

THE EFFECT OF TiO₂ NANOPARTICLES (DOPED OR NOT WITH Ag, ENCAPSULATED OR NOT IN LYPOSOMES), ON THE SPLEEN ULTRASTRUCTURE IN *Mus musculus* SPECIES, EXPOSED AT A STRESS FACTOR (X-RAYS)

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Abstract. The present experiment was performed on young females of *Mus musculus*, 22-24 g each, intraperitoneal injected with a suspension of titanium dioxide (five injections of 0.5 ml each, one at two days, with 0.01% TiO₂ or TiO₂-Ag suspension). The TiO₂ nanoparticles of anatase crystallization form, 10-20 nm size, were conjugated or not with 1% Ag, encapsulated or not in liposome. A day after the third injection, half of the animals received a sublethal dose of X-rays (2.58 Gy; the stress factor). A day after the last injection, the animals were sacrificed through the section of the carotid artery. Ultrastructural investigations were performed at the spleen level. The analysis of the ultrastructural features from the spleen level enabled the following observations: the single action of the TiO₂ or TiO₂-Ag nanoparticles induced an inflammatory process, but in the presence of X-rays, they manifested a slight protective effect; the TiO₂-Ag nanoparticles, encapsulated in liposome, manifested a strong radioprotective effect, but the endocapsulation process is not optimal and has to be improved; the presence of a protective effect at the spleen level can suggest that the TiO₂-Ag nanoparticles can be used to enhance the organism resistance in case of carcinogenic treatment (in animal or in homo).

INTRODUCTION

Initially, the TiO₂ nanoparticles were considered an inert material. Subsequently, it was pointed that TiO₂ nanoparticles can induce lesions in the genetic matter (DNA and chromosomes), as well as at the ultrastructural level (Corneanu et al., 2012). The studies performed in different research centres established that they can migrate and accumulate in different organs and induce oxidative stress and cell death. The biological investigations pointed out different biological effects of the TiO₂-Me nanoparticles, depending on the metal type (silver, gold, copper, platinum, a/o), nanoparticle size, their single presence or doped with other element, activation or not by UV rays, encapsulation or not in different bioactive materials, a/o (Corneanu et al., 2007; Yonezawa et al., 2009; Corneanu et al., 2012, a/o). In a previous experiment performed on *Mus musculus* (Corneanu et al., 2012),

it was analyzed the interaction of TiO₂ nanoparticles (single or doped with a metal), with the hepatic cell.

In the present paper, it was studied the effect of TiO₂ nanoparticles, conjugated or not with silver, encapsulated or not in liposomes, at the spleen level, toward a stress factor (a sublethal dose of X-rays). As analysis element, were used the ultrastructural modifications at the spleen level. The obtained results underline that the TiO₂-Ag nanoparticles, encapsulated in liposomes enhanced the organism resistance to some stress factors and, thus, can be used in the carcinogenic treatment.

MATERIAL AND METHOD

The experiment was performed on young *Mus musculus* females, 22-24 g each, intraperitoneally injected (one at two days) with 0.5 ml from an aqueous solution of a 0.01% bioactive solution. The bioactive substance was a suspension of 0.01% TiO₂ nanoparticles of 10-20 nm size, anatase form crystallization, doped (conjugated) or not with 0.01% Ag aqueous solution, encapsulated or not in liposome. The animals from the Control variant did not receive the injection or were injected with the same amount of distilled water. As stress factor, it was used a sublethal dose of x-rays (5.28 Gy). A day after the third injection, half of the animals from the experimental variant were irradiated (the entire body), at a SEIFERT, ERESO MF-1 installation (Germany), at the following parameters: 185 kV, 5 mA, 1 mm Al filter, dose debit 52.8 R/min, total doses being of 528 R (5.28 Gy). After the X-irradiation, there were administered (in the same conditions), two intraperitoneal injections. A day after the fifth injection, all the animals were sacrificed through the section of the jugular venous or carotid artery.

The biological matter, from different organs was sampled for the electron microscopy analysis. In this experiment, the analyses were performed at spleen level. Small pieces of about 1 mm³ of spleen (white and red pulp), from all the experimental variants, were sampled for ultrastructural investigations. The processing of the biological matter for the electron microscopy investigations was performed according to the classical work protocol: prefixing in a 2.7% glutaraldehyde solution (2 ½ h); postfixing in a 1% Millonig solution (1 ½ h); embedding and infiltration in Epon 812. The serial sections of about 80-90 nm thickness were performed at ultratome, then contrasted with uranyl acetate and lead citrate. The examination was performed at an TEM JEM JEOL-1010 electron microscope in the Electron Microscopy Centre, Babeş-Bolyai University (Cluj-Napoca). The chosen images were successively captured and preserved in the data basis, by means of Megaview III Soft Image Analysis software. The obtained images were processed using the Wiever Imaging Analysis programme, being transformed in tiff images with a 16 pixels resolution.

RESULTS AND DISCUSSIONS

Spleen, electron microscopy analysis

Control variant.

The white pulp presented a characteristic structure for normal mice. It contained lymphocytes of variable size, disposed around the periarterial lymphoid sheaths and splenic lymphatic follicles. Many lymphocytes were in mitotic division (Fig. 1), at this level being present proliferative processes. In the germinative centre of the lymphatic follicle, there predominated big lymphocytes, with bulk nucleus and fine-granulated chromatin and evident nucleus. The cytoplasm was rich in aggregate ribosomes, which were similar to some polysomes. The mitochondria presented evidenced cristae and profiles of the endoplasmic reticulum. Numerically, the small lymphocytes were more reduced in comparison with the big or middle lymphocytes. They presented a big nucleus with two

nucleoli and heterochromatin condensed in blocks, little cytoplasm but they were rich in cellular organelles that appeared in an incisure of the nucleus. The middle-sized lymphocytes presented an intermediate structure between big and small lymphocytes. Between lymphocytes, there were stellated reticulate cells, with numerous prolongers. The marginal area of the pulp was rich in lymphocytes and plasmocytes. The plasmocytes presented a big nucleus, with peripheral heterochromatin blocks and a widely represented endoplasmic reticulum. In the white pulp of the spleen, there were also present eosinophils and basophils, as well as megakaryocytes, involved in the thrombocyte generation. In the marginal pulp area, there were present macrophages involved in the phagocytosis and removal of the antigenic rests. The red pulp from the young mice presented venous sinuses normally structured and interstitial spaces populated especially with haematies and, rarely, migrated macrophages, lymphocytes or plasmocytes.

Control, X-rays.

The mice irradiation with a unique sub-lethal dose of 5.28 Gy conducted to some deep adulteration at the spleen level. There took place an accentuated depletion, a rarefication of the lymphocytes and other cell components at the spleen level, while other components presented an adulterated structure. The organism response was represented through enhanced division rate of the cells. The adulteration and degeneration of the cells from the white and red pulp was evident through the presence of the pyknotic nuclei, cytoplasm balloonization and vacuolization and the broken plasmalemma lymphocyte, a/o (Fig. 2). As the lymphocyte population was rarefied, the reticulate cells and their prolongation were evident. The splenic macrophages were very solicited in the inclusion and digestion of the destroyed lymphocytes, to be destroyed also, their content being released in the extracellular area. Some haematies from red pulp presented an adulterated structure and a diffuse shape. The megakaryocytes presented deformed nuclei and adulterated cytoplasm, the synthesis function and liberation of thrombocytes being adulterated.

TiO₂, undoped.

The undoped TiO₂ administered at the animals from this experiment determined the appearance of some metabolic reversible structures of the nucleus, as **bodyguard** (Fig. 3) at the spleen level. The bodyguard structure was represented through constitutive heterochromatin, disposed on inner nuclear envelope, for the protection of the gene involved in cell survival. This structure was described by Hsu (1973), Corneanu et Crăciun and al. (1999), a/o. The cell population, which supported a deletion, was characterized through the reduction of the lymphocyte number. Moreover, the lymphocytes presented a polymorphism for their nuclei shape. The cells maintained their integrity, the destruction of the plasmalemma being observed especially at the red pulp level. At this level, the ageing cells are destroyed, together with some haematies. The presence of the neutrophils and eosinophils, at the red pulp level, indicated a reaction of the organism similar with the presence of an inflammatory process, determined by the presence of an exogenous substance (TiO₂ nanoparticles). The presence of TiO₂ nanoparticles determined the reduction of the mitotic rate of lymphocytes.

TiO₂ undoped, irradiated.

The presence of TiO₂ nanoparticles at the time of mice irradiation, manifested a radioprotective effect. The cell population from the spleen white pulp was slightly rarefied, being present both lymphocytes with normal structure and lymphocytes with moderately affected structure. In some regions, there were present pronounced cell rarefiers, together with adulterations of the lymphocytes. The macrophages presented an intense activity of phagocytate and destroyed the ageing lymphocytes or dead lymphocytes.

The X-irradiation promoted the lymphocytes multiplication, numerous mitoses being present. In the red pulp, there were also present neutrophils and a part of the lymphocytes were balonized, especially at the cytoplasm level. Under X-irradiation influence, the red cells from the red pulp presented a diffuse structure. The protective effect of the TiO₂ nanoparticles vs. -irradiation was underlined also by the ultrastructural features of the megakaryocytes. Thus, in the variant irradiated in the absence of a radioprotective substance (TiO₂ nanoparticles), the all megakaryocytes presented adulterate ultrastructure; when TiO₂ nanoparticles were present during the X-irradiation time, in the spleen, there were present both megakaryocytes with adulterated ultrastructure and megakaryocytes with normal ultrastructure (Fig. 4).

TiO₂-Ag, unirradiated (TiO₂ nanoparticles doped with silver).

The TiO₂-Ag doped nanoparticles represented an exogenous substance, a stress factor and determined a reaction from the organism (Fig. 5). In the white pulp of the spleen, there were predominant the small lymphocytes, ageing, among which there were pyknotic lymphocytes, which can be destroyed. The lymphocytes of medium size and the big lymphocytes (the young lymphocytes), were in a small number, the small number of lymphocytes in the mitotic division being explicable. The majority of the lymphocytes presented an adulteration degree. In the red pulp, among the red cells, there was present a small number of lymphocytes, neutrophils, as well as plasmocytes with dilated endoplasmic reticulum. The megakaryocytes presented a normal structure. TiO₂-Ag induced a slowdown of the lymphocyte mitotic division. Thus, it took place the ageing of the existent cells, together with their structure adulteration. This process was obvious at the nuclei level, which presented a tendency for picnotization.

TiO₂-Ag, X-irradiation.

The concomitant action of the two exogenous factors (TiO₂ nanoparticles and X-rays), led to the reduction of their negative action, recorded at their single action (Fig. 6). There occurred the mitotic division of the young lymphocytes (of big size), which were more frequent, the lymphocytes of medium size having polymorphic nuclei and slightly adulterated ultrastructure. At the red pulp level, more heamaties were in different degradation stages and some plasmocytes and macrophages endured an adulteration process with vacuolization, having thus cells rarefaction. The normally structured megakaryocytes, delivered thrombocytes.

TiO₂-Ag, encapsulated in liposome, without X-irradiation.

The encapsulation of the TiO₂-Ag nanoparticles in the liposome presented effects of mosaic type: in some areas, it did not induce significant modifications, while in other areas, there were recorded some fragmentary effects. In the areas without significant modifications, the recorded aspects were similar with those recorded at the administration of TiO₂-Ag non-capsulated in the liposome (Fig. 7). In the white pulp, the small lymphocytes with polymorph nuclei were predominant, as well as lymphocytes with pyknotic or degenerated nuclei. There were some areas the structure of which is normal and lymphocytes are in mitotic division. This process can occur due to a deficient encapsulation. The red pulp of the spleen presented a normal structure, between heamaties being present numerous small and medium lymphocytes, with normal structure. The presence of some polymorphonuclear leucocytes and eosinophils suggested the existence of an inflammatory process, determined (probably) by a deficient encapsulation of the TiO₂-Ag nanoparticles. There were also present young megakaryocytes in development together with others in intense activity, having in the cytoplasm many mitochondria, endoplasmic reticulum, dense cytoplasmic matrix and vesicles with an electron-dense matter. These components will be in the thrombocytes structure.

TiO₂-Ag, encapsulated in liposome, X-irradiation.

The presence of the TiO₂ nanoparticles, doped with silver (TiO₂-Ag), encapsulated in liposome, manifested a strong protective effect at the red pulp from the spleen, as well as

a zonal protective effect in the white pulp of the spleen (Fig. 8). In some areas of the white pulp, the small lymphocytes were predominant, accompanied by lymphocytes of medium size, usually with polymorph nuclei. Some lymphocytes presented a ballooned cytoplasm and the others presented pyknotic nuclei, and in the reticulate cells, there were present vesiculations. In other areas, especially near the red pulp, lymphocytes of middle size were predominant while some big, normally structured lymphocytes were in mitotic division. In the red pulp, between hematies, there was a small lymphocyte number, some in division. Others were in disintegration; the macrophages are very active, in cytoplasm being presents lysosomes and cell rests in the digestion phase, as well as polymorphonuclears in an intense activity.

CONCLUSIONS

- *The animals from the Control variant presented normal features of the spleen.
- *The singular action of a stress factor (a sublethal dose of X-rays) induced severe adulterations at the spleen level.
- *The singular presence of the TiO₂ doped or not with silver (TiO₂-Ag or TiO₂) determined a response from the spleen, similarly with the response induced by the presence of an inflammatory agent: neutrophils and eosinophils were present in the red pulp; cell population supported a depletion, the lymphocytes number being very small; inhibitory effect over the division of the lymphocytes, a/o.
- *At the X-irradiation, TiO₂ / TiO₂-Ag attenuated the destructive effects of the X-rays, permitting a reduction of the lesions amount from the spleen.
- *TiO₂ – Ag nanoparticles encapsulated in liposomes presented strong radioprotective effect, but the capsulation process was not optimal, requiring an amelioration.
- *The actual method for the inclusion in liposomes of doped TiO₂-Ag (TiO₂-Me) nanoparticles was deficient, being affected areas especially from the white pulp. Probably, TiO₂-Ag/liposomes presented efficacy at the red pulp level and in the neighbouring areas from the white pulp.
- *The irradiation of the mice in the absence of protecting agent induced severe adulterations at the spleen level: rarefaction of the cell organelles (especially in lymphocytes), enhancement of the mitotic division rate (a possibility explanation for their numeric reduction is related to destructions, adulterations and degeneration of some lymphocytes through cytoplasm ballooning, vacuolization and rupture of plasmalemma, a/o). Moreover, the splenic macrophages can be also destroyed. Also, the megakaryocytes were not capable for the deliverance of a normal amount of thrombocytes.
- *The TiO₂ undoped presence in the animal irradiation presented a protecting effect: the cell population was rarefied, but the induced changes were limited and reversible; irradiation induced the intensification of the mitotic activity at lymphocytes to compensate the destruction induced as a result of the X-rays action. The protected macrophages of TiO₂ presence were in intense activity, removing the ageing and destroyed lymphocytes; part of the megakaryocytes presented a normal structure, as a result of the protection offered by TiO₂ nanoparticles presence.
- *TiO₂-Ag induced a slowdown of the mitotic division in lymphocytes, with the consequence of ageing of the existent lymphocytes, concomitant with their structure adulteration (pyknosis tendency of their nuclei).
- **TiO₂-Ag presence in the X-irradiation time of the animals reduced the destructive effects of the X-rays, being present some adulterations. The X-irradiation induced the enhancing of a multiplication rate of the lymphocytes. The adulterations were recorded especially in the red pulp. The megakaryocytes were less affected and presented a normal structure and delivered the thrombocytes.

*The presence of TiO₂-Agencapsulated in liposomes, in the white pulp, conducted to the protecting effect in some areas, while in other areas, the aspects are similar with the non-encapsulated TiO₂ effect. This process is probably a result of deficient encapsulation of TiO₂-Ag. The red pulp presented a normal structure, being present young megakaryocytes in growth, together with others in an intense activity, with many cell organelles in the cytoplasm, as well as vesicles with electron-dense content, components from the next thrombocytes. The presence of some polymorphonuclears leucocytes and eosinophils supports the existence of an inflammatory process, determined (probably) by a deficient encapsulation of the TiO₂-Ag.

*In the white pulp, there were areas in which small lymphocytes were predominant together with average lymphocytes with reversible adulterations. In other areas, near the red pulp, the big and average lymphocytes with normal structure were predominant, some cells being in mitotic division. In the red pulp, among degraded haematies, there were lymphocytes with normal structure, in mitotic division, as well as some degraded lymphocytes. The macrophages were in an intense phagocytosis activity, together with some polymorphonuclears.

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Figures.

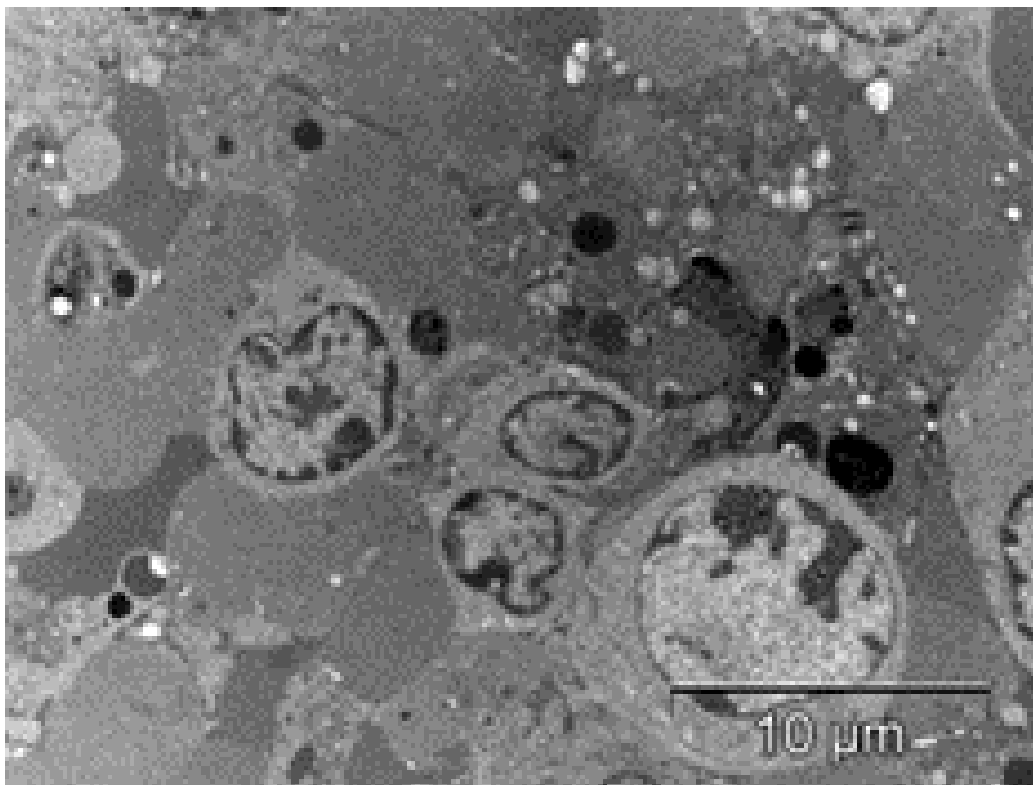


Fig. 1. Control variant. Lymphocytes in mitotic division (metaphase).



Fig. 2. Control, X-rays variant. Adulteration induced by X-rays in lymphocyte.

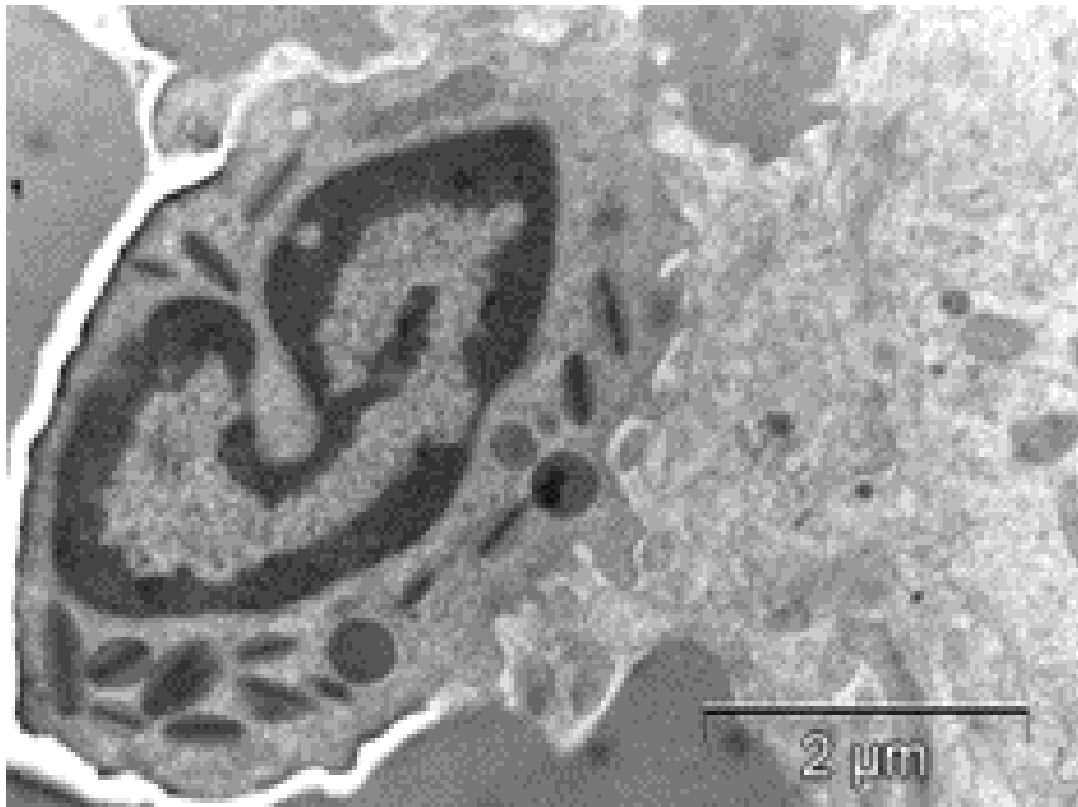


Fig. 3. TiO₂ variant. *Bodyguard* metabolic structure on the inner nucleus envelope.

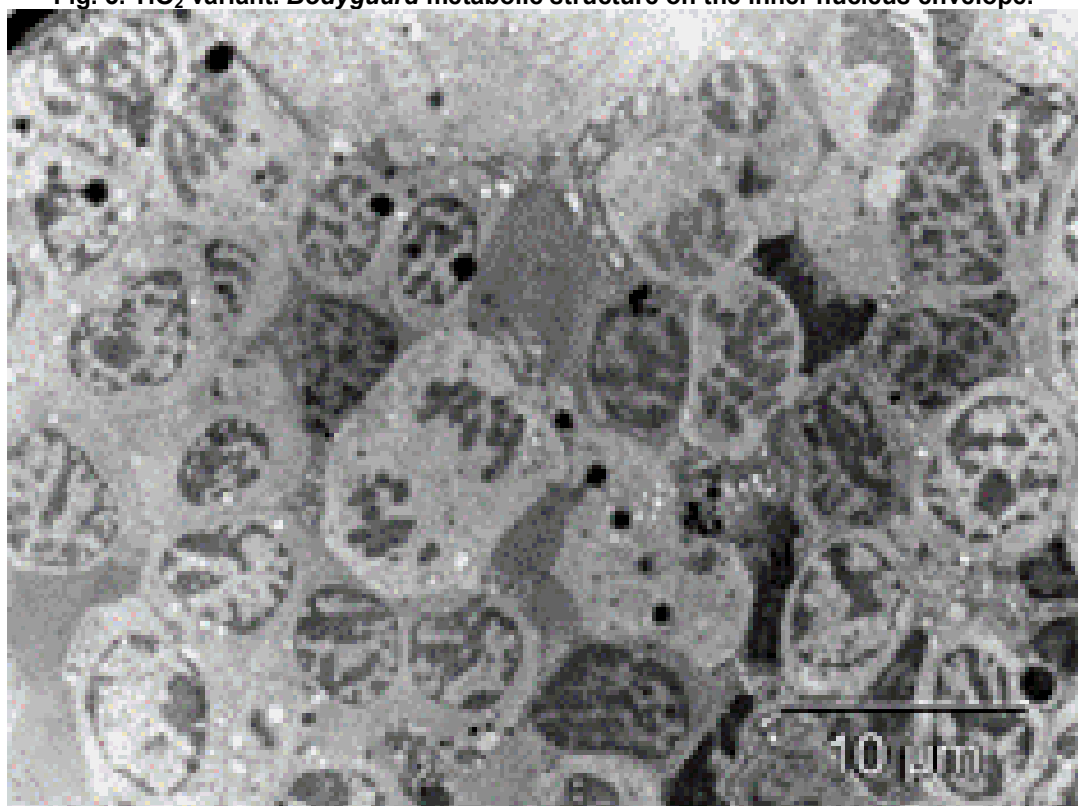


Fig. 4. TiO_2 – X-rays variant. Protective effect on the lymphocyte division, induced by TiO_2 presence during the X-irradiation.

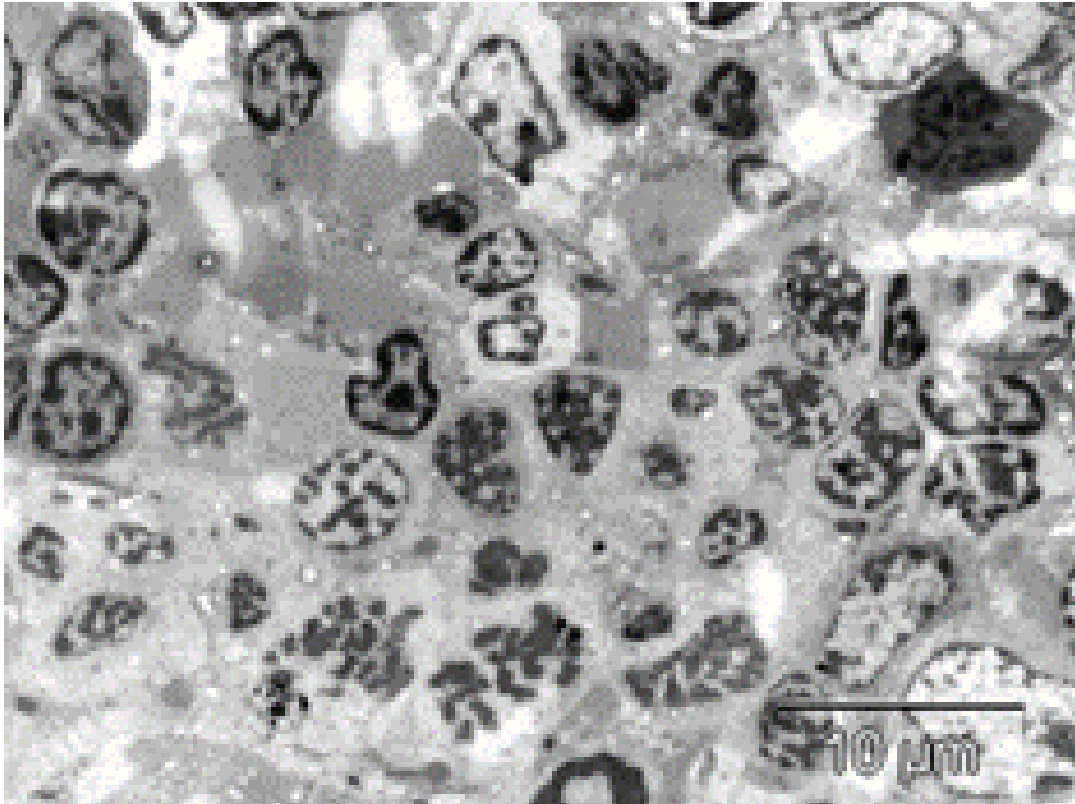


Fig. 5. TiO_2 –Ag variant. The reduction of the mitotic division rate.

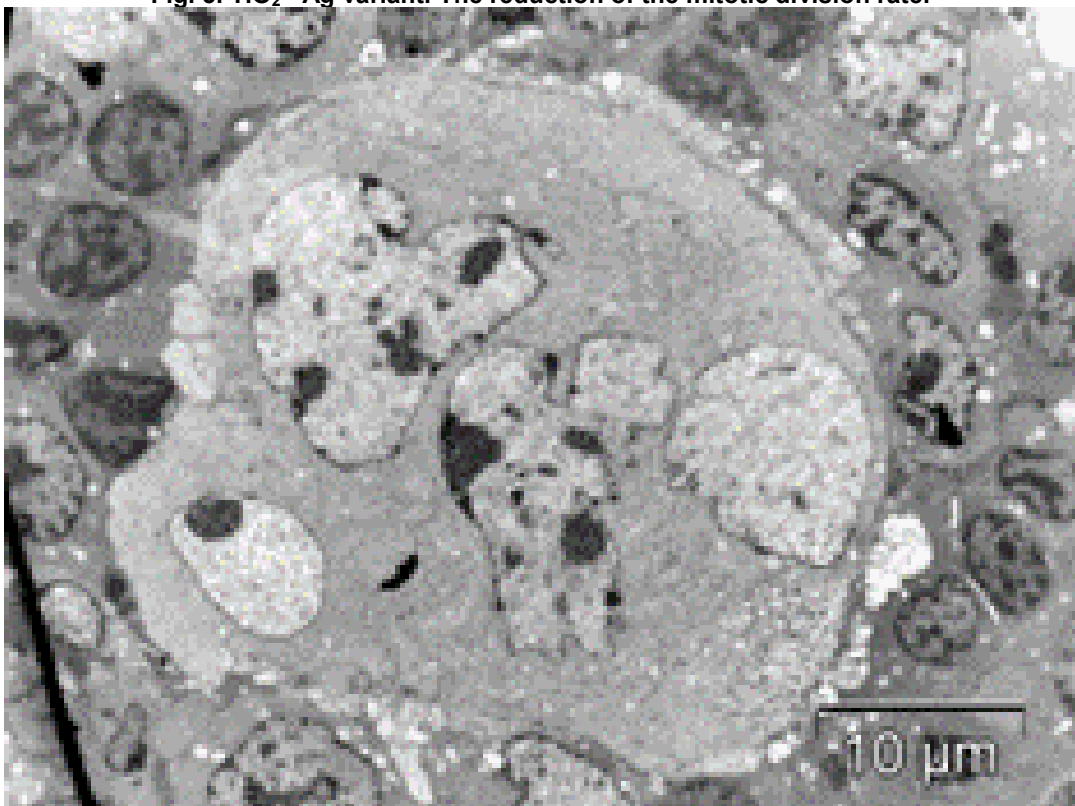


Fig. 6. TiO_2 – Ag – X-rays variant. Megakaryocytes with a normal structure and thrombocyte deliver.

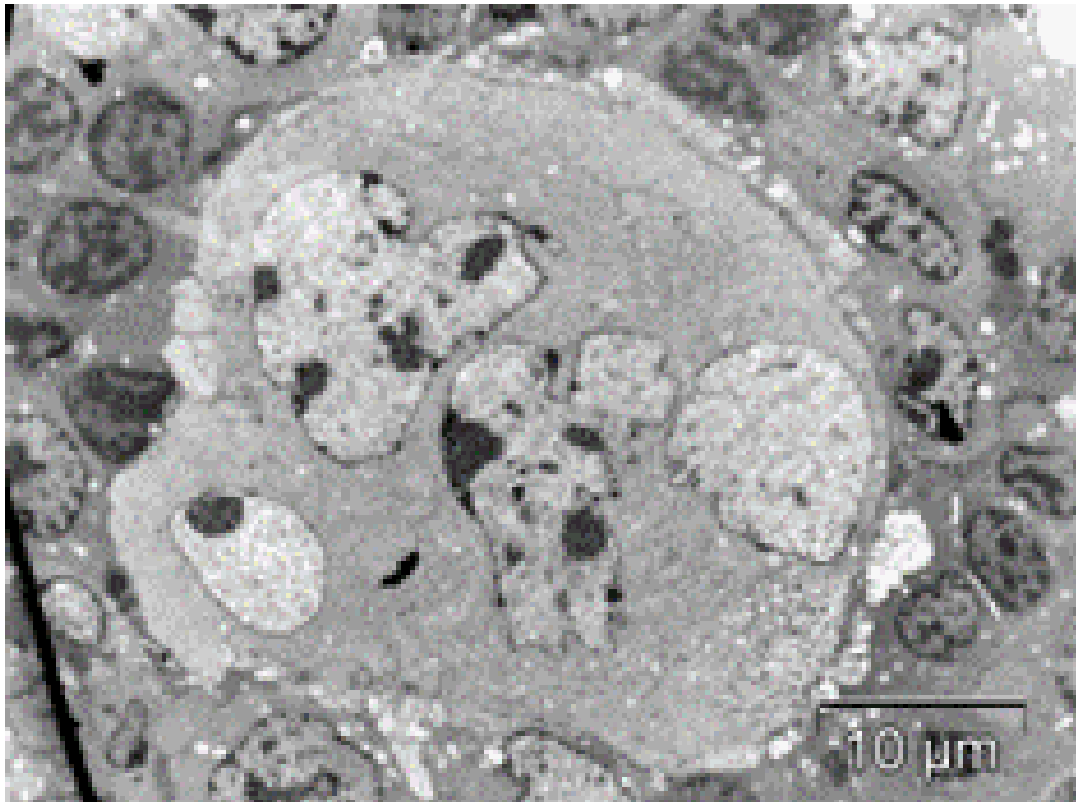


Fig. 7. TiO_2 – Ag, liposome variant. Aspects similar with an inflammatory process.

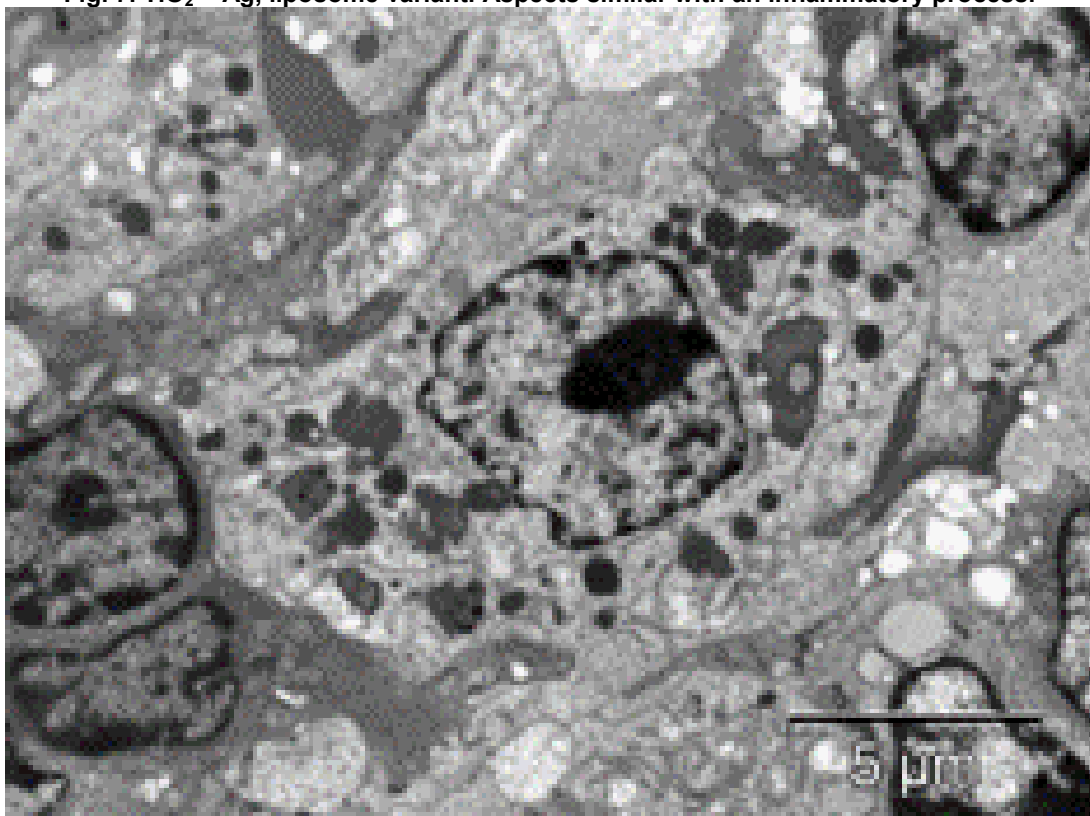


Fig. 8. TiO_2 – Ag, liposome –X-rays variant. Aspects of a strong radioprotective effect.