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Paneth cells are also target of the ribotoxic lectin nigrin b

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Summary. Ribosome-inactivating lectins (RILs) are A-B type toxins like ricin whose molecular target is the large rRNA of eukaryotic ribosome. Administration of lethal doses of the RIL nigrin b isolated from elderberry (*Sambucus nigra* L.) bark triggers specific intestinal derangement. The aim of the present research was to explore the early effects of a lethal dose of nigrin b (16 mg/kg body weight) on the small intestine using light and electron microscopy to ascertain intestinal epithelium changes. 6 h after nigrin administration, the small intestine crypts began to show signs of damage with cells appearing at different stages of apoptosis. 16 h after injection crypts appeared more impaired, including the derangement of Paneth cells. The novelty of our results is that the Paneth cells in the small intestine in addition to stem cells are the early cellular targets for nigrin b.

Key words: Paneth cells, Nigrin b, Lectin, RIP, RIL

Introduction

Plant ribosome-inactivating proteins (RIPs) are common enzymes that act on ribosomes and promote translation arrest (Stirpe, 2004; Girbés et al., 2004). They are considered part of the plant defense system against insects (Peumans and Van Damme, 1995) and may be induced in plants to counteract viruses (Girbés et al., 1996) and fungi attacks (Corrado et al., 2005). RIPs are classified as type I (A chain), and type II (A-B chains). The A chain is responsible for N-glycosidase activity on the A_{4234} present in a highly conserved active site (Chambery et al., 2007), which leads to the arrest of protein synthesis. The B chain displays lectin activity (Stirpe, 2004), which allows for the union of the type II RIPs to cell membrane, making possible their internalization by endocytosis. Interest in RIPs is mainly due to their toxicity, for instance ricin and related highly toxic proteins (Lord et al., 1994; Lord and Spooner, 2011), and also to their use in drug targeting for anti-cancer immunotoxins and conjugate preparation with anti-tumour activity (Ferreras et al., 2011a; Muñoz et al., 2012). Among type 2 RIPs or ribotoxin with lectin activity (henceforth, ribosome-inactivating lectins or RILs), the ribotoxin nigrin b was isolated from *Sambucus nigra* L. (Girbés et al., 1993) and found to internalize following an endosomal pathway (Battelli et al., 2004). Administration of nigrin b to mice promoted dose-dependent severe intestinal derangement that at concentrations higher than 10 mg/kg body weight (i.v.) could lead to death (Battelli et al., 1997). At lower doses, 5 mg/kg body weight (i.v.), nigrin b triggers a reversible effect on crypts (Gayoso et al., 2005). This should be taken into account for its potential use in pharmacokinetic characterization of drugs and nutrient

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Abbreviations: Electron microscopy (E.M.), Phosphate buffer (BP), Ribosome-inactivating lectin (RIL), Ribosome-inactivating protein (RIP), Sodium dodecyl sulphate (SDS), Terminal deoxyribonucleotidyl transferase-mediated d-UTP-nick-end labelling (TUNEL).

absorption studies. Light microscopy analysis of the effects of intravenous administration of nigrin b to mice revealed serious and specific dose-dependent intestine derangement. In a previous light microscopy histological analysis, we reported (Gayoso et al., 2005) that mice treated with 16 mg/kg body weight nigrin b (i.v.) revealed severe alteration of small intestine stem cells and transit-amplifying compartment (TAC) present in the crypts, displaying apoptotic-like morphology, including cell shrinkage and increased cytoplasmic eosinophilia (Gayoso et al., 2005; Ferreras et al., 2011b). However, Paneth cells, which are located underneath TAC in the crypts (Díaz-Flores et al., 2006; Clevers and Bevins, 2013), seemed to be spared.

The aim of the present work was to ascertain by light and electron microscopy (E.M.) analysis whether in addition to transit-amplifying compartment cells, Paneth cells are also affected by a lethal intravenous dose of nigrin b at early times after administration.

Materials and methods

Nigrin b preparation and administration

Highly purified nigrin b was prepared from elderberry bark by the affinity chromatography procedure to ensure the maximal activity as described elsewhere (Girbés et al., 1993; Jiménez et al., 2013a). Purity was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Nigrin b was administered as 0.1 mL of a solution containing the indicated amount of nigrin b in 0.1 M phosphate-buffered saline, pH 7.4.

Subjects

Female Swiss mice were housed individually in plastic cages and fed *ad libitum* with free access to water under a 12 h light-dark cycle. Management of the animals followed European Communities Council guidelines (2010/63/EU) for laboratory animal care and experimentation.

Survival study

For the survival evaluation, Kaplan-Meier plots were obtained from three different groups of animals treated with 16 mg/kg body weight in three different ways, namely intravenous (n=7), intraperitoneal (n=14) and by oral gavage (n=7).

Light and electron microscopy analysis

For histological analysis, eight animals were treated with 16 mg/kg body weight intravenously, another eight were injected the same dose intraperitoneally, and another eight the same dose by oral gavage. Finally, a control group of four animals was injected with saline solution via i.v. and sacrificed 16 h later.

After being anesthetized with a mixture of ketamine (10 mg/kg) and xylacine (80 mg/kg), animals were intracardially perfused with 1% paraformaldehyde (Sigma-Aldrich Química S.A., Spain) plus 1% glutaraldehyde (TAAB, Aldermaston, UK) in 0.1 M phosphate buffer (PB) pH 7.4. Half of the animals in each group were sacrificed at 6 h after treatment and the other half at 16 h after treatment. Samples of duodenum were taken from 1 cm away of pylorus, left overnight in the same fixative, washed in PB, and post-fixed in 1% OsO_4 (Sigma-Aldrich Química S.A., Spain), in PB. After gradual dehydration in ethanol series, pieces were embedded in low-viscosity epoxy resin (Spurr, 1969) (TAAB, Aldermaston, UK). Sections of 1 μm (semi-thin) were stained with toluidine blue in 1% sodium tetraborate, studied under light microscopy and selected zones were analysed under electron microscopy. Semi-thin and ultra-thin (30 nm; silver reflected colour) sections were obtained in an ultramicrotome (LKB 8800 Ultratome IV, Bromma, Stockholm, Sweden) and after staining with lead citrate (Reynolds, 1963) were examined in a JEM 10/11 HR transmission electron microscope. One additional sample od duodenum of each group was paraffin embedded and studied for apoptosis detection by kit-DeadEnd Fluorimetric TUNEL System (Promega, USA).

Results

Intravenous administration of 16 mg/kg body-weight of nigrin b killed all animals within 2 days. By contrast, i.p. administration of the same dose of nigrin b killed all the animals within 10 days and the oral administration did not affect the mice at all (Fig. 1).

Lesions caused by intraperitoneal administration

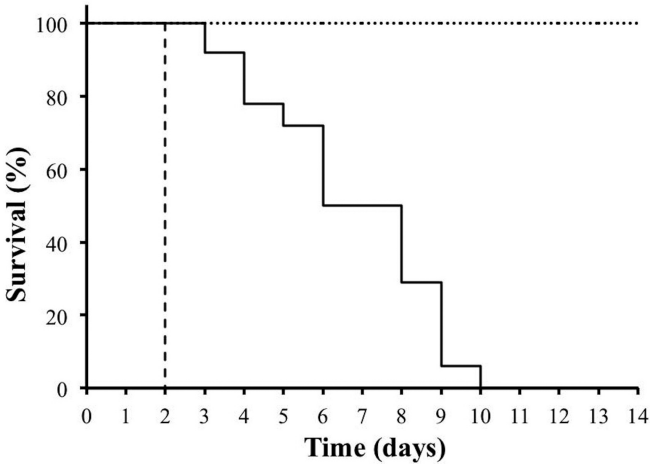

Fig. 1. Kaplan–Meier plots of the dose-dependent toxicity of nigrin b to mice. Groups of female mice were treated with 16 mg/kg body weight either by intravenous way (dashed line; n=7), intraperitoneal way (solid line; n=14) or by oral gavage (dotted line; n=7). Survival was assessed at the indicated times.

were similar to those found with intravenous injection, but to a lesser degree, therefore we will present findings from i.v. administration only. Control animals showed normal histological structure of intestinal villi and crypts (Fig. 2A,B). As early as 6 h after the administration of nigrin b (i.v.) the general structure of the small intestine was mostly preserved (Fig. 2C). The villi appeared well structured showing hyper-cellularity in the core, with vessels filled with red blood cells most probably due to vascular leaking of the vein endothelial cells. The crypts appeared swollen with a large number of cells, most of them showing signs of apoptosis characterised by

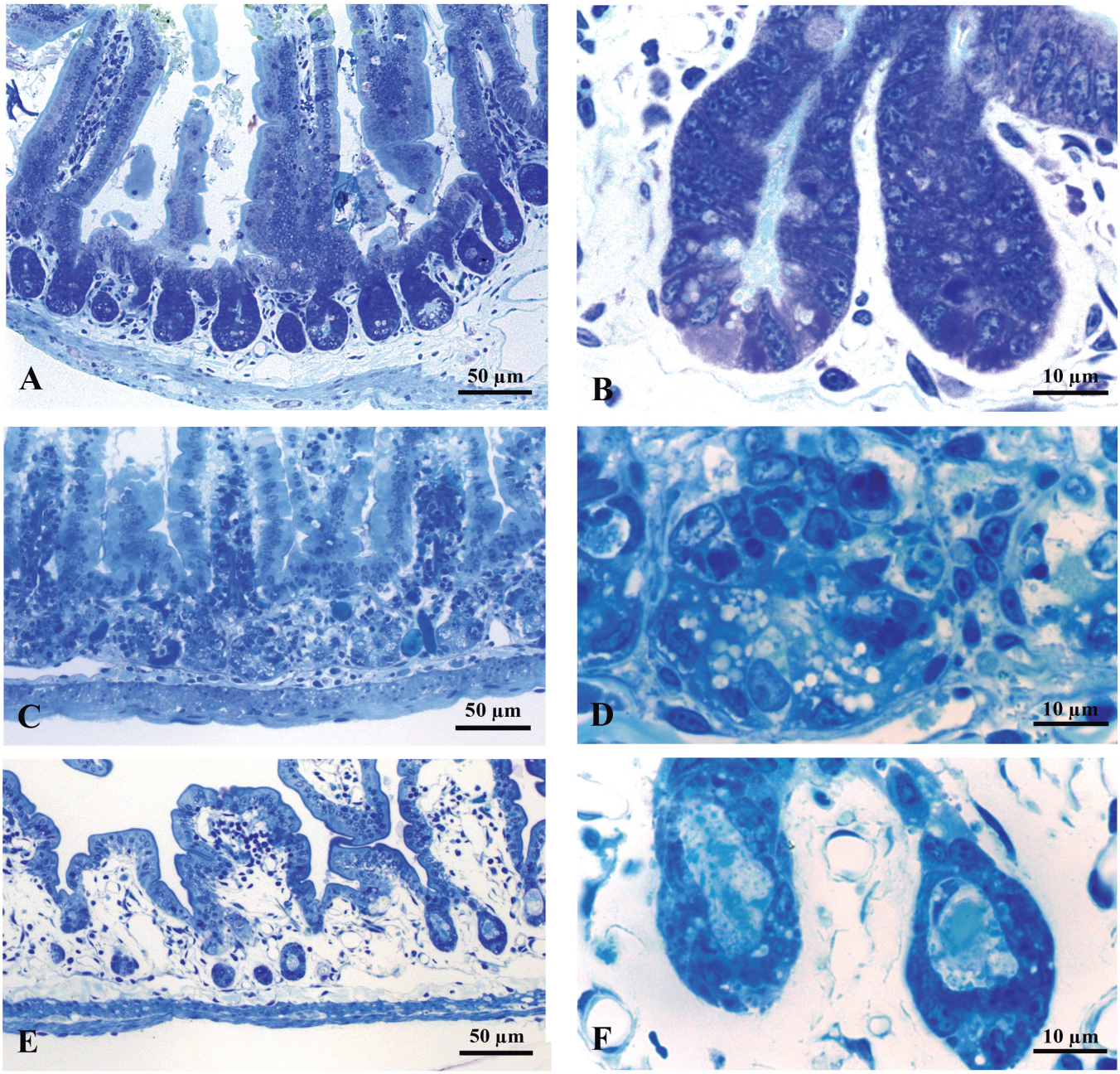

Fig. 2. Small intestine mucosa of mice after intravenous injection of saline solution **(A and B)**, and 16 mg/kg nigrin b **(C-F)**. Control animals show normal histological structure **(A and B)**. After 6 h of the nigrin b administration, the structure of the small intestine mucosa is largely preserved **(C),** although the crypts appear thickened **(D)**. After 16 h of the nigrin b administration, the intestinal mucosa appears atrophic **(E)**, the crypts having lost a large number of cells, including Paneth cells, which appear at different stages of apoptosis **(F)**. Semithin sections stained with toluidine blue.

condensation of chromatin to form peripheral masses leading to nuclear destruction by formation of apoptotic bodies and positivity to TUNEL method (Fig. 3A,B). Paneth cells were also evident at the bottom of the crypts, appearing more resistant, but some also showed different stages of apoptotic death (Fig. 2D).

16 h after administration of 16 mg/kg body-weight of nigrin b (i.v.) cell destruction was more severe than at 6 h. Under these conditions, the villi lost their characteristic organization appearing atrophic and remained as masses of loose connective tissue surrounded by intestinal epithelium devoid of goblet cells (Fig. 2E). The crypts lost most of their cells and Paneth cells were still present, although they finally underwent apoptosis (Figs. 2F, 3C,D).

E.M. analysis revealed swelling of the rough and smooth endoplasmic reticulum and even destruction of the cell membrane in both enterocytes and Paneth cells at 6 h after treatment (Fig. 4A,B). Dilation of rough endoplasmic reticulum in Paneth cells included a large number of vesicles that increased in size as the cells degenerated (Fig. 4A). The nuclei showed a number of alterations such as chromatin aggregation and different stages of apoptosis (Fig. 4B). 16 h after nigrin b administration the enterocytes preserved the microvilli structure and the characteristic junction complexes as well as their abundant glycocalix (Fig. 4C). Paneth cell nucleus clearly evidenced several breaks in their envelope that appeared together with the condensation process of their chromatin (Fig. 4D).

Discussion

Our data reveal that in contrast with the high toxicity displayed via i.p., and especially via i.v., nigrin b administration by oral gavage did not show any

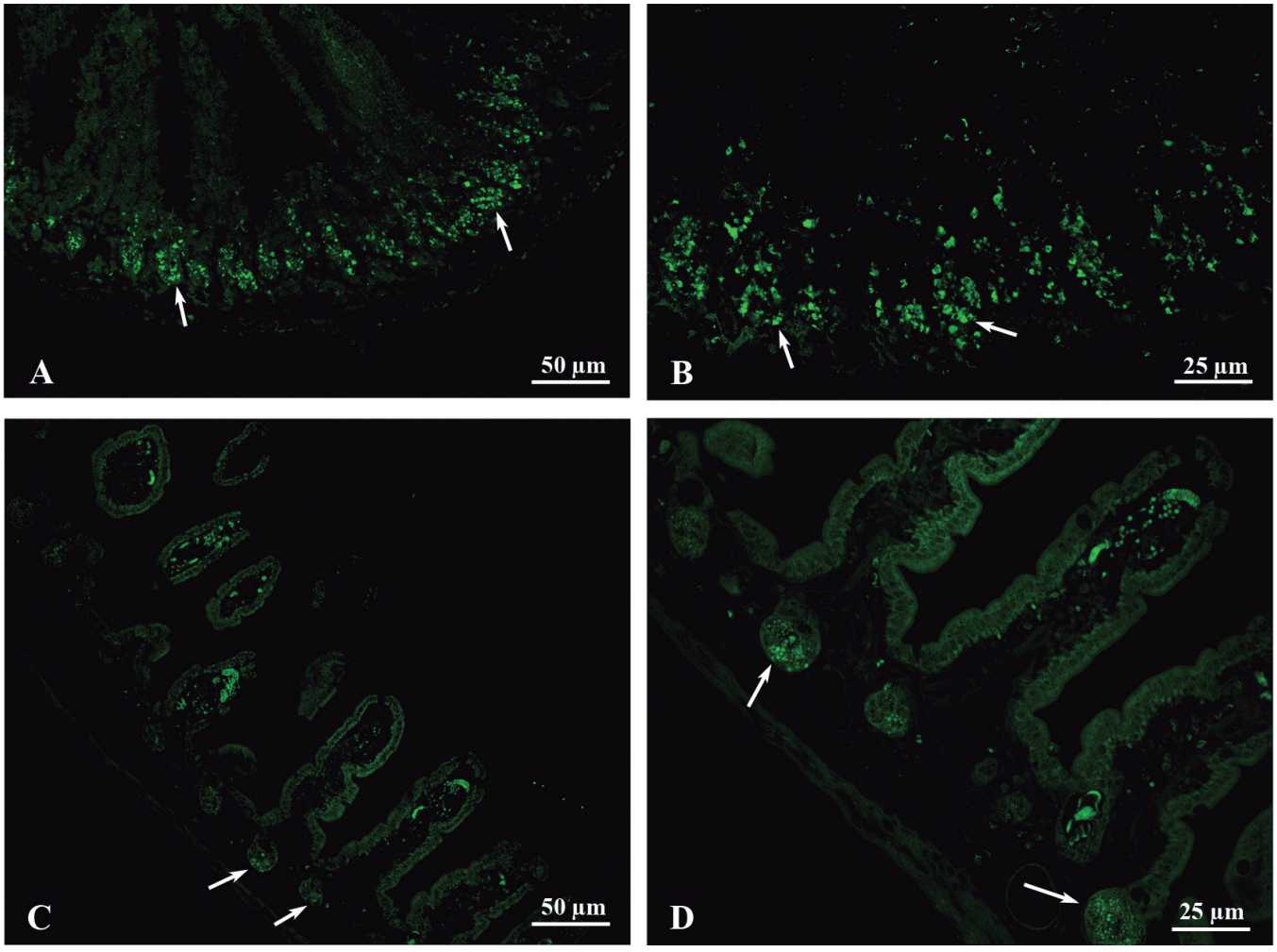

Fig. 3. TUNEL staining in small intestine mucosa of mice after administration of an intravenous injection of 16 mg/kg nigrin b. Apoptotic cells (arrows) are present in Lieberkühn's crypts at 6 h **(A and B)** and 16 h **(C and D)** after treatment.

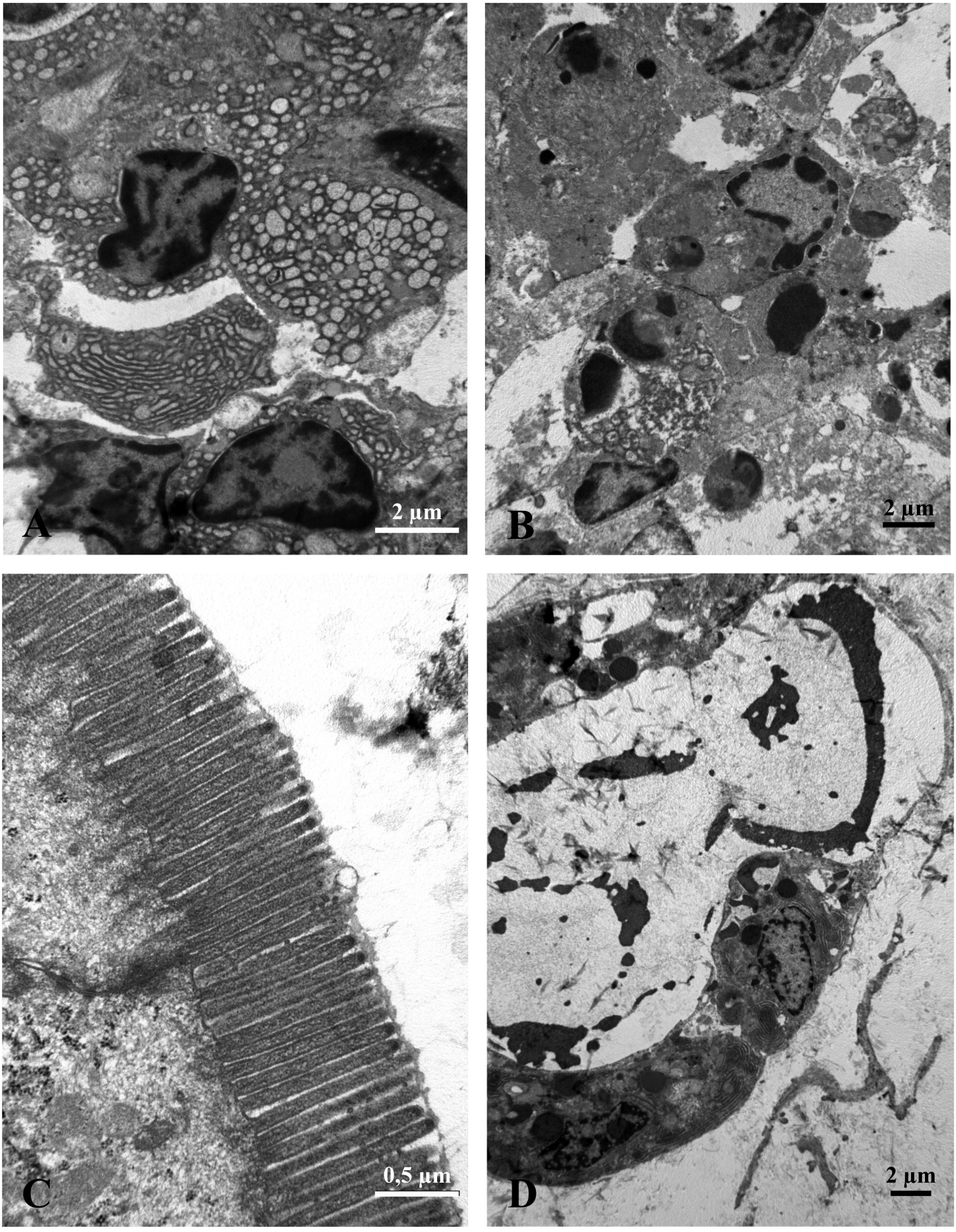

Fig. 4. Electron micrographs of small intestine mucosa of mice after administration of an intravenous injection of 16 mg/kg nigrin b. 6 h after nigrin b administration the affected Paneth cells in the small intestine show abundant, fragmented and dilated cisternae of rough endoplasmic reticulum as well as peripheral nuclear chromatin condensation **(A and B).** After 16 h of the nigrin b administration, enterocyte microvilli were well preserved **(C)**, whereas Paneth cells showed evident signs of apoptotic death **(D)**.

intestinal damage at the utilized dose.

Sub-lethal concentration effects of nigrin b are reversible, meaning that the intestinal stem cell niche was not totally eliminated, thus allowing the rapid cycling of the stem cells to support crypt regeneration through functional crosstalk with other cell types such as Paneth cells, pericryptal myofibroblasts, and epithelial cells (Sato et al., 2011; Yeung et al., 2011). That transient effect of nigrin b observed with sub-lethal concentration is reflected in the ability of the small intestine mucosa to take up nutrients. So, sub-lethal nigrin b administration promoted an important transient and reversible reduction of plasma vitamin B6 from a single oral dose (Jiménez et al., 2013b). At a lethal concentration, such as the one tested in this report, the stem cells appear completely destroyed and epithelial continuity disappears, which further leads to animals death. Along with these transit-amplifying progenitors, Paneth cells also appear affected by these lethal concentrations of nigrin b. The administration of such concentrations results in the loss of the epithelium of the small intestine due to the absence of physiological cell replacement in the crypt-villus growth axis.

Mutations of a stem cell, a progenitor or a differentiated cell of the normal epithelium may originate intestinal tumours, which grow, leading to the formation of an adenoma. Thereafter, one or more cells of the adenoma may suffer a further malignant transformation which ultimately results in an intestinal cancer tumour mass (Barker et al., 2009). Since the effects of sub-lethal nigrin b are exerted on the transit-amplifying compartment, treatment of intestinal tumour-bearing mice with that lectin would prove helpful towards studying the development and growth of intestinal tumours. In fact, it has been reported that nigrin b also promotes apoptosis of small intestine crypt cells in intestinal tumour producer APC/Min+ mice (Ferreras et al., 2011a).

As regards the mechanism of action, the effects of nigrin b do not seem to be those expected merely from an arrest of protein synthesis triggered by a typical translation inhibitor since the concentration of nigrin b required to inhibit translation is higher than is required to affect cell viability (Muñoz et al., 2007). This, together with the early effects exerted by nigrin b on mice seen in the present experiments, supports the notion that the effects of nigrin b might be pleiotropic and probably comprise translation arrest along with the promotion of other different mechanisms that trigger apoptosis. In this line, another ribotoxin like the highly toxic ricin was shown to induce release of macrophage-derived cytokines that might play an important role in ricin pathogenesis (Licastro et al., 1993). Additionally, it has been recently reported that type 2 RIPs cytotoxicity is mediated by the three signalling cascades encompassed with the term unfolded protein response, a mechanism activated in response to endoplasmic reticulum stress (Horrix et al., 2011), which has been recently associated, in Paneth cells, with inflammatory bowel diseases (Adolph et al., 2013).

In conclusion, the present study mainly indicates that intravenous administration of a lethal dose of the ribotoxin lectin nigrin b to mice starts to exert specific toxic effects on the small intestine at least as early as 6 h after the administration. Nigrin b acts by promoting apoptosis of all cell types in the small intestine crypts. A research line is open in which further work may address the potential usefulness of sub-lethal nigrin b to trigger the ablation of transformed cells present in the intestinal crypts that lead to the development of intestinal cancer.

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