, Tahamont MV, Malik AB. Prevention of increased pulmonary vascular permeability after pancreatitis by granulocyte depletion in sheep. Am Rev Respir Dis. 1982;126(5):904-8.

13. Willemer S, Feddersen CO, Karges W, Adler G. Lung injury in acute experimental pancreatitis in rats. I. Morphological studies. Int J Pancreatol. 1991;8(4):305-21.

14. Aho HJ, Nevalainen TJ. Experimental pancreatitis in the rat. Light and electron microscopical observations on early pancreatic lesions induced by intraductal injection of trypsin, phospholipase A2, lysolecithin and non-ionic detergent. Virchows Arch B Cell Pathol Incl Mol Pathol. 1982;40(3):347-56.

15. Shrivastava P, Bhatia M. Essential role of monocytes and macrophages in the progression of acute pancreatitis: World J Gastroenterol. 2010 Aug 28;16(32):3995-4002.

16. Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L. Acute inflammatory response in spinal cord following impact injury. Exp Neurol. 1998;151(1):77-88.

17. Malleo G, Mazzon E, Genovese T, Di Paola R, Muia C, Crisafulli C, et al. Effects of thalidomide in a mouse model of cerulein-induced acute pancreatitis. Shock. 2008;29(1):89-97.

18. Campanella M, Sciorati C, Tarozzo G, Beltramo M. Flow cytometric analysis of inflammatory cells in ischemic rat brain. Stroke. 2002;33(2):586-92.

19. Kerfoot SM, Kubes P. Overlapping roles of P-selectin and alpha

4 integrin to recruit leukocytes to the central nervous system in experimental autoimmune encephalomyelitis. J Immunol. 2002;169(2):1000-6.

20. Tjoa T, Strausbaugh HJ, Maida N, Dazin PF, Rosen SD, Noble-Haeusslein LJ. The use of flow cytometry to assess neutrophil infiltration in the injured murine spinal cord. J Neurosci Methods. 2003;129(1):49-59.

21. Li K, Feng JY, Li YY, Yuece B, Lin XH, Yu LY, et al. Anti-inflammatory role of cannabidiol and O-1602 in cerulein-induced acute pancreatitis in mice. Pancreas. 2013;42(1):123-9.

22. Perides G, Weiss ER, Michael ES, Laukkarinen JM, Duffield JS, Steer ML. TNF-alpha-dependent regulation of acute pancreatitis severity by Ly-6C(hi) monocytes in mice. J Biol Chem. 2011;286(15):13327-35.

23. Ogawa M. Acute pancreatitis and cytokines: "second attack" by septic complication leads to organ failure. Pancreas. 1998;16(3):312-5.

24. Barone FC, Hillegass LM, Tzimas MN, Schmidt DB, Foley JJ, White RF, et al. Time-related changes in myeloperoxidase activity and leukotriene B4 receptor binding reflect leukocyte influx in cerebral focal stroke. Mol Chem Neuropathol. 1995;24(1):13-30.

25. Sandoval D, Gukovskaya A, Reavey P, Gukovsky S, Sisk A, Braquet P, et al. The role of neutrophils and platelet-activating factor in mediating experimental pancreatitis. Gastroenterology. 1996;111(4):1081-91.

26. Schober A, Weber C. Mechanisms of monocyte recruitment in vascular repair after injury. Antioxid Redox Signal. 2005;7(9-10):1249-57.

27. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010;327(5966):656-61.

28. Park P, Haas M, Cunningham PN, Bao L, Alexander JJ, Quigg RJ. Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes. Am J Physiol Renal Physiol. 2002;282(2):F352-7.

29. Azab B, Jaglall N, Atallah JP, Lamet A, Raja-Surya V, Farah B, et al. Neutrophil-lymphocyte ratio as a predictor of adverse outcomes of acute pancreatitis. Pancreatology. 2011;11(4):445-52.

30. Qin Y, Pinhu L, You Y, Sooranna S, Huang Z, Zhou X, et al. The role of fas expression on the occurrence of immunosuppression in severe acute pancreatitis. Dig Dis Sci. 2013;58(11):3300-7.