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In vivo toxicity of the ribosome-inactivating lectin ebulin f in elderly mice

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Summary. Ebulin f is a ribosome-inactivating protein (RIP) present in green fruits of the dwarf elder (*Sambucus ebulus* L). Since dwarf elder fruits are used for food and as a medicine, we assessed the study of toxicological effects and safety of ebulin f in elderly mice, comparing these results with those reported in young animals and with other RIPs. Female Swiss mice aged 6 and 12 months of age were intraperitoneally injected with a single dose from 1.4 to 4.5 mg/kg ebulin f. Heart, stomach, intestines, lung, kidney, liver, spleen, pancreas, adrenal gland, uterus, ovary and brain were studied. Histology analysis was carried out by staining with hematoxylin and eosin and Masson's trichrome observed with a light microscope, or apoptosis detection by TUNEL method observed with a confocal laser microscope. Treated animals injected with the lower dose could recover their weights, but after 14 days half of them died. The higher dose caused a progressive loss of body weight leading to death. In the animals of the experimental groups it was found atrophy of Lieberkühn's crypts, pneumonia, nephronal degeneration, myocardial atrophy, centrolobular hepatic necrosis, splenic white pulp necrosis foci and increased rate of apoptosis in the intestines and liver, in which apoptoses were mainly located in the vicinity of the lobular central vein. We conclude that ebulin f affects vital organs in elderly mice.

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Introduction

Ribosome inactivating proteins (RIPs) are a group of proteins causing inactivation of eukaryotic ribosome (Barbieri et al., 1993) and classified in types 1, 2 and 3, the latter being a special group containing very few examples (Fabbrini et al., 2017). Type-1 RIPs is constituted by a single polypeptidic chain and those of type 2 are mainly A-B dimeric proteins whose A-chain is catalytic, equivalent to the single chain in type-1 RIPs, while the B-chain shows lectin properties, because of which these proteins are also known as ribosome inactivating lectins (RILs). Both chains are bound by a disulfide bond. RIL enzymatic activity causes hydrolysis of the N-glycosidase bond linking adenine 4326 to the ribosome-phosphate backbone of the 28 rRNA of the large ribosome subunit (Endo and Tsurugi, 1987; Girbés et al., 2004; Stirpe, 2004; May et al., 2013). Elderberry RIPs have also been reported to act on DNA and polynucleotides (Barbieri et al., 2004; Iglesias et al., 2010). The lectin activity present in the B-chain allows them to get into the endosome compartment by binding to specific sugars of receptors in the cell surface (Stirpe, 2004). Most RIPs are of plant origin, although some have been extracted from bacteria and algae (Stirpe, 2004). In plants, RIPs have been proposed to play a protective role against insects, viruses and fungi (Peumans and Van Damme, 1995; Vandenbussche et al., 2004). During the ripening process, fruits show a

decrease in RIPs until disappearing, while a progressive decrease in the leaves has also been found as these become senescent (Barbieri et al., 1993; Stirpe, 2004; Jiménez et al., 2014).

The pioneering works by Peumans and Van Damme's group opened the field of elderberry (Sambucus) lectins (Nsimba-Lubaki et al., 1986; Peumans et al., 1991; Van Damme et al., 1996), and during the last two decades a number of RIPs have been isolated from different parts of these shrubs (Tejero et al., 2015), offering a promising research line for their nutritional, pharmaceutical and medical applications. The first RILs found in the elders, namely ebulin and nigrin b-SNA V were isolated by Girbés et al. in 1993, the former from the dwarf elder (*Sambucus ebulus* L.) (Girbés et al., 1993a) and the latter from the black elder (*Sambucus nigra* L.) (Girbés et al., 1993b). They both are included in the type-2 RIPs like ricin, abrin, volkensin, SEA, viscumin, etc. (Dang and Van Damme, 2015; Tejero et al., 2015). Since then, different isoforms of these RILs have been extracted from the *Sambucus* genus plants (Van Damme et al., 1997; Tejero et al., 2015). Specifically from the dwarf elder, different isoforms of ebulin, which binds to D-galactose, have been described, namely ebulin l (from leaves), ebulin f (from green fruits), ebulin blo (from blossoms), and ebulins r1 and r2 and SEA I (from rhizome). Among them, ebulin f displays the highest activity (IC50 of 0.03M) on protein synthesis in rabbit reticulocyte lysates (Girbés et al., 1993a).

Although much less toxic than other type-2 RILs, such as ricin, abrin and volkensin, ebulin and nigrin have been proved to show certain *in vivo* toxicity (Gayoso et al., 2005; Jiménez et al., 2013a; Garrosa et al., 2015). This point is quite relevant due to the use of many parts of *Sambucus* shrubs as food or medicine, mainly in the Mediterranean region and Middle East. Archaeological remains of seeds and charcoals of *Sambucus* have been recovered from the Neolithic age and very abundantly at certain chalcolithic sites (Kaack and Austed, 1998; Martin et al., 2008; Veberic et al., 2009). Since Hippocrates and Dioscorides it is known their medicinal use. Among the pharmacological effects reported so far, we can mention the improvement of lipid profile (Ivanova et al., 2014), antibiotic (Salehzadeh et al., 2014), anti-ulcerogenic (Yesilada et al., 2014), antiparasitic (Rahimi-Esboei et al., 2013), anti-inflammatory (Schwaiger et al., 2011) and wound-healing (Süntar et al., 2010). The putative responsible compounds for these actions are polyphenols and anthocyanidins (Tejero et al., 2015). Of special interest is cyanidin-3-O-glycoside, which has been reported to cause inhibition of cancer cell proliferation (Ding et al., 2006; Marczylo et al., 2009).

Hence, taking into account the nutritional and medicinal properties of the elders, a detailed study of the characterization and toxicity of their compounds is mandatory. We have reported in previous papers the *in vivo* toxicity of nigrin b (Gayoso et al., 2005) and ebulin in young mice (Jiménez et al., 2013a). Considering the possible differential effect that toxins may exert along the life cycle, we also achieved a preliminary study of ebulin toxic effects on lungs and intestines in elderly mice when administered via i.p. We reported ebulin f to cause pneumonia and promotion of apoptosis both in the small and in the large intestines; the committed precursor cells of the transit amplifying compartment (TAC) in Lieberkühn's crypts being its primary target, leading to the loss of enterocytes and atrophy of the crypts (Garrosa et al., 2015). In the present paper we aim to complete the *in vivo* toxicity study of intraperitoneal administration of ebulin f by analyzing its dose-specific effect throughout the different organs of the body in elderly mice and compare the results with those reported in younger animals and with those obtained with other RILs.

Materials and methods

Materials

The reagents utilized were of the highest purity available, most of them purchased in Sigma-Aldrich (Madrid, Spain). Isoflurane was purchased from Esteve Veterinaria (Barcelona, Spain). The chromatographic supports for protein isolation were purchased from GE Healthcare Europe GmbH (Barcelona, Spain). The affinity chromatographic support was acid-treated Sepharose 6B and was prepared as described elsewhere (Jiménez et al., 2013b). Dwarf elder green fruits were harvested in Barruelo del Valle (Valladolid, Spain) during July and August and stored frozen at -20°C until use.

Isolation of ebulin f

Ebulin f was isolated from green fruits as described elsewhere (Jiménez et al., 2013b) since they contain the largest amount of the toxin (Citores et al., 1998). Briefly, 200 g of frozen fruits collected in July were minced and ground in a mortar to obtain a paste, which was extracted overnight with 800 mL of extraction buffer (280 mM NaCl containing 5 mM sodium phosphate, pH 7.5). The extract was filtered and centrifuged to obtain a clear solution. It was then chromatographed through acid-treated Sepharose 6B (AT-Sepharose 6B) to obtain D-galactose-binding proteins which were released from the resin by 0.2 M lactose buffer. D-galactose binding proteins were separated by chromatography on Superdex 75 (26/60; GE). The purified proteins were dialyzed and concentrated at 2.5-4.0 mg/mL in water. The purity of the ebulin f was assessed by SDS-PAGE and mass spectrometry (Jiménez et al., 2013b).

Animals and experimental groups

Treatments, experiments and euthanizing the mice were conducted according to the European Communities

Council guidelines (Directive 2010/63/EU of the European Parliament and of the Council of 22nd September 2010 on the protection of animals used for scientific purposes) concerning the protection of laboratory animals. A total of 50 female Swiss mice were used: four experimental groups (n=7) of 6-month-old, two experimental groups (n=7) of 12-month-old animals plus two control groups (n=4) of the referred ages. The number of animals per group was reduced to minimize unnecessary killing. Mice obtained from our university facilities were housed individually in plastic cages in a temperature-controlled room, and fed *ad libitum* with free access to water in a 12 h light-dark cycle.

Treatment with ebulin f

Ebulin f dissolved in 0.1 M phosphate buffer saline (PBS) pH 7.4 was administered in a volume of 60 μL adjusted at the desired concentration and injected into the experimental animals. Controls were injected with the same solution but without ebulin f. The doses used for 6-month-old animals were 1.4, 2.1, 2.8 or 4.1 and those for 12-month-old ones were 1.4 and 2.1 mg/kg ebulin f. After death, animals were immediately taken for histological analysis. Two weeks after administration of the toxin, all the surviving animals were anesthetized with isoflurane and then sacrificed by decapitation. The dead animals were then taken into account to construct the corresponding Kaplan-Meyer plots.

Histological analysis

Several organs were removed from the dead animals, and fixed by immersion in 4% PBS-buffered paraformaldehyde. They were: heart, stomach, small and large intestines, lung, kidney, liver, spleen, pancreas, adrenal gland, uterus, ovary and brain. Four animals were used for the histological analysis. Samples of each of these organs were processed for paraffin embedding, and 7 μm-thick slices were prepared by slicing with a Minot microtome and further stained for light microscopy with hematoxylin and eosin, Masson's trichrome or studied for apoptosis detection using the kit-DeadEnd Fluorimetric TUNEL System (Promega, USA). Photomicrographs were taken with a Zeiss digital camera in an Axiophot Zeiss photomicroscope, while for TUNEL slides a Leica SPE-TCP confocal laser microscope was used.

Statistical analysis

Numeric data were statistically analyzed by means of one-way ANOVA and Tukey post hoc test. The significance level was established at $p<0.05$.

Results

Animals injected with 2.1 mg/kg of body weight and lower suffered two reductions in their body weight in both age groups. The first occurred one day after treatment and the second around the sixth day after treatment as previously reported (Garrosa et al., 2015). Mice injected with 1.4 mg/kg were able to recover their weights, but after 14 days half of them died. Doses higher than 2.1 mg/kg caused a progressive loss of body weight and led to death the sooner the higher dose utilized. The days before death, on the contrary to control animals, the experimental ones remained quiet in a corner of the cage, not showing diarrhea or abnormal aspect of ears and extremities.

Both in control and experimental animals, regardless of the dose, venous congestion was a common finding in many organs of the body, such as lungs, kidneys, liver, spleen, pancreas, ovaries and adrenal glands. As well, it was a common finding, both in control and experimental animals, the scarce number of ovary follicles and the presence of large amounts of lipofuscine granules, especially in the spleen (Fig. 1A) and adrenal medulla. In the white pulp of the spleen of animals treated with 2.8 mg/Kg and higher, numerous small foci of necrosis could be observed (Fig. 1B).

Atrophy and degeneration of some cardiac muscle fibers appeared in experimental animals, extensive loss of cardiomyocytes being found with the highest dose utilized (Fig. 2). This loss accounted for macroscopic evidence of heart atrophy in these animals, whose heart weight loss was evaluated at 25% ($P<0.05$).

Experimental animals injected with 2.1 mg/kg doses and higher showed scattered tubular degenerations and nephrocyte necrosis (Fig. 1D). In addition, atrophy of renal glomeruli could be seen in experimental animals of any group as well as in 12-month-old control animals, but not in 6-month-old control animals.

Centrolobular necrosis could be seen in the liver of animals treated with 2.8 mg/Kg and higher (Fig. 1F). In addition, an increased number of apoptotic cells was observed in the liver of the animals injected with 2.1 mg/kg and higher (Fig. 3). These apoptoses were mainly located in the vicinity of the central vein of the classical lobule and were more frequently encountered as the dose injected was higher (Fig. 4).

Discussion

Atrophy of Lieberkühn's crypts, loss of enterocytes and increased number of apoptotic cells have also been described to occur in young animals (Jiménez et al., 2013a), but using doses higher than 2.1 mg/kg. Using this 2.1 mg/kg body weight dose, however, we have found damage in elder animals in our experiment. This fact could be explained by a higher sensitivity to the toxin in these elder mice, in which their resistance to hazards is reduced. The increased rate of apoptosis found in the intestinal epithelium affecting the TAC suggest a specific action of ebulin f from the serous side of the intestinal epithelium breaking the restricted microenvironment supplied in their niche located above

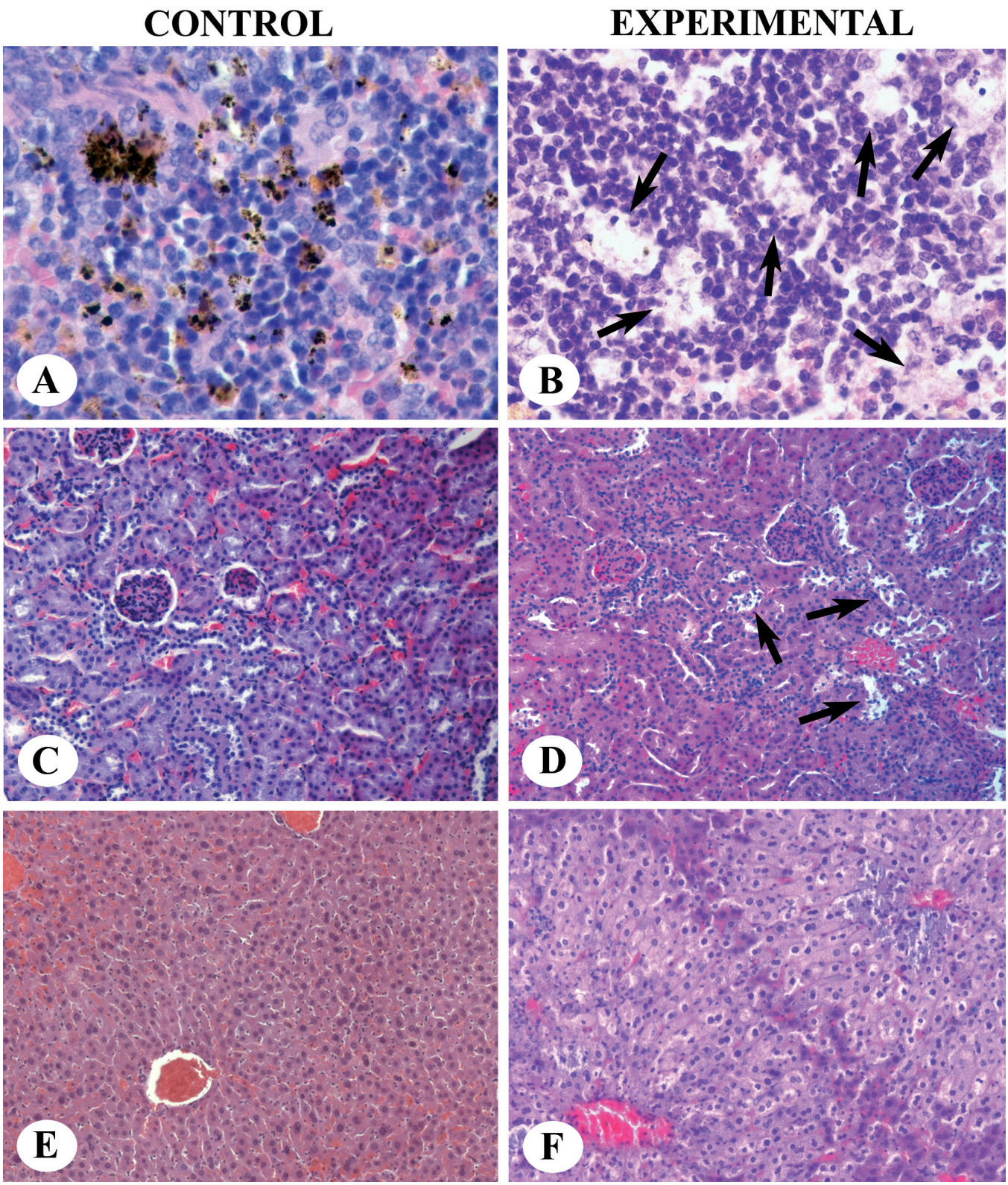

Fig. 1. A. Normal spleen of a 12-month-old control mouse showing numerous lipofuscin granules. **B.** Abundant small necrotic foci in the white pulp (arrows) of a 6-month-old mouse injected with 2.8 mg/Kg ebulin f. **C.** Renal cortex of a 6-month-old control mouse. **D.** Nephronal necrosis (arrows) in a 6-month-old mouse treated with 2.1 mg/Kg ebulin f. **E.** 6 month-old control murine liver. **F.** Two hepatic classical lobules showing centrolobular necrosis in a 6-month-old mouse injected with 2.8 mg/Kg ebulin f. A longitudinal band corresponding to the center of zone 1 of the hepatic acinus appears well preserved, in which hepatocytes are best supplied by the terminal branches of the hepatic artery and porta vein. Hematoxylin and eosin. A, B, x 340; C-F, x 110.

Paneth cells in the crypt (Díaz-Flores et al., 2006), and subsequently leading to the derangement of the villi.

The lower resistance to hazards present in more aged animals may also account for the pneumonia caused by ebulin in elder mice, since younger mice did not show pneumonic foci even with higher doses such as 5 mg/kg (Jiménez et al., 2013a). A discussion of this fact can be found in our previous article, where we reported the presence of pneumonia to be independent of the dose of ebulin (Garrosa et al., 2015).

Regarding spleen, Battelli et al. (1990) reported a mild affection of the red pulp which varied from focal to extensive necrosis after i.p. injection of the type-1 RIPs saporin 6 or gelanin. Except for congestion, we have not found changes in the red pulp; however, small foci of necrosis were found in the white pulp, our findings being more consistent with those reported by Stirpe et al. (1987) after saporin treatment.

Atrophy of some renal glomeruli and degeneration of proximal convoluted tubules were observed to occur both in control and experimental animals of 12 months of age, but not in 6-month-old mice, therefore these changes are attributable mostly to advanced age. Nevertheless, doses of ebulin 2.8 mg/kg and higher caused scattered necrosis of nephrons both in 6- and 12-month-old animals. The nephrotoxic effects are feasible since ebulin has low enough molecular size to be filtered through the renal glomerulus and presumably reabsorbed by the cells of the proximal convoluted tubules leading to the necrosis of nephrocytes. Necrosis of convoluted tubules have also been described to occur after administration of lethal doses of other RIPs (Stirpe et al., 1987; Battelli et al., 1990).

Hepatic damage is a common feature provoked by multiple toxins, some of them –like paracetamol and carbon tethrachloride- causing centrolobular necrosis. Ebulin might also cause direct damage since ebulin uptake by the hepatocytes is favoured by the receptors for galactosyl-terminated glycoproteins present in the hepatocyte surface (Ashwell and Harford, 1982). Nevertheless, the pattern of the lesions observed in the liver showing centrolobular necrosis, taken together with the cardiomyocyte degenerations, suggest that a circulatory disorder is also a factor to account for hepatotoxicity pathogenesis. The myocardial atrophy subsequent to the cardiomyocyte loss would cause heart failure leading to cell death closer to the central vein, while hepatocytes in the proximity to blood supply coming from both the hepatic artery and porta vein, that is zone 1 of the hepatic acinus, would resist. The increased number of apoptotic cells observed in the liver after ebulin f treatment is similar to that occurred in the intestines and the fact that these apoptoses were more frequently encountered in acinar zone 3, that is closer to the lobular central vein, supports this hypothetic influence of the blood supply. Battelli et al. (1990, 1996) have also found hepatic damage with other RILs, but without reporting a centrolobular pattern; however, Stirpe et al. (1987) did report an atrophy of hepatocytes surrounding the central vein after i.v. administration of the type-1 RIP saporin to mice.

The increased rate of apoptoses observed both in intestines and liver support an apoptosis promoting action of ebulin as a cellular mechanism to explain its toxicity together with the polynucleotide action and the arrest of protein synthesis, all the more considering that other related RILs like nigrin require higher concentration to inhibit translation than that causing

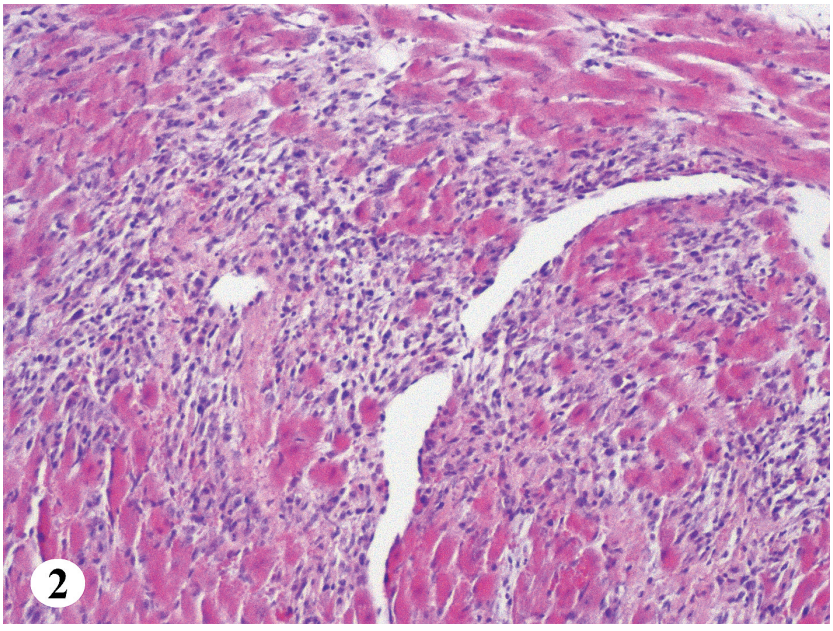

Fig. 2. Histological section of the myocardium of a 6-month-old mouse injected with 4.1 mg/Kg ebulin f showing ample area of cardiomyocyte degeneration. Hematoxylin and eosin. x 200.

death (Benítez et al., 2005). In addition, it has recently been reported that type-2-RIP cytotoxicity is mediated by the unfolded protein activated in response to endoplasmic reticulum stress (Horrix et al., 2011), and that the lectin domain might induce autophagy (Shang et al., 2015).

Ricin, a type-2 RIP, is considered to be one thousand times more cytotoxic than ebulin, causing much more severe damage in liver and spleen. Congestion has also been reported to occur after ricin treatment (Strocchi et al., 2005); however, liver damage caused by ricin seems to be different since ricin primarily affects sinusoidal cells bringing about the formation of trombi (Derenzini et al., 1976), a fact not observed in our study. The much higher toxicity of ricin is based in the great capacity of its B-chain to bind to the polysaccharide of the glycocalix (Pascal et al., 2001; Lord and Spooner, 2011) and after entering the cytoplasm, it seems to follow a retrograde transport from endosomes to the trans-Golgi network and final transfer to the rough endoplasmic

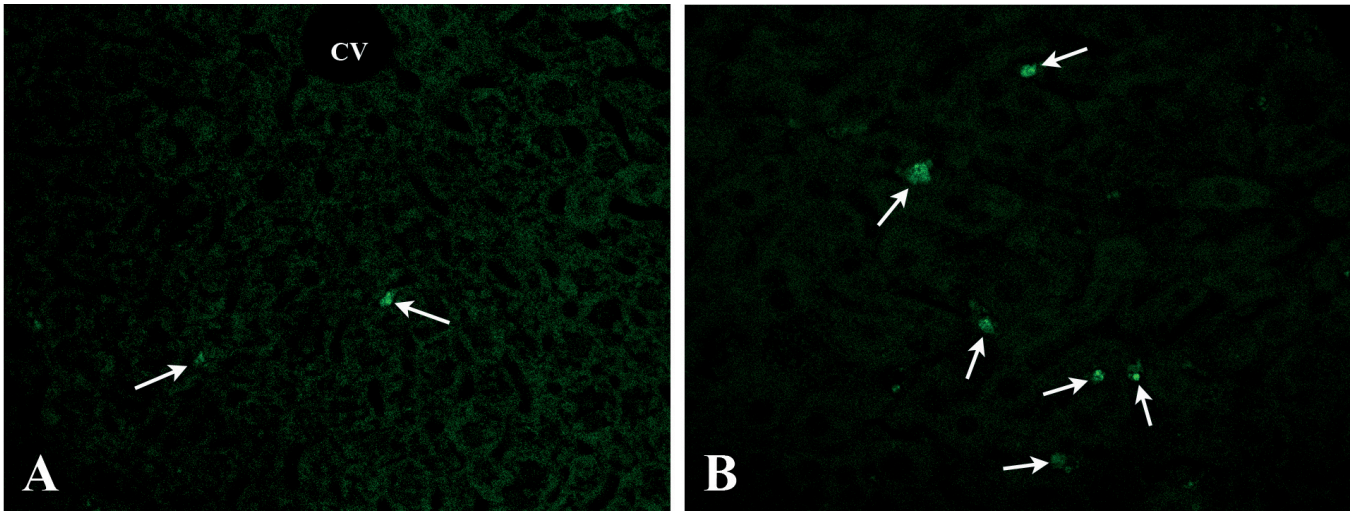

Fig. 3. Confocal microscope images of the livers of 12-month-old mice showing apoptoses (arrows). A higher rate of apoptoses can be observed in an experimental animal treated with 2.1 mg/Kg ebulin f **(B)** with regard to the control **(A)**. CV, Central vein. TUNEL method. x 300.

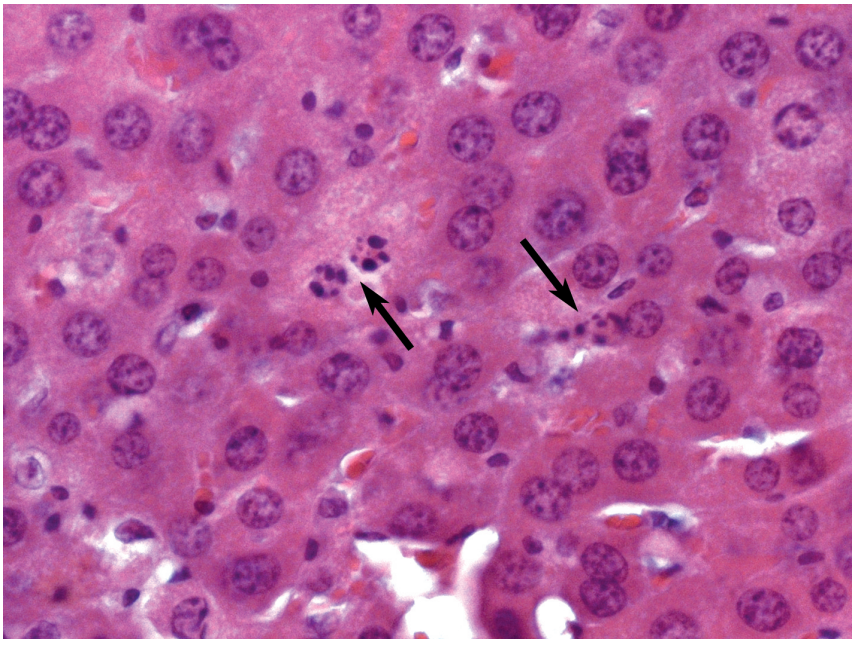

Fig. 4. Cariorrexis (arrows) showing apoptotic bodies close to the central vein in the liver of a 12-month-old mouse injected with 2.1 mg/Kg ebulin f. Hematoxylin and eosin. x 580.

reticulum, whereas ebulin goes from endosomes to phagolysosomes, where it is degraded into inactive products (Battelli et al., 2004).

In conclusion, our findings offer data to consider ebulin f as a toxin at doses of 1.4 mg/kg i.p. causing damage not only in the small and large intestines, as it was reported in young animals, but also in other organs such as heart, liver, lungs, kidneys and spleen. These organic changes will give rise to alterations in the circulatory system and of the absorptive functions, degradation and excretion of nutrients, and the damage of the mentioned vital organs would cause such an impairment of vital functions that leads to death. Hence, an important concern raises up due to the nutritional and potential medicinal uses of dwarf elder fruits, and since ebulin f appears especially harmful for elder animals, we could say that "the elders must mind the elders". In this regard, Jiménez et al. (2014) have reported that boiling the aqueous extracts of the plant notably increased the pepsin sensitivity of ebulin f to a safe level. Anyhow, the toxicity level of ebulin f, as is the case of nigrin b-SNA V, being much lower than that of the so-called toxic type-2 RIPs, render them suitable for the construction of immunotoxins and conjugates for targeted therapy (Tejero et al., 2015).

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