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Spleen and bone marrow megakaryocytes as targets for inhaled vanadium

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Summary. An increased incidence in ischemic and thromboembolic events in the population of cities with rising air suspended particle pollution has suggested the interaction of some of the components of these particles in the coagulation system. A previous report from our laboratory identified thrombocytosis as a consequence of the subacute and chronic inhalation of vanadium. With this preceding information we decided to evaluate the effects of this element in the spleen and bone marrow in a mouse experimental model. CD-1 male mice inhaled V_2O_5 0.02 M for one hour twice a week for twelve weeks. The spleen and bone marrow were processed for light microscopy. The increase in quantity and size of megakaryocytes (MKs) in the exposed group in both organs was striking. Also, modifications in the cytoplasm, granule content and nuclear ultrastructure were evident. Our results indicate the influence of vanadium on megakaryopoyesis, an effect which could be the onset of the thrombocytosis previously reported by our group. The modifications in MKs described here suggest that inhaled vanadium could induce megakaryocytic proliferation, which may result in increased production of platelets and increased risk for thromboembolic events.

Key words: Megakaryocytosis, Vanadium, Transition metals, Inhalation exposure

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Introduction

Vanadium (V) has become an important element in recent years and has increased in relevance as an air pollutant (Fortoul et al., 2002). It is a component of Residual Oil Fly Ash (ROFA) (Samet et al., 1999) and its main entrance into the body is by inhalation (Brook et al., 2004; Nemmar et al., 2004). A predominant source of this element in the atmosphere is the increased combustion of fuel products, such as those derived from Venezuelan or Mexican oil, which have a high vanadium concentration (Nriagu, 1998; Ivancsits et al., 2000). In previous reports from our group an increased metal concentration in lungs from autopsy cases, comparing three decades from 1960-1990 has been described (Fortoul et al., 1996); one of the analyzed elements was vanadium (V), which in recent years has shown a marked increment (Fortoul et al., 2002).

We have also previously shown thrombocytosis in a mouse experimental model, as a consequence of inhalation of vanadium pentoxide (Gonzalez-Villalva et al., 2006). A few other reports indicate interaction of V with the hematopoietic system (Zaporowska et al., 1992; Beutler et al., 2001). Consequently, we decided to explore the behavior of platelet producing cells – megakaryocytes – in the spleen and bone marrow of our animal model.

Materials and methods

CD-1 male mice weighing 33±2g were housed in hanging plastic cages under controlled light conditions (12 h light/dark regime), and fed with Purina rat chow and water *ad libitum*. The experimental protocol was in accordance with the Animal Act of 1986 for Scientific Procedures. Inhalations were performed as described by Avila-Costa et al. (2005). One hundred and four mice

were placed in an acrylic box, inhaling 0.02 M V_20_5 (Sigma, St Louis USA). For one hour twice a week over twelve weeks. Control mice (one hundred and four) inhaled only the vehicle - deionized water- for the same period of time. Eight exposed mice and eight controls were killed between the first inhalation (24h) and at intervals of one week up to twelve weeks of vanadium pentoxide inhalation exposure. Animals were anesthetized with sodium pentobarbital and perfused via aorta with saline containing 10% formaldehyde in phosphate buffer. The spleen and some tissue from the bone marrow were proccesed for paraffin wax embedding and stained with Hematoxylin-Eosin. In order to evaluate increases in megakaryocyte numbers, 100 fields at 400x from tissue from controls and exposed animals were assessed using an Olympus CX21FS1 light microscope. To evaluate their increase in size, 250 megakaryocytes were assessed at 400x, with the same light microscope with a graticule.

Concentrations of V_20_5 in the chamber were quantified as follows: a filter was positioned at the outlet of the ultranebulizer during the whole inhalation time at a flow rate of 10 L/min. After each exposure, the filters were removed and weighed; the element was quantified following the same protocol as with tissue samples. Six filters for each inhalation were evaluated (Fortoul et al., 2002). Samples were analyzed using a graphite furnace Atomic Absorption Spectrometer (Perkin Elmer Mod. 2380). The light source came from a hollow cathode lamp. Formaldehyde and the blanks were also analyzed to identify metal contamination from this source. Accuracy was assured by three random determinations of seven different standard solutions, prepared with the same chemical reactives used during the metal analysis. For V_20_5, the wavelength was 318.4 nm, the detection limit was 0.37 ppm and the slit 0.7 nm. Each sample was analyzed in triplicate.

ANOVA with Tukey's posttest was performed, considering differences statistically significant between controls and exposed animals at $p<0.05$.

Results

V_2O_5 concentration in the inhalation chamber

The average concentration of V in the chamber was 1436 $\mu g/m^3$ during the whole experiment (data non shown).

Spleen weight

No statistical difference was observed throughout the experiment comparing controls vs exposed mice as shown in Figure 1.

Histological findings

The spleen of control mice contained two well delimited components, as expected; the white and the red pulp. The white pulp was composed of lymphocytes organized into lymphoid nodules with central arterioles. Splenic cords were readily observed in the red pulp, with scattered megakaryocytes present (Fig. 2A). At higher magnification megakaryocytes with lobulated nuclei were readily evident (Fig. 2B).

In exposed animals, white pulp was increased with larger and better defined follicles with reactive germinal centers composed of tightly arranged lymphocytes (Fig. 2C). At higher magnification the increase in size and in quantity of megakaryocytes was particularly apparent (Fig. 2D). These subjective estimates were confirmed when megakaryocytes were measured, showing in exposed animals an increase in number (from 2.1±0.12 to 3.8±0.12 μm per field) (Fig. 3). When MK diameter was compared, controls exhibited 25±0.35 μm, in contrast with 36±0.52 μm in the exposed animals, remaining constant regardless of the length of the exposure (Fig. 4).

In bone marrow, the same morphological modifications in the megakaryocytes described in the spleen were observed, such as irregular nuclei with prominent nucleoli (Fig. 5). There was an increase in megakaryocyte count (Fig. 6), as well as MK diameter (Fig. 7) in exposed animals, with similar values to those obtained for the spleen.

Discussion

In a previous report we demonstrated thrombocytosis in a mouse inhalation model of vanadium exposure, speculating on the possibility of an associated clonal event, since we identified several supportive criteria referred to by Schafer (2004), Tefferi et al. (2007) and Tefferi and Vardinam (2008) such as: absence of underlying disease, megaplatelets, and morphological changes in megakaryocytes.

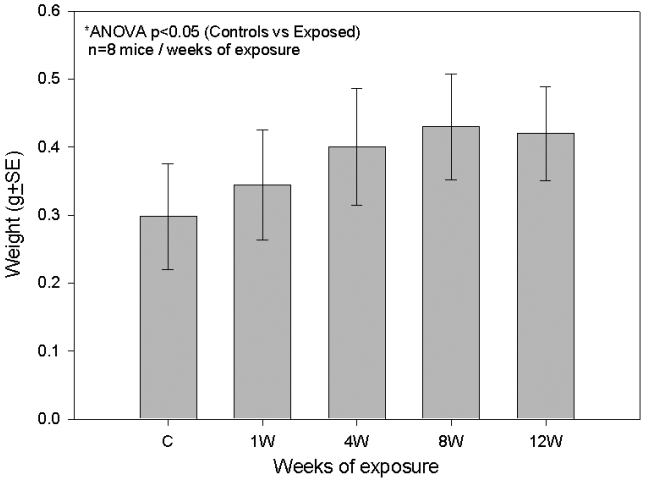

Fig. 1. Spleen weight comparison between controls and vanadium-exposed mice from one to 12 weeks.

A neoplastic chronic myeloproliferative disorder with predominant megakaryocyte hypercellularity in bone marrow and thrombocytosis is well recognized in clinical Haemato-Oncology (Tenedini et al., 2004). Some of the changes observed are the abnormal proliferation of neoplastic MKs and persistent thrombocytosis, along with elevated numbers of bone marrow MKs and morphologically and functionally abnormal circulating platelets. Disregulation of megakaryotopoyesis in ET has been found to involve defective binding of Thrombopoyetin (TPO) by platelets and MKs due to reduced or abnormal expression of the Thrombopoyetin receptor (Mpl); this results in increased levels of plasma TPO and increased sensitivity of MKs to TPO, in turn leading to MK hyperproliferation (Teofili et al., 2002; Tenedini et al., 2004).

Megakaryocytes are the cells responsible for the production of platelets, and this production is determined in part by MK size, number and maturation state, as well as the production of several cytokines. Our morphological findings suggest that vanadium induces, in our model, changes compatible with a myeloproliferative disorder, since the other findings such as splenomegaly, giant platelets and thrombocytosis also suggest it (Gonzalez-Villalva et al., 2006; Hao et al., 2006).

A survey of published literature has revealed no information about vanadium and myeloproliferative disorders. Reports of megakaryocytosis in mice are usually in transgenic specimens which have helped to elucidate the participation of diverse factors in megakaryocytosis, such as STAT3 over-expression

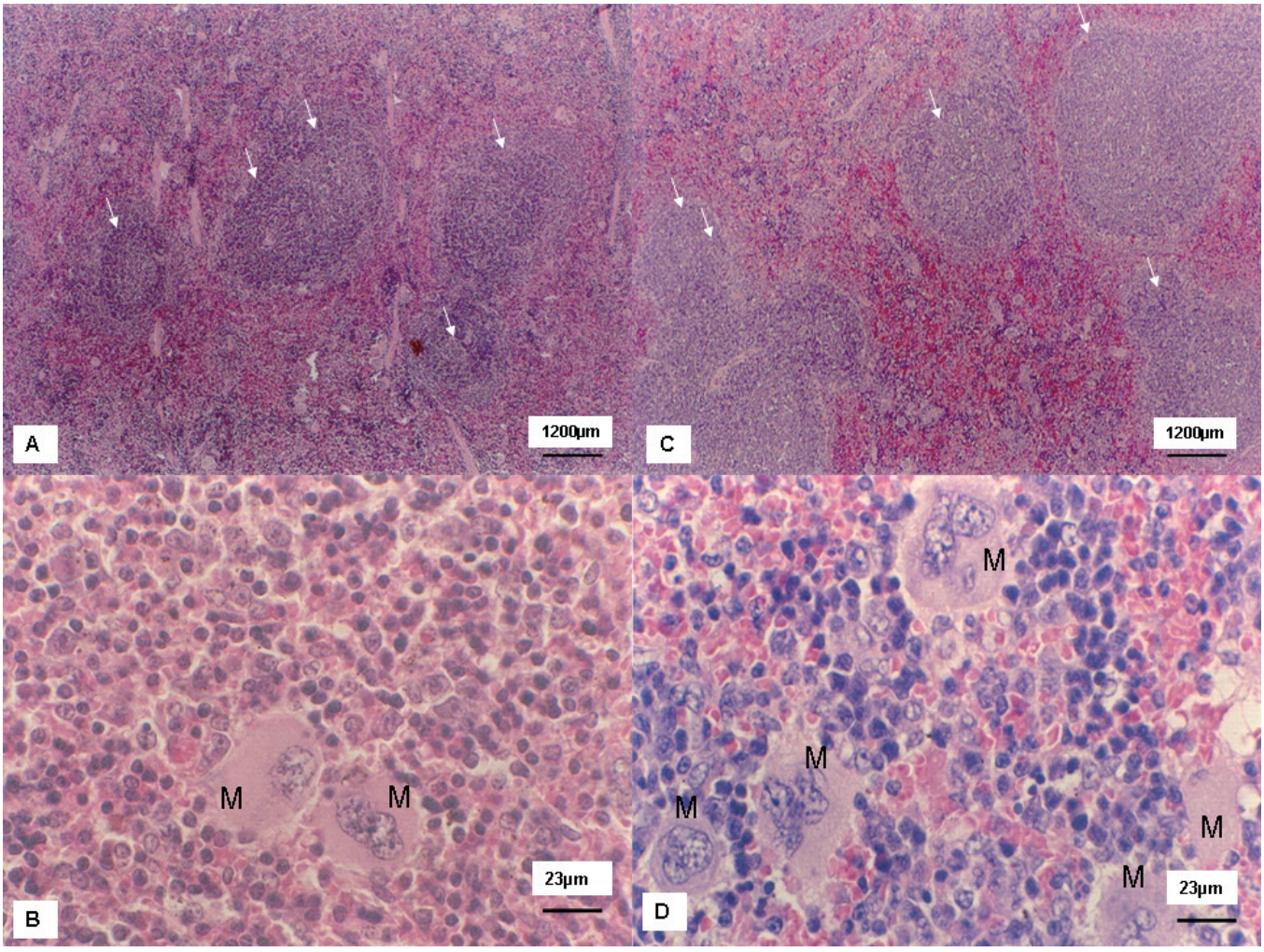

Fig. 2. Spleen photomicrograph comparing controls vs exposed mice. **A.** In control mice well demarcated lymphoid nodules are observed and some megakaryocytes are easily identified (arrows). **B.** At higher magnification megakaryocytes with regular borders and a multilobular well defined nucleus (M) characterized control animals. **C.** In exposed animals an increase in size of the lymphoid follicles and the presence of megakaryocytes were evident (arrows). **D.** In exposed mice, larger megakaryocytes (M) are visible with multilobulated nucleus and more prominent nucleoli. Also, an increase in number was appreciated. (H.E.).

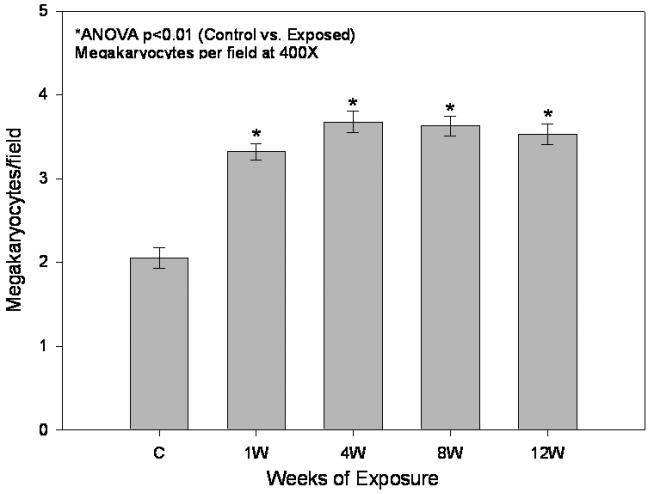

Fig. 3. Megakaryocyte count per field in spleens from mice, contrasting control vs exposed after vanadium inhalation. An increase in megakaryocyte count occurred after the first week of inhalation with a significant increase after longer exposures.

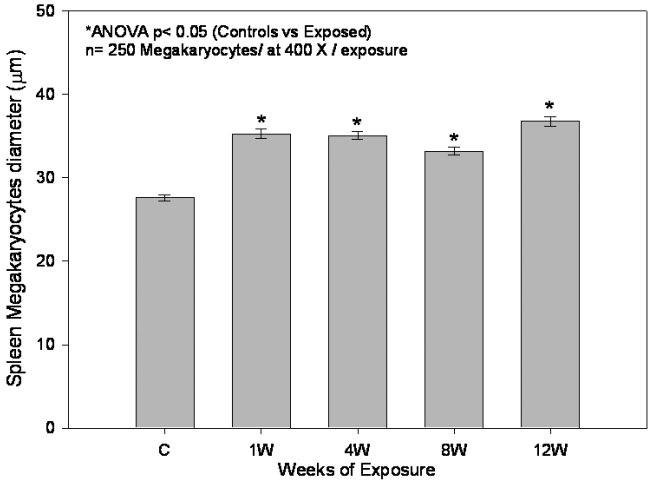

Fig. 4. Megakaryocyte diameter and exposure time in spleens from mice, contrasting control vs exposed after vanadium inhalation. An increase in megakaryocyte size was observed after the first week of inhalation, with a steady increase with further exposures.

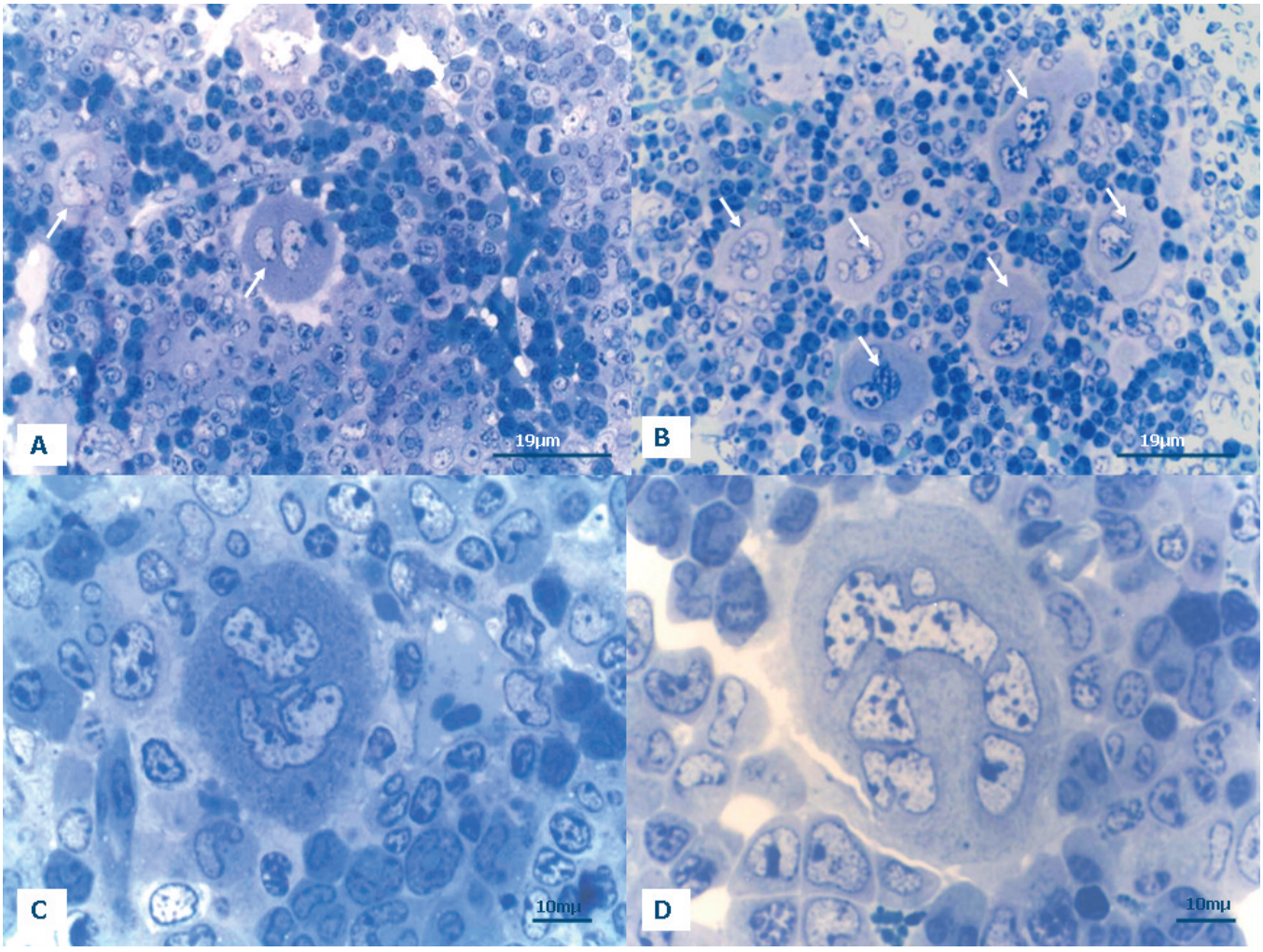

Fig. 5. Bone Marrow megakaryocytes in semi-thin sections. **A** (Arrow) and **C** show control sections with round and well defined megakaryocytes with irregular nucleus and similar to those found in the spleen **(B)** and **(D)** show megakaryocytes in exposed animals with larger and more irregular shaped nuclei (Toluidine blue).

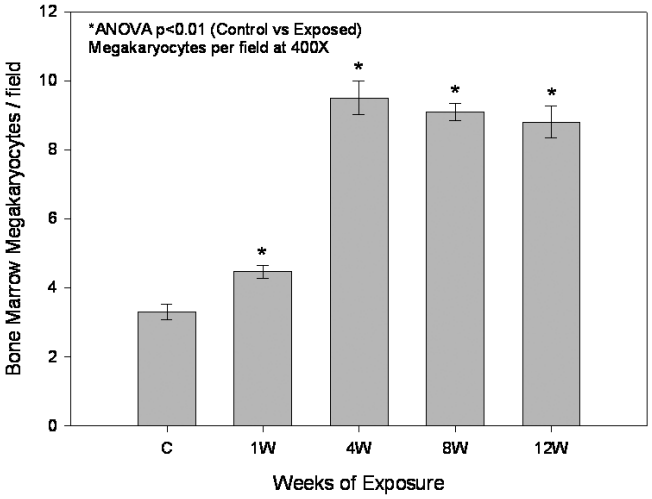

Fig. 6. Megakaryocyte count per field in bone marrow from mice, contrasting control vs exposed after vanadium inhalation. An increase in megakaryocyte count was found after the first week, but this was more evident at week 4.

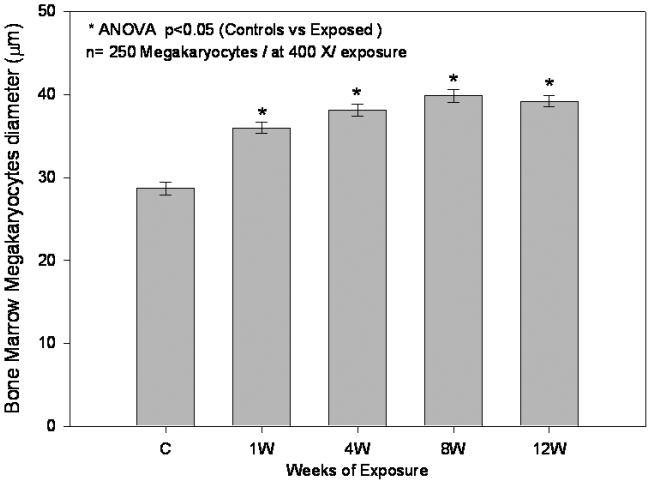

Fig. 7. Megakaryocyte diameter and exposure time in bone marrow from mice, contrasting control vs exposed after vanadium inhalation. An increase in megakaryocyte size was observed after the first week of inhalation, behaving similarly to findings in the spleen.

(Kirito et al., 2002), JAK2 (Steensma, 2006) and GATA1 mutations (Vannucchi et al., 2005a,b), *B-Raf* kinase mutation (Kamata et al., 2005) and mutation in c-Myb (Metcalf et al., 2005). GATA-1 acquired mutation has been mentioned by Li et al. (2005) who propose this acquired mutation as a hyperproliferative factor for MKs (Suna, 2006). In this report we used mice without genetic manipulation, so we might speculate that the effects observed could be associated with activation or inhibition of some of the molecular mechanisms by which metals commonly exert toxicity. Such a mechanism is the generation of Reactive Oxygen Species (ROS). Metals and ROS have been demonstrated to affect a number of receptors and genes, including growth factor receptors, src kinase, ras signaling, mitogen-activated protein kinases, and nuclear transcription factors NF-κB, AP-1, p53, NFAT, and HIF-1. The activation or inhibition of some of these genes or growth factors should be analyzed in the further investigation of possible interactions of vanadium with myeloid cells in our inhalation model. The roles that ROS may play in this animal model are also potential areas for further research (Leonard et al., 2004).

Megakaryocytes arise from pluripotent hematopoyetic stem cells capable of differentiation into all of the cell lines that reside in the bone marrow and produce blood cells. MK differentiation and proliferation are regulated by a number of cytokines. However, TPO is regarded as the primary regulator of MK maturation, including cellular enlargement and nuclear poplyploidisation. Changes in these features have been observed in this study. TPO binding to its receptor, Mpl, results in the activation of a wide spectra of signaling events, including the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), ras/raf/MAP kinase and PI-3 kinase/Akt pathways. These pathways have been studied in genetically modified cells or knock-out mice, as mentioned above. The activation of *JAK2* phosphorylates Mpl, which activates a series of signaling cascades that end in maturation, proliferation and an increase in platelets. This increase in proliferation/maturation correlates with augmented intensity and duration of mitogen-activated protein kinase (MAPK) activity, which is required for maturation of MK (Lannutti et al., 2006) and is a target for V (Samet et al., 1998).

Aguirre et al. (2005) mentioned that after the administration of a single intraperitoneal dose of sodium orthovanadate, an increase in erythropoiesis was shown by morphological bone marrow analysis. Also, over-expression of GATA-1 and Bcl-xL has been reported, variations that we should explore in our chronic exposure model (Aguirre et al., 2005).

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