EVALUATION OF APOPTOSIS AND NECROSIS OF PERITONEAL MACROPHAGES IN RATS AFTER INJECTION OF ZINC CHELATES INTO ABDOMINAL CAVITY

VAVROVA EVA[1], SLADEK ZBYSEK[1], KOPEL PAVEL[2], KOMINKOVA MARKETA[2], ADAM VOJTECH[2]
[1]Department of Morphology, Physiology and Animal Genetics
[2]Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
CZECH REPUBLIC

eva.vavrova.umfgz@mendelu.cz

Abstract: Aim of this study was to evaluate the influence of zinc chelates injected into abdominal cavity on viability of peritoneal macrophages. Three organic acids (EDTA, DTPA, NTA) were used as zinc carriers. 24 female rats were employed in this study. The rats were divided into 5 groups: 6 received intraperitoneal injection of 2 mL 40 mM Zn-EDTA (group E), 6 received intraperitoneal injection of 2 mL 40 mM Zn-DTPA (group D), 6 rats received intraperitoneal injection of 40 mM 2 mL Zn-NTA (group N), 3 rats received 2 mL of normal saline (group K4–6) and 3 rats (group K1–3 for control) were intact. On the day after injection all rats were sacrificed and peritoneal lavages were performed and cell viability analysis was done. The macrophages were divided in two morphologically different groups – group of smaller monocytes-like macrophages (ML) with kidney-shaped nuclei and pseudopodia on their surface, and a group of macrophages with spherical nuclei and many vacuoles in the cytoplasm (vacuolized macrophages, VM). Apoptosis of ML macrophages of the peritoneum in rats administrated with Zn-DTPA was almost similar to apoptosis of cells in intact animals. This means that properties of this chelate are very close to homeostasis of rats´ abdominal cavity. The apoptosis significantly increased in group E compared to K1–3. There was a significant difference between groups K1–3 and K4–6. As for necrosis the values for K1–3 and Zn-DTPA are again very close. The most damage of cells was caused by Zn-EDTA chelate. Apoptosis of vacuolized macrophages was significantly higher in groups K4–6, E and N. Necrosis of vacuolized macrophages was significantly higher in groups K4–6 and N. The Zn-DTPA chelate looks to be the mildest carrier for Zn into the organism. The present study showed that the zinc-organics acid chelates are not toxic or irritating tissues after being injected into rat´s abdomen. The Zn-DTPA had the smallest influence to the peritoneal macrophages.

Key Words: macrophages, peritoneal lavage, chelate, apoptosis, necrosis

INTRODUCTION

The peritoneal cavity provides an easily accessible site for the harvesting of moderate numbers of resident, non-manipulated macrophages (Zhang et al. 2008). Macrophages play an important role in the immune system. These cells originate from the myeloid cell lineage (Wynn et al. 2013) and are present in all tissues. Macrophages show substantial morphological and phenotypic heterogeneity and have diverse physiological functions. They participate in proper tissue development and homeostasis. After injury or pathogen invasion, they recognize danger signals, change their morphology and start to secrete cytokines and other immunomodulators to attract circulating immune cells and coordinate the immune response (Moon et al. 2013). Under inflammatory conditions, circulating monocytes migrate into target sites and differentiate into inflammatory dendritic cells and macrophages (Geissmann et al. 2010). They mediate pathogen and damaged cell clearance, trigger specific immune responses and, lastly, terminate inflammatory processes. Macrophages also coordinate resolution, tissue re-modelling and repair (Mosser et al. 2008, Zhang, Mosser 2008). Depending on local or systemic stimuli like the presence of specific cytokines, macrophages perform different tasks and can

stimulate or inhibit various aspects of tissue metabolism. In addition, the same cells can repeatedly change their functional phenotype and adjust their activity to current demands determined by the local microenvironment (Porcheray et al. 2005, Biswas et al. 2012). When stimulatory factors disappear, macrophages return to their basal, resting state, and such functional plasticity is unique among the cells of the whole immune system. Zinc is a fundamental nutritional component required for normal development and maintenance of the immune function in humans and animals.

A wide range of pathologies develop as a consequence of zinc deficiency, such as growth defects, hypogonadism, dermal and immune alterations, and neurological dysfunctions (Maret, Krezel 2007). Zinc protects tissues from reactive oxygen radicals. The present study considers the use of zinc-organic acid (Zn-EDTA, Zn-DTPA, Zn-NTA) chelates as a source of zinc for organisms via determination of influence of intraperitoneal administration of zinc chelates to peritoneal macrophages viability.

MATERIAL AND METHODS

Animals and reagents

This study employed 24 female rats, each weighing 210-240 g. All rats were maintained in an air conditioned area and were provided with water and laboratory chow ad libitum. The rats were divided into 5 groups: 6 received intraperitoneal injection of 2 mL Zn-EDTA (group E), 6 received intraperitoneal injection of 2 mL Zn-DTPA (group D), 6 rats received intraperitoneal injection of 2 mL Zn-NTA (group N), 3 rats received 2 mL of normal saline (group K4–6) and 3 rats (group K1–3 - control) were intact. The dosage of chelates was 2 mL in each rat and was arbitrarily determined by considering the fact that the maximum of intraperitoneal injection in animals is 10 $mL.kg^{-1}$ (Turner et al. 2011). The concentrations of the chelates were selected based on prior cytotoxic tests. The dosage of chelates and normal saline was the same. Injection of chelates and normal saline was carried out by an experienced member of the research team.

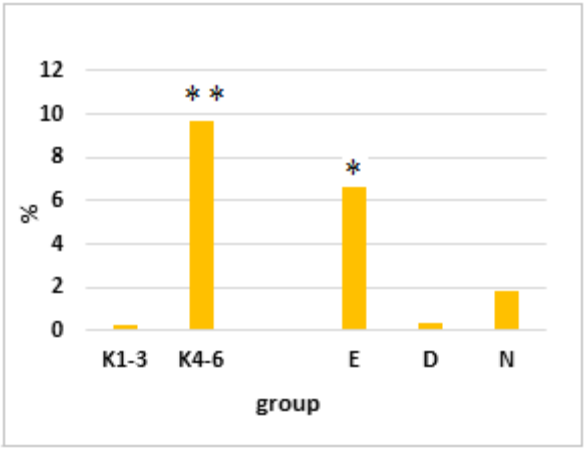
Each rat was anesthetized with ether. Prior to injection, each rat was placed in a supine position and the skin was scrubbed with 99.9% alcohol. A 23-gauge needle attached to a 2 mL syringe was inserted on the right lower side of the navel and 2 mL chelate or normal saline was injected into the peritoneal cavity. The entire procedure was performed under general anaesthesia in each rat. On the day after injection all rats were sacrificed and peritoneal lavages were performed. 18-gauge needle attached to a 20 mL syringe was inserted on the left lower side of the navel and 20 mL of normal saline was introduced into the abdominal cavity. A gentle massage of the abdomen was done and then at least 13 mL of material from each rat was aspired. All the samples were put into centrifuge at 1500 rpm for 10 minutes so we got 24 compact masses of peritoneal cells. We used 1.5 mL of supernatant to re-suspend each pellet.

Cell viability analysis

3 mL of CellWash were added to each sample and another centrifugation for 10 minutes at 1500 rpm followed. The pellets were re-suspended again. Macrophage apoptosis and necrosis was detected, using flow cytometry, according to the protocol provided with the Annexin-V-FITC Apoptosis Detection Kit (Sigma Aldrich, USA). The cells were analysed using the BD LSR Fortessa Flow Cytometer (Becton Dickindon, San Jose, USA) by counting 1500000 events. In quadrant analysis, the percentage of apoptotic and necrotic Final dot plots was evaluated using BD FACSDiva software (Becton-Dickinson, San Jose, USA). The results were evaluated by Student´s pair T-test. P values were considered statistically significant if $P<0.05$, $P<0.01$ and $P<0.001$. The data were processed using GraphPad Prism® - a commercial scientific 2D graphing and statistics software by GraphPad Software, Inc., California.

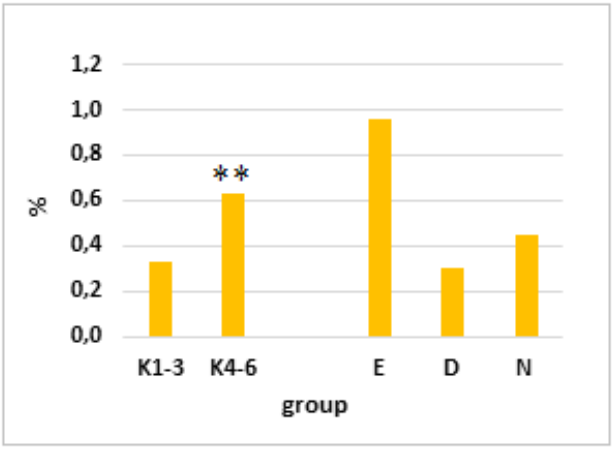
RESULTS AND DISCUSSION

Figure 1 Peritoneal macrophage apoptosis



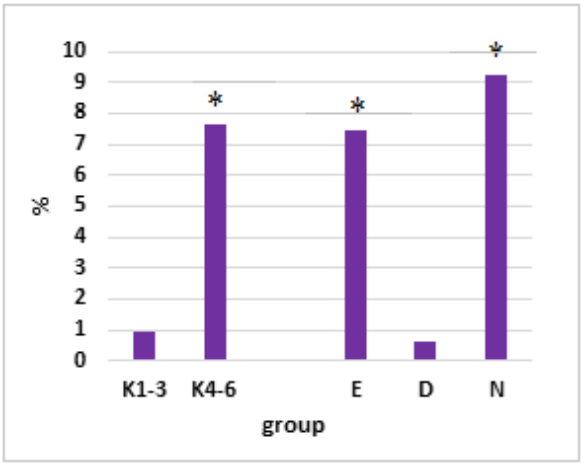
*Significant differences: control group (K1–3) compared to saline group (K4–6) and experimental groups E, D, N (*P<0.05, **P<0.01).*

Figure 2 Peritoneal macrophage necrosis



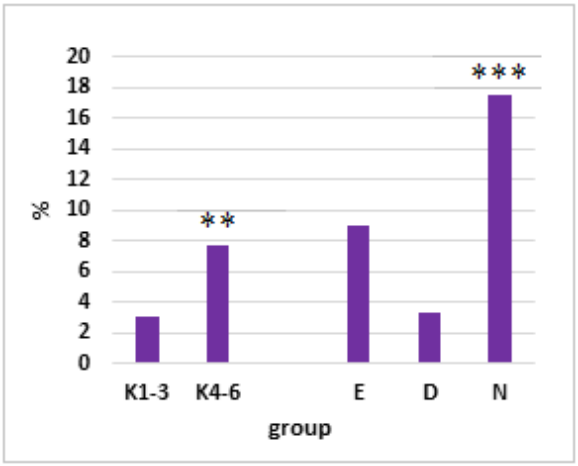
*Significant differences: control group (K1–3) compared to saline group (K4–6) and experimental groups E, D, N (**P<0.05).*

Figure 3 Apoptosis of vacuolized macrophages



*Significant differences: control group (K1–3) compared to saline group (K4–6) and experimental groups E, D, N (*P<0.05).*

Figure 4 Necrosis of vacuolized macrophages



*Significant differences: control group (K1–3) compared to saline group (K4–6) and experimental groups E, D, N (**P<0.01, ***P<0.001).*

Of the 24 rats, no infection or other complications in the abdominal wall were found. There were no colour changes of peritoneum or other macroscopic abnormalities. The macrophages were divided in two morphologically different groups – group of smaller monocytes-like macrophages (ML) with kidney-shaped nuclei and pseudopodia on their surface, and a group of macrophages with spherical nuclei and many vacuoles in the cytoplasm (vacuolized macrophages, VM).

Apoptosis of ML macrophages of the peritoneum in rats administrated with Zn-DTPA (group D) was almost similar to apoptosis of cells in intact animals (K1–3). This means that properties of this chelate are very close to homeostasis of rats´ abdominal cavity. The apoptosis significantly increased in group E compared to K1–3 ($P<0.01$). There was a significant difference between groups K1–3 and K4–6 ($P<0.001$).

As for necrosis the values for K1–3 and Zn-DTPA are again very close. The most damage of cells was caused by Zn-EDTA chelate.

Apoptosis of vacuolized macrophages was significantly higher in groups K4–6, E and N ($P<0.05$) compared to control group (K1–3). Necrosis of vacuolized macrophages was significantly higher in groups K4–6 and N ($P<0.05$ resp. $P<0.001$). The Zn-DTPA chelate looks to be the mildest carrier for Zn into the organism.

There is a large difference between the control groups. This corresponds with the fact, that "normal saline" or "physiological saline" frequently is used as neutral and physiological fluid. But it is not a physiological solution at all. The osmolality of this fluid is slightly higher than that of body fluids, the concentrations of sodium and chloride (both 154 $mEq.L^{-1}$) are higher. Furthermore, the pH of 0.9% NaCl solution is acidic (5.0–6.0). The first description of tissue toxicity of 0.9% NaCl solution dates back to the beginning of the 20th century (Cushing 1901). Intraperitoneal injection of the unphysiological and bioincompatible fluid may damage the mesothelial cells that line the abdominal cavity which may influence macrophages adhered to the abdominal wall.

CONCLUSION

The present study showed that the zinc-organics acid chelates are not toxic or irritating tissues after being injected into rat´s abdomen. The Zn-DTPA had the smallest influence to the peritoneal macrophages. The apoptosis and necrosis of these cells was almost the same in group K1–3 (control) and the group which received 2 mL of Zn-DTPA. There were significant differences in apoptosis and necrosis between in groups K1–3 and K4–6.

ACKNOWLEDGEMENT

The research was financially supported by the MENDELU IGA TP/01/2015.

REFERENCES

Biswas S. K., Chittezhath M., Shalova I. N., Lim J. Y. 2012. Macrophage polarization and plasticity in health and disease. *Immunological Research*, 53(1-3): 11–24.

Cushing H. 1901. Concerning the poisonous effect of pure sodium chloride solutions upon the nerve-muscle preparation. *American Journal of Physiology,* 6: 77–90.

Geissmann F., Manz M. G., Jung S., Sieweke M. H., Merad M., Ley K. 2010. Development of monocytes, macrophages, and dendritic cells. *Science,* 327(5966): 656–661.

Maret W., Krezel A. 2007. Cellular zinc and redox buffering capacity of metallothionein/thionein in health and disease. *Molecular Medicine,* 13(7–8): 371–375.

Moon M. L., McNeil L. K., Freund G. G. 2011. Macrophages make me sick: how macrophage activation states influence sickness behavior. *Psychoneuroendocrinology,* 36(10): 1431–1440.

Mosser D. M., Edwards J. P. 2008. Exploring the full spectrum of macrophage activation. *National Review of Immunology* 8(12): 958–969.

Porcheray F., Viaud S., Rimaniol A.C., Léone C., Samah B., Dereuddre-Bosquet N., Dormont D., Gras G. 2005. Macrophage activation switching: an asset for the resolution of inflammation. *Clinical and Experimental Immunology* 142(3): 481–489.

Turner P. V., Brabb T., Pekow C., Vasbinder M. A. 2011. Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. *Journal of the American Association for Laboratory Animal Science : JAALAS*, 50(5): 600–613.

Wynn T. A., Chawla A., Pollard J. W. 2013. Macrophage biology in development, homeostasis and disease. *Nature,* 496: 445–455.

Zhang X., Gocalves R., Mosser D. M. 2008. The Isolation and Characterization of Murine Macrophages. *Current Protocols in Immunology,* Chapter 14, Unit 14.1.

Zhang X., Mosser D. M. 2008. Macrophage activation by endogenous danger signals. *Journal of Pathology,* 214(2): 161–178.