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Biochemical and morphological changes in mouse liver induced by mistletoe toxins

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ABSTRACT

Natural compounds are often characterized by high biological activity and sometimes toxicity. This also applies to compounds contained in the herb mistletoe.

The objective of this study was to investigate short-term effects (up to 48 h) of mistletoe toxins on mouse hepatocytes. Standardized mistletoe extract Iscador P was given to female mice as a single injection (0.1 mg/kg b.w., 1 mg/kg b.w., or 2 mg/kg b.w). Activities of lysosomal hydrolases: acid phosphatase, cathepsins D and L, N-acetyl-β-D-hexosaminidase, β-D-glucuronidase, β-D-glucosidase and cytosolic proteases: arginine and leucine aminopeptidases were analyzed in the liver fractions 24 and 48 h after the injection. The morphology of hepatocytes was examined by light and transmission electron microscopy.

Iscador P caused a decrease in the activity of all lysosomal hydrolases (except cathepsins) in the lysosomal pellet, and an increase in the activity of both aminopeptidases and β-D-glucuronidase in the cytosol. However, despite membranotropic properties of the viscotoxins, we did not find a significant labilising effect on the lysosomal membranes. Only β-D-glucuronidase activity was relocated to the supernatant of lysosomal fraction. Microscopic examinations revealed that hepatocyte mitochondria were enlarged and increased in number, whereas the surface of the rough endoplasmic reticulum was decreased significantly.

1. Introduction

The plant kingdom is an unlimited source of biologically active substances with therapeutic potential (Atanasov et al., 2015; Yeung et al., 2018). Phytochemicals are products of a secondary plant metabolism and serve survival functions for the organisms producing them (Demain and Fang, 2000). A lot of plant-derived compounds are already used in modern medicine - about 25% of drugs prescribed worldwide are of plant origin (Hostettmann et al., 1998). In traditional medicine it has been very common to use plant/multi-plant extracts whose therapeutic effects may result from the additive action of several active compounds (Hostettmann et al., 1998). To ensure a constant quality and therapeutic efficacy, it is, however, essential that such extracts should be standardized for their content of bioactive constituents (Hostettmann et al., 1998; Lipińska et al., 2019).

Mistletoe is widely used in medicine. For decades, standardized aqueous mistletoe extracts have been used as a complementary treatment for benign and malignant tumors in human oncology (Ziegler, 2009). No severe adverse events have been reported during many years of use by thousands of patients which confirms the information on the safety of the mistletoe extracts (Gorter et al., 1998; Maldacker, 2006; Pelzer and Tröger, 2018; van Wely et al., 1999). Clinical trials have shown an increase the survival rate and a reduction in side effects caused by conventional oncological therapy (surgery, chemo-, hormone- or radiotherapy) when complementary treatment with mistletoe extracts was introduced and no adverse interactions with anticancer agents were reported (Maldacker, 2006; Pelzer and Tröger, 2018; Thronicke et al., 2018). Currently, attempts are being made to use the herbal drug also in veterinary medicine. There have been reports of effective treatment of animals, e.g., of equine sarcoid in horses and of fibrosarcoma in cats, but there is a lack of scientific data on efficacy (Christen-Clottu et al., 2010; Klocke et al., 2008).

Mistletoe extracts contain a large variety of cytotoxic and immunomodulatory substances with potential effectiveness against cancer

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cells (Twardziok et al., 2017). The main biologically active components of mistletoe extracts are toxic proteins viscumins and viscotoxins, and substances of non-protein nature such as triterpenes and flavonoids (Maier and Fiebig, 2002; Twardziok et al., 2017). Viscumins are mistletoe lectins. Three isoforms of mistletoe lectins have been identified, namely ML-I, ML-II and ML-III. They belong to the ribosome-inactivating proteins type II (RIPs II) family (Eck et al., 1999; Pevzner et al., 2005). Like all RIPs II, viscumins are heterodimeric glycoproteins (approx. 66 kDa) composed of two subunits (A and B) bound together by disulphide bonds. The toxin subunit A possesses a highly specific N-glycosidase activity (EC 3.2.32.22) and cleaves a single adenine residue at position A4324 of highly conserved loop structure in eukaryotic 28S rRNA of the major ribosomal 60S subunit (Abuharbeid et al., 2004; Büssing et al., 1999; de Virgilio et al., 2010; Eck et al., 1999; Pevzner et al., 2005). This results in the ribosome's inability to bind elongation factor and in the irreversibly blocking of protein synthesis in the cell (de Virgilio et al., 2010; Eck et al., 1999). The toxin subunit B has lectin properties and highly specific bind to appropriate carbohydrate (ML-I to galactose, ML-III to N-acetylgalactosamine, whereas ML-II to both sugars) receptors on the cell surface, inducing uptake of the toxic subunit A via receptor-mediated endocytosis, which, after translocation into cytosol, irreversibly modifies ribosomes (Heinzerling et al., 2006; Pevzner et al., 2005). It is believed that viscumins are especially highly cytotoxic to tumour cells due to their cancer-specific glycosylation patterns, which are the target for viscumins. New research proves that RIPs attack not only rRNA but also several other adenine containing substrates (Baskar et al., 2012).

Another important component of mistletoe extracts are viscotoxins - small (46 amino acids, approx. 5 kDa), amphiphil, cationic, cysteine-rich basic polypeptides structurally related to the family of α- and β-thionins (Heinzerling et al., 2006; Romagnoli et al., 2000; Tabiasco et al., 2002). They have been shown to be cytotoxic to bacterial, fungal and animal cells (Giudici et al., 2006). To date, seven isoforms (A1, A2, A3-the most abundant and cytotoxic, B, C1, 1-PS and U-PS) have been described (Giudici et al., 2006; Romagnoli et al., 2000). Studies on the mechanism of viscotoxins action suggest that most of the observed toxic effects probably result from direct interaction of viscotoxins with the target cell membrane and disruption of its integrity, which is followed by disturbances in cell homeostasis (Coulon et al., 2002; Romagnoli et al., 2000). The exact mechanism is not known yet (Giudici et al., 2006; Romagnoli et al., 2000; Tabiasco et al., 2002). Numerous studies indicated that both viscumins and viscotoxins induce oxidative stress (Büssing et al., 1999; Giudici et al., 2006).

One of the oldest standardized mistletoe preparations is Iscador® (WELEDA) extracted from mistletoe plants growing on different host trees like oak (Iscador Qu special), apple (Iscador M special) and pine (Iscador P) (Maier and Fiebig, 2002). All extracts are biologically and biochemically standardized (Maier and Fiebig, 2002). Iscador Qu contains the most lectins (375 ng/ml), Iscador M slightly less (250 ng/ml), whereas Iscador P contains only trace amount of lectins, but a relatively high concentration of viscotoxins (Maier and Fiebig, 2002). Iscador P induces necrosis rather than apoptosis as seen for Qu and M, probably through viscotoxins (Ramaekers et al., 2007).

Even though mistletoe extracts have been in clinical use for decades, their mechanism of action on cells has been poorly understood. In the present study we analyzed the effect of a single injection of Iscador® P (WELEDA AG, Germany) on degradative processes of mouse liver cells 24 and 48 h after *in vivo* exposure to the doses of 0.1 mg, 1 mg and 2 mg (/kg/b.w.). Additionally, a morphological study of liver cells was conducted.

We decided to use Iscador P in our experiment because there is little data in the literature about *in vivo* studies with its use. The majority of studies use Iscador Qu and Iscador M, whereas Iscador P has a different composition based mainly on viscotoxins, which certainly also translates into a different mechanism of action on cells.

According to the producer the active substance in Iscador P is *Viscum album* L. ssp. *austriacum* (mistletoe) grown on *Pinus sylvestris* L. (Scots pine), fresh herb, fermented liquid extract (1:5) (Public Assessment Report Scientific discussion. Iscador P 0.01 mg, 0.1 mg, 1 mg, 10 mg, 20 mg, solution for injection. The Medical Products Agency Läkemedelsverket). Pine mistletoe extract contains a range of substances with potential pharmacodynamic activity, among which viscotoxins (high concentration), viscumines (trace amount) and other biologically active compounds such as alkaloid-like compounds (tyramine, phenylethylamine, choline and acetylcholine), numerous flavonoids (quercetin), phenylpropanoids (caffeic acid, ferulic acid and sinapic acid), vaster proteins and peptides (e.g. viscumamide), amino acids, several enzymes, phytosterols (β-sitosterol, stigmasterol and their respective glycosides), triterpenes (β-amyrin, β-amyrin-acetate, betulinic acid, oleanolic acid, ursolic acid), mono-, oligo- and polysaccharides (methylester of 1→α4 galacturonic acid, rhamnogalacturonanes), polyalcohols (1D-1-O-methyl-muco-inositol, mannitol, quebrachitol, pinitol and viscumitol) and minerals (Pfüller, 2000). All these ingredients may contribute to the pharmacodynamic properties of the extract, but the role of these different compounds is currently not known.

According to literature data use of Iscador® P represent a safe and effective treatment for skin cancers (Christen-Clottu et al., 2010; Werthmann et al., 2017). Current epidemiologic data for both non-melanoma and melanoma skin cancers proved that skin cancer is exceedingly common and the incidence is increasing at an alarming rate. It is probably due to the excessive sun/UV light exposure, increased longevity, ozone depletion, genetics and in some cases, immune suppression (Leiter and Garbe, 2008). The risk for both types skin cancers increases with age (for melanoma the average age is about 60). With regards to the gender, there is a slight male predilection, which increases with age (Apalla et al., 2017). However, excessive use of tanning beds and other cosmetic treatments with UV light by women may contribute to an increase of the incidences of skin cancer in the future.

2. Materials and methods

2.1. Animals and treatment procedure

Fifty individuals of 16-month-old Swiss-Albino female mice (36 ± 1g body weight) originating from the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences in Jastrzebiec were used in this study. Female animals were chosen in order to counteract the existing strong gender bias in pharmacologic research (since so far most of medical and drug research is conducted mainly on men patients and male animals, and up to now there is much less studies exploring pharmacologic effects in female animals or patients). The mice were housed in standard cages at 21 °C with a 12 h light-dark cycle and fed standard pelleted mice food Murigram (Agropol, Motycz, Poland) and water *ad libitum*. During the 7-day preliminary quarantine mice were observed for signs of illness. All mice were deemed clinically healthy and included in the study. They were allocated randomly to five groups, 10 individuals per group. The mice serving as a control were injected intraperitoneally with 0.9% NaCl, while the mice from the experimental groups were injected with Iscador® P (WELEDA AG, Germany) in doses of 0.1 mg/kg b.w., 1 mg/kg b.w. and 2 mg/kg b.w. The treatment protocol was based on human treatment schedules applied in cancer patients (van Wely et al., 1999), adapted to the body weight of mice. The Iscador® P therapy was well tolerated by the animals. None of the mice showed any disease symptoms. After due time (24 h and 48 h) from treatment the animals were killed and segments of liver were immediately taken and processed for biochemical and morphological studies. Liver was chosen as a study focus, since it represents the major organ involved in the metabolism of xenobiotics, and therefore it is often a target of diverse toxins (including compounds that are converted to toxic metabolites as a result of a local conversion by the liver). Moreover, Iscador P is used in cancer therapy, and the liver is one of the

most common sites for metastatic disease, accounting for 25% of all metastases to solid organs.

All procedures were performed according to the guiding principles for the care and use of research animals and were approved by the 2nd Local Ethics Commission for Experimentation with Animals No 27/2009.

2.2. Preparation of subcellular fractions of liver

All the procedures were carried out at 0–4 °C. The mouse livers after perfusion with PBS were homogenized in a medium of PBS containing 250 mM sucrose, in a ratio of 1 g of tissue to 7 ml sucrose solution. The homogenates were transferred into centrifuge tubes and fractionated by differential centrifugation. After a low-speed centrifugation (1000 × *g* for 10 min), the homogenates were centrifuged at 20000 × *g* for 20 min to obtain a crude lysosomal fraction (pellet, CLF) and a supernatant of the lysosomal fraction. Both pellet and supernatant of CLF were collected separately. The lysosomal pellet was re-suspended in 5 ml of 0.1% TRITON X-100 to release latent lysosomal enzymes. Both fractions (re-suspended pellet and supernatant) were stored at −20 °C until use for analysis. Prior to the enzymatic analysis, both fractions were centrifuged for 2 min at 12000 × *g* to remove debris and insoluble material. The enzyme activities were determined for each fraction.

2.3. Determination of the enzymatic activity of some lysosomal and cytosolic hydrolases

Enzymes' activities were assayed by spectrophotometric assessment of specific product as described below. The total activity of cathepsins D (EC 3.4.23.5) and L (EC 3.4.22.15) was assayed according to Langner et al. (1973) using 2% azocasein in 6 M urea as a substrate (product measured at 366 nm). The activity of lysosomal acid phosphatase (EC 3.1.3.2) was assayed according to Hollander (1971) using a synthetic substrate p-nitrophenyl phosphate (product p-nitrophenol measured at 420 nm). Activities of N-acetyl-β-D-hexosaminidase (EC 3.2.1.52), β-D-glucuronidase (EC 3.2.1.31) and β-D-glucosidase (EC 3.2.1.21) were determined according to Barrett and Heath (1977) using as substrates p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-β-D-glucuronid and p-nitrophenyl-β-D-glucopyranoside, respectively (the product p-nitrophenol measured at 420 nm). The activities of cytosolic leucyl aminopeptidase (EC 3.4.11.1) and arginyl aminopeptidase (EC 3.4.11.6) were detected using as substrates L-leucine-2-naphtylamide hydrochloride and L-arginine-2-naphtylamide hydrochloride, respectively. Their activities were measured as Fast Blue BB salt derivatives at 540 nm by the method of McDonald and Barrett (1986). All the substrates were produced by SERVA GmbH & Co (Heidelberg, Germany) and Sigma-Aldrich (Taufkirchen, Germany). The total protein level was determined by a modified Lowry's method (Kirschke and Wiederanders, 1984) using bovine serum albumin as a standard. The specific enzyme activities were expressed as μmoles of product per mg of total protein. Statistical significance of differences was evaluated by the Student's t-test for unpaired data using the STATISTICA software (Ver. 10. StatSoft Company, 2011); $p < 0.05$ was considered a significance threshold.

2.4. Light and electron microscopy examination of liver samples

The part of the right lobe of the liver was excised immediately after the animal death, cut into approx. 1 mm^3 cubes and fixed by infiltration with 3% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) for 2 h at 4 °C. After the samples were fixed, they were washed overnight in 0.1 M sodium cacodylate buffer (at 4 °C), and then post-fixed in 2% osmium tetroxide for 1 h at 20 °C. For epoxy resin embedding, samples were dehydrated using a graded series of ethanol (50%–100%), then transferred to propylene oxide, infiltrated in a mixtures of propylene oxide and epoxy resin, embedded in the pure EPON 812 epoxy resin in silicone embedding mold, and finally thermally polymerized (Marzella and Glaumann, 1980).

Before preparing ultrathin (50–60 nm) sections for TEM, thicker semi-thin sections (500–1000 nm) were cut from EPON blocks for light microscopy (LM) observation. They were transferred on a microscope slide and stained with a 1% toluidine blue, and then studied with a ZEISS Axio Scope.A1 light microscope. Ultra-thin sections were double contrasted with uranyl acetate followed by lead citrate, and viewed on a TESLA BS500 transmission electron microscope. Electron micrographs were taken with a slow scan camera (Variospeed 1K-SSCCD camera, TRS, Germany). All the reagents were produced by SPI Supplies (West Chester, PA, USA).

Morphometric analysis of lysosomes (primary lysosomes, autophagic vacuoles/secondary lysosomes) and mitochondria were performed in 100 randomly chosen electron micrographs from each animal group. The average numbers of organelles per cell were calculated for each animal group. The data were analyzed by the nonparametric *Mann–Whitney U* test using the STATISTICA software (Ver. 10. StatSoft Company, 2011); $p < 0.05$ was considered a significance threshold.

3. Results

3.1. Mistletoe extract affected activities of lysosomal and cytosolic hydrolases

The activity of some lysosomal enzymes, namely acid phosphatase, β-D-glucuronidase, β-D-glucosidase, N-acetyl-β-D-hexosaminidase, cathepsin D & L, and cytosolic leucine and arginine aminopeptidases was estimated in both the lysosomal fraction and in the corresponding supernatant of lysosomal fraction isolated from mouse liver homogenates. The tissue material was obtained 24 and 48 h after the mice treatment with a single dose of Iscador P: 0.1 mg, 1 mg or 2 mg (/kg/b.w.).

In general, the applied treatment significantly decreased the activity of all the analyzed glycosidases and acid phosphatase in the lysosomal fractions with regard to all doses and times for acid phosphatase, β-D-glucuronidase and N-acetyl-β-D-hexosaminidase, whereas in the case of β-D-glucosidase only 24 h after treatment with doses of 1 mg and 2 mg (Fig. 1A–D). A reduction in peptidases activity was noted only 24 h after the administration of the 0.1 mg dose (for leucyl aminopeptidase) and 1 mg dose (for arginyl aminopeptidase) (Fig. 1F–G). An increase in enzyme activity in lysosomal fraction was found only 48 h after the injection in the case of β-D-glucosidase (Fig. 1D) and arginyl aminopeptidase (Fig. 1F).

Analysis of the enzyme activities in the supernatant of lysosomal fraction showed that of all the studied lysosomal enzymes, only the β-D-glucuronidase activity increased - 24 and 48 h after the dose of 1 mg (Fig. 1B), and the β-D-glucosidase activity slightly decreased – 24 h after the 2 mg dose (Fig. 1D). In contrast, more significant changes were noted in the case of aminopeptidases, especially 24 and 48 h after the dose of 1 mg – the activity of both peptidases increased significantly (Fig. 1F–G). Cathepsins D & L activity did not change significantly either in the lysosomal fraction or in the corresponding supernatant of liver (Fig. 1E).

3.2. Mistletoe extract affects the morphology of hepatocytes

To reveal the effect of mistletoe extract on the morphology of hepatocytes, firstly the epon semi-thin sections of the liver were analyzed by light microscopy (Fig. 2), and then the ultra-thin sections were analyzed by transmission electron microscopy (TEM) (Fig. 3).

Fig. 2A shows a correct histological structure of the liver from the control animal. There are visible normal hepatocytes of central zone of the hepatic lobule (zone 3) (the same zone of the lobule was used as a reference in the following micrographs) and numerous Ito cells.

Fig. 2B–D presents hepatocytes of mice treated with mistletoe extract. In these cells we observed areas of empty cytoplasm around the nucleus and the general increase in cytoplasmic brightening, which

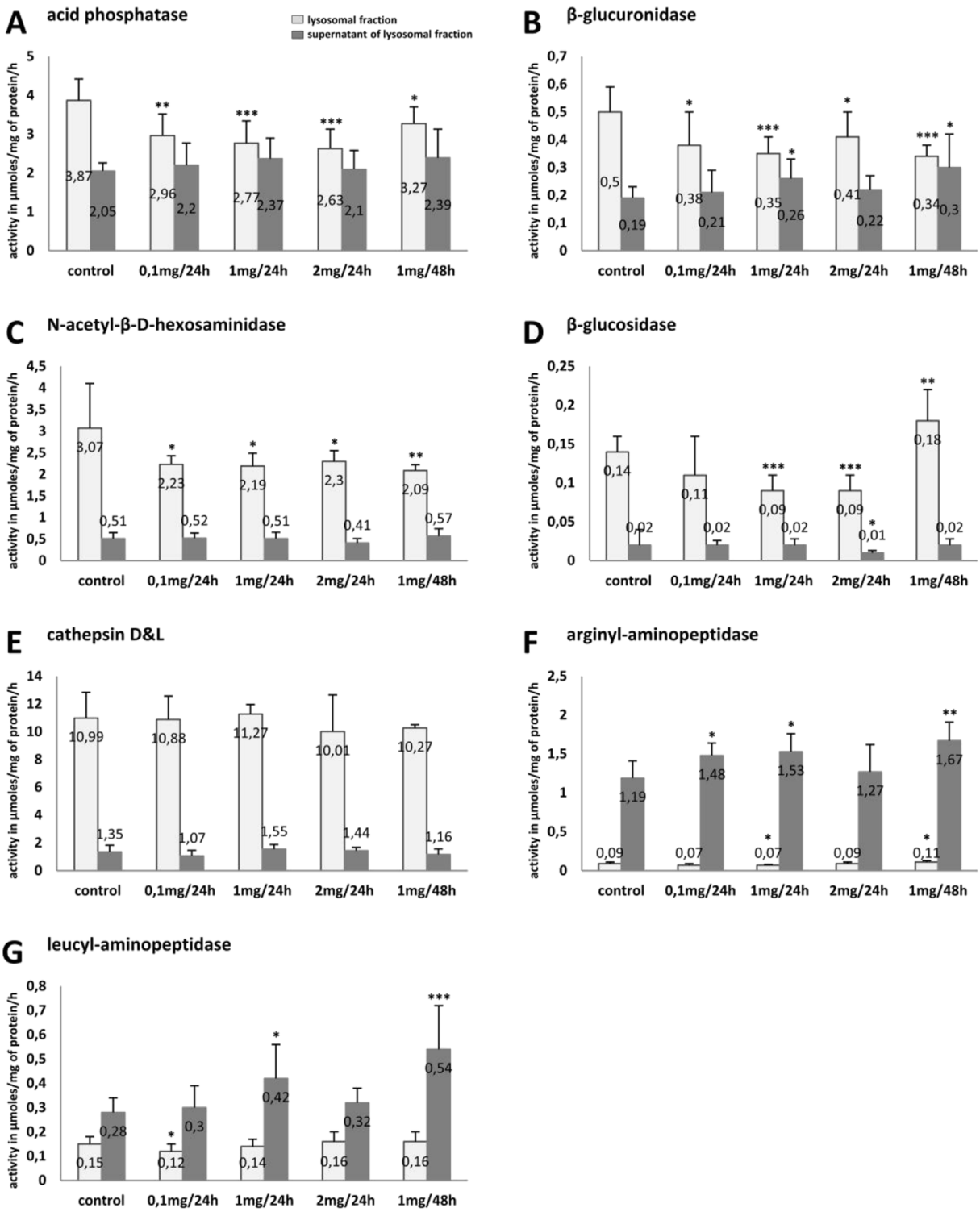

Fig. 1. The specific activity of lysosomal hydrolases (A–E) and cytosolic aminopeptidases (F–G) in the both fractions of liver homogenates of mice 24 h after treatment of Iscador P in dose 0.1 mg/kg b.w., 1 mg/kg b.w., 2 mg/kg b.w. and 48 h after treatment with a 1 mg/kg b.w. dose. Results are expressed as change in relation to control non-treated animals; showed are mean values ± SD (n = 10), asterisks represent statistical significance of changes (*p < 0.05, **p < 0.01, ***p < 0.001).

markedly elevated after the 2 mg dose. The liver cells cytoplasm became pale-staining as a result of the reduction in the organelle quantity (it is confirmed by ultrastructural studies – Fig. 3). Interestingly, the areas of empty, rarefied cytoplasm were almost invisible 48 h after the treatment (Fig. 2E).

In the second step, the ultrastructure of hepatocytes was analyzed. The ultrastructural analysis was performed in the same areas of liver tissue which were analyzed by light microscopy. Fig. 3A shows a representative micrograph of hepatocyte from a control mouse with a typical "normal" structure. Inside hepatocytes, fragment of nucleus and numerous cytoplasmic organelles are visible, including many orthomorphic mitochondria separated by numerous canals of rough

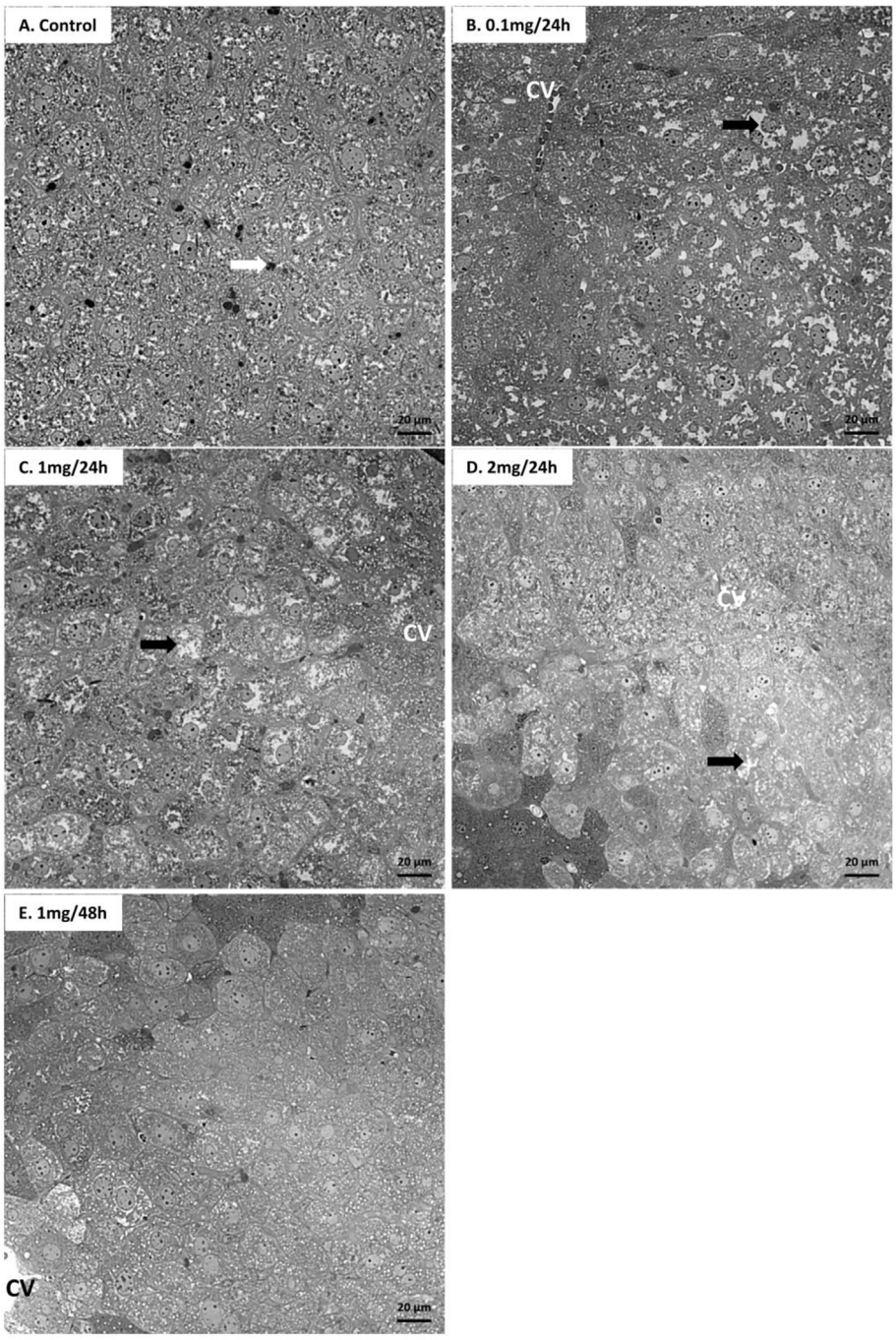

Fig. 2. Morphology of liver revealed by light microscopy analysis of toluidine blue stained epon semi-thin sections from control mouse (A); mouse treated 24 h with dose 0.1 mg/kg b.w. (B), 1 mg/kg b.w. (C) or 2 mg/kg b.w. (D); mouse treated 48 h with dose 1 mg/kg b.w. (E). Black arrows indicate empty pale-staining cytoplasm, white arrow – Ito cell, CV— central vein (magnification 400×, scale bar = 20 µm).

endoplasmic reticulum, and peroxisomes and a few (on average, 5 in the image field) small primary lysosomes. Mouse treatment with a dose of 0.1 mg induced changes in the morphology of mitochondria – the mitochondrial matrix became clear due to the swelling. In addition, we observed a reduction of rough endoplasmic reticulum canals and their concentration in one place, and consequently, appearance of areas of empty cytoplasm without organelles (Fig. 3B). Increasing the dose to 1 mg and 2 mg resulted in intensification of the above-described changes (Fig. 3C–D), and additionally in the increase in the average number of mitochondria per cell (especially after the dose of 2 mg, Fig. 4C) and in the dose-dependent increase in the size of mitochondria (Fig. 4D). However, the mitochondrial internal ultrastructure is preserved and does not reveal any impairment of the inner and outer mitochondrial membranes and matrix (Fig. 3C–D). It is noteworthy that the mitochondria are in close contact with each other (they are not separated by rough endoplasmic reticulum canals), which promotes the fusion. Fig. 3C shows the moment of mitochondrial fusion. The strongest changes in ultrastructure, especially of mitochondria, were observed 48 h after injection, when additionally the enlarged and swollen mitochondria appeared (to 2 µm, on average; to 5–8 µm maximum; Figs. 3E and 4D). These mitochondria tightly fill the empty spaces created after the disappearance of the rough endoplasmic reticulum.

Our ultrastructural studies did not show an increase in the number of autophagic vacuoles and/or secondary lysosomes (Fig. 3A–E,

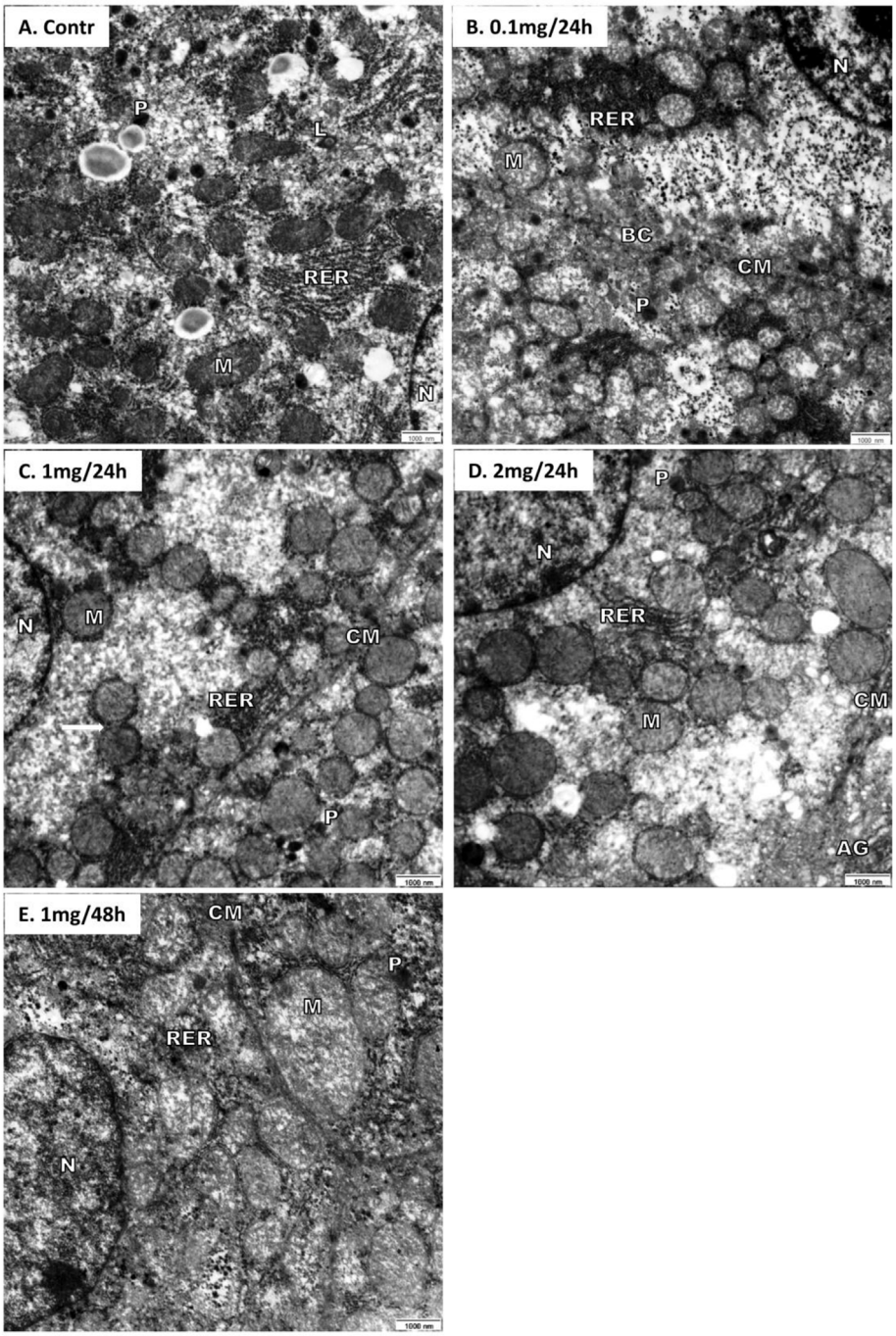

Fig. 3. Ultrastructure of hepatocytes in control mouse (A) or 24 h after treatment with doses: 0.1 mg/kg b.w. (B), 1 mg/kg b.w. (C), 2 mg/kg b.w. (D) and 48 h after treatment with a 1 mg/kg b.w. dose (E). N— nucleus; M-mitochondria, RER-rough endoplasmic reticulum; L-lysosomes, P— peroxisomes, AG- Golgi apparatus, CM-cell membrane, BC— bile canaliculus, white arrow-fusion; scale bar = 1000 nm (A–E).

Fig. 4A). On the contrary, we even noticed a statistically significant reduction in the number of primary lysosomes, most pronounced 24 h after the 1 mg and 2 mg doses (Fig. 3A–E, Fig. 4B).

In conclusion, the changes in hepatocyte morphology regarding mitochondria and the rough endoplasmic reticulum and the appearance of organelle-free cytoplasm areas appear to be morphological indicators of mistletoe extract toxicity.

4. Discussion

The maintenance of cellular homeostasis requires precise control of both the rate of protein synthesis and its degradation (Dai et al., 2013; Princiotta et al., 2003). Under normal conditions, proteins are constantly turning over, i.e. they are hydrolyzed to amino acids and replaced with newly synthesized proteins (Lecker et al., 2006). At a steady state, rates of protein synthesis and degradation are balanced (Blommaart et al., 1997), because both processes are subject to constant dynamic control by amino acids and insulin (Kanazawa et al., 2004). It is known that alterations of protein degradation/biosynthesis may regulate protein biosynthesis/degradation through a feedback mechanism (Dai et al., 2013).

The intracellular degradative systems must have a sufficient baseline capacity to eliminate damaged or defective molecules produced by cells under normal conditions and, in the reserve, a sufficient excess

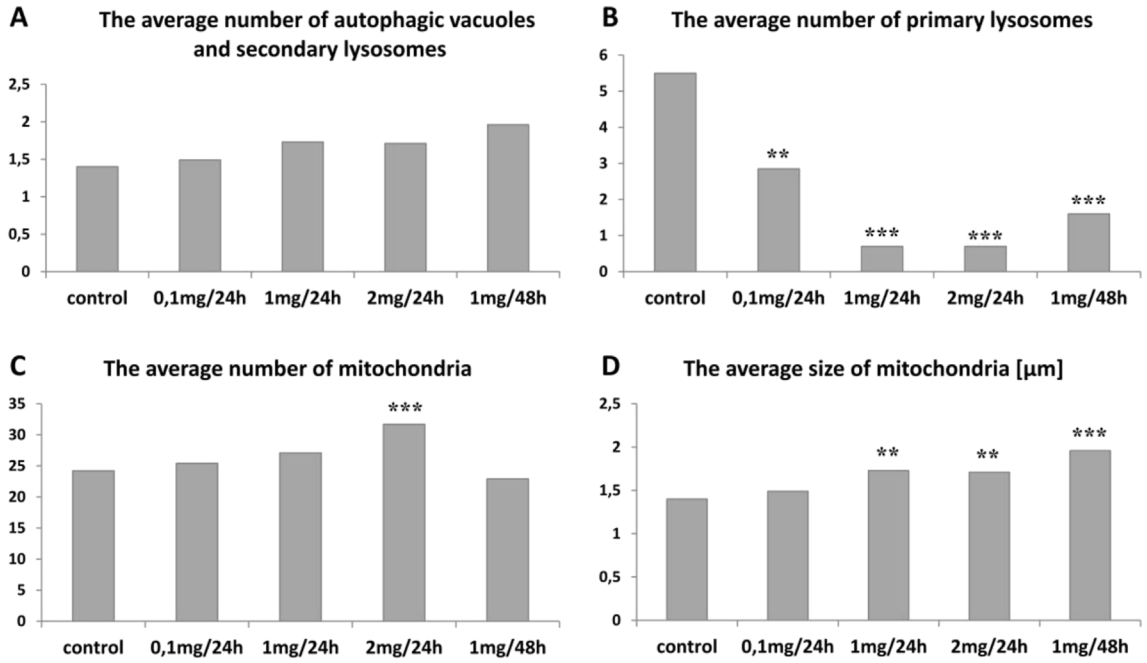

Fig. 4. Changes in the average number of mitochondria, primary and secondary lysosomes per cell, and changes in the average size of mitochondria in hepatocytes of Iscador P - treated mice *vs* control. The significance of differences was tested by *Mann-Whitney U* test.

capacity to cope with damage induced by pathological factors that usually rapidly increase intracellular protein turnover (Princiotta et al., 2003). Therefore, intracellular degradation is considered an important homeostatic process (Lecker et al., 2006). In the eukaryotic cell there are two major degradation routes: the autophagy – lysosome pathway and the ubiquitin - proteasome system (Appelqvist et al., 2013; Korolchuk et al., 2009). In the liver, proteasomes are responsible for the degradation of a small pool of proteins with a high turnover rate (e.g. regulatory proteins), whereas the lysosomes degrade a wide range of long-lived proteins and other macromolecules (Blommaart et al., 1997; Jóźwik et al., 2018). The lysosomal catabolism involves a well-ordered action of a mixture of hydrolytic enzymes, like proteases, esterases and glycosidases degrading oligosaccharides sequentially from the outer non-reducing end (Jóźwik et al., 2016; Winchester, 2005).

Both degradative systems are involved in the most aspects of normal physiology and implicated in a wide range of pathological states (Korolchuk et al., 2009). It has been well established that the autophagy –lysosome system is a very dynamic, highly reactive structure, which plays an important role in cell adaptive reactions to a wide range of stress factors by restoring of the biochemical homeostasis (Jóźwik et al., 2009; Śliwa-Jóźwik et al., 2002). Changes in the autophagy-lysosome system are widely used to monitor the effect of different agents on cell function and as indicators of cell injury. Several methods are available to measure lysosomal functional status (Moore et al., 2004). In our study, we measured the activity of some lysosomal enzymes collectively implicated in the intracellular degradation, namely acid phosphatase – "marker" enzyme for lysosomes, glycosidases such as β-D-glucuronidase, β-D-glucosidase and N-acetyl-β-D-hexosaminidase, as well as peptidases such as endopeptidases (cathepsin D & cathepsin L) and cytosolic exopeptidases (leucine and arginine aminopeptidases) in both the lysosomal fraction and in the corresponding supernatant of lysosomal fraction isolated from mouse liver homogenates. The tissue material was obtained 24 and 48 h after the mice treatment with a single dose of Iscador P: 0.1 mg, 1 mg or 2 mg.

In general, the applied treatment significantly decreased the activity of all the analyzed glycosidases and acid phosphatase in lysosomal fraction; with regard to all doses and times for acid phosphatase, β-D-glucuronidase and N-acetyl-β-D-hexosaminidase, whereas in case of β-D-glucosidase only 24 h after treatment with doses of 1 mg and 2 mg (Fig. 1A–D). The reduction in aminopeptidases activity was noted only 24 h after the administration of the 0.1 mg dose (for leucyl aminopeptidase) and 1 mg dose (for arginyl aminopeptidase) (Fig. 1F–G). The increase in enzymes activity was found only 48 h after the injection in the case of β-D-glucosidase (Fig. 1D) and arginyl aminopeptidase (Fig. 1F).

The decrease in enzyme activities may indicate intensification of degradation processes as a result of the applied treatment. According to Marzella et al. (1982), initially, the enzymes activity increases in parallel with the induction of degradation, but it later decreases with the advanced stage of the sequestered cell organelles degradation (Wieczorek et al., 2018). With more advanced stages of lysosomal degradation, the activity seems to decline due to enzyme reserves consumption (Marzella et al., 1982) and possibly their insufficient synthesis (or synthesis block). Our ultrastructural studies did not confirm the intensification of autophagic processes in hepatocytes (Fig. 3). We did not observe an increase in the number of autophagic vacuoles and/or secondary lysosomes (Fig. 4A). However, it should be noted that in the liver, autophagic vacuoles are characterized by a high turnover rate (they are formed and degraded with a half-life of 8 min), and their volume in the cell at a given moment is low. Therefore, it is difficult to show them on the electronograms, even when autophagic flux is high (Blommaart et al., 1997). On the contrary, we even noticed a statistically significant reduction in the number of primary lysosomes (Fig. 4B). Thus, the decrease in enzymes activity can be rather the result of the lysosomal enzymes synthesis blockage (due to a general reduction in the biosynthesis of cellular proteins) or their direct inactivation by both mistletoe toxins (mainly viscotoxins) and the free radicals generated by them. As mentioned earlier, viscumins are effective inhibitors at the translation step of protein synthesis (Abuharbeid et al., 2004; Büssing et al., 1999; de Virgilio et al., 2010; Eck et al., 1999; Pevzner et al., 2005). Viscotoxins can inhibit protein synthesis, too - probably through direct interaction with mRNA or at the initial translation level (Stec, 2006). van Wely et al. (1999) observed increased urea levels and a slightly decreased total protein caused by a minor decrease in albumin level in patients treated with Iscador Qu Spezial. These results suggest a slighthly increase in protein breakdown and a decrease

of protein synthesis in the liver.

Our ultrastructural studies revealed a significant reduction of rough endoplasmic reticulum compartment (Fig. 3C–D), which could be a confirmation of reduced protein synthesis, especially of secretory, membrane and lysosomal proteins. On the other hand, Piñeiro et al. (1995) reported that thionins are able to form disulfide bonds with other cysteine-rich proteins (with free -SH groups or -SS- bridges), and thus irreversibly inhibiting certain thiol-dependent enzymes or proteins that are active only in their reduced form, including cytochrome *c* and β-glucuronidase, both *in vitro* and *in vivo* (Piñeiro et al., 1995; Śliwa-Jóźwik et al., 2002). Thus, various mechanisms should be considered when interpreting our results.

Lysosomal degradation is ATP dependent process. Each stage of autophagy requires energy supply (Blommaart et al., 1997). Under normal conditions, the ATP concentration in the hepatocytes is strongly buffered (Blommaart et al., 1997). However, pathological factors, such as mistletoe toxins, can induce mitochondrial dysfunction, resulting in reduced ATP production and bioenergetic failure. Our ultrastructural studies have shown that mitochondria was the most sensitive structure to mistletoe toxins. We observed the time and dose-dependent enlargement of the mitochondria (to 2 μm, on average, with maximum to 5–8 μm; Fig. 3C–E, Fig. 4D), and at the same time an increase in the total number of mitochondria (Fig. 3C–E, Fig. 4C). However, the mitochondrial internal ultrastructure is preserved and does not reveal any observable impairment of the inner and outer mitochondrial membranes and matrix, which usually accompany degeneration of these organelles. It seems that the mitochondria tightly fill the empty spaces created after the disappearance of the rough endoplasmic reticulum. The strongest changes in mitochondrial morphology were observed 48 h after drug administration, when additionally the swollen mitochondria appeared (Fig. 3E). It is likely that large mitochondria are formed by the fusion of normal mitochondria. Büssing et al. (1999) revealed profound changes in mitochondria morphology of leukocytes treated with viscotoxin, too. However, they observed additional changes in other cytoplasmic and nuclear structures. These authors suggested that viscotoxin-induced mitochondrial defects may result from excessive production of reactive oxygen species (ROS) (Büssing et al., 1999). Mitochondria are highly dynamic organelles. They constantly change their morphology to fit the bioenergetic requirements of the cell, especially in a state of metabolic or environmental stresses (Lysek-Gladysinska et al., 2018; Westermann, 2012; Youle and van der Bliek, 2012). Currently, it is believed that the dynamics of the mitochondrial compartment is controlled by two opposing processes such as mitochondrial fusion and fission (Westermann, 2012; Youle and van der Bliek, 2012). Recently, several reports have appeared that draw attention to the importance of mitochondrial fusion in conditions of high energy demand in the cell (Westermann, 2012). Mitochondrial fusion allows efficient mixing of mitochondrial content and complementation of gene products, which restores respiratory activity (Westermann, 2012; Youle and van der Bliek, 2012). Fused mitochondria may be more metabolically efficient and can cope with increased energy demand during stress conditions (Westermann, 2012; Youle and van der Bliek, 2012). Thus, the sudden need for ATP is the reason for the induction of mitochondrial fusion (Westermann, 2012). *In vitro* studies have shown that various pathological factors, especially some drugs that inhibit protein synthesis (e.g. actinomycin D, cycloheximide) as well as starvation can induce mitochondrial fusion, termed stress-induced mitochondrial hyperfusion (SIMH) (Tondera et al., 2009; Westermann, 2012; Youle and van der Bliek, 2012). Prolonged stress ultimately leads to mitochondrial fragmentation (fission) and to apoptosis (Youle and van der Bliek, 2012). Both mitochondrial fusion and fission processes are regulated by proteolysis and posttranslational modifications (Youle and van der Bliek, 2012). Fig. 3C–D shows numerous rounded mitochondria in close contact with the neighboring ones, while in the control cell (Fig. 3A), mitochondria are separated from each other by numerous rough endoplasmic reticulum cisterns. Fig. 3C shows the moment of mitochondrial fusion. We have also observed numerous cases of mitochondrial fission, which probably influenced the increase in the number of mitochondria despite their fusion. In addition to changes in mitochondrial morphology, we observed a significant reduction of rough endoplasmic reticulum canals and the presence of areas of empty, rarefied cytoplasm characterized by the reduction of organelles (Fig. 3B–D). Empty, pale-staining cytoplasm was also seen in semi-thick sections (Fig. 2B–D).

Interestingly, 48 h after the treatment, no cytoplasmic pale-staining (clear) areas were observed both in the light and electron microscope studies, because a lot of swollen and enlarged mitochondria filled the cell tightly (Figs. 2E and 3E).

Changes in the morphology of mitochondria may be the result of both the membranotropic properties of viscotoxins and reactive oxygen species generated by them, as well as disorders in protein biosynthesis and proteolysis. Autophagy is required for maintaining a healthy mitochondrial compartment by elimination of old and damaged mitochondria (Youle and van der Bliek, 2012).

Due to the presence of highly active hydrolytic enzymes in lysosomes, they are potentially harmful to the cell (Boya, 2012). Any damage to the lysosomal membrane may trigger a release of acid hydrolases from the lysosomal lumen into the cytoplasm, inducing uncontrolled degradation of cellular components, which in turn may induce cell damage (Boya, 2012). Lysosomal membrane permeabilization (LMP) is induced by a plethora of distinct agents, including especially those which generate reactive oxygen species and by lysosomotropic compounds with detergent activity (Boya and Kroemer, 2008). Since viscotoxins induce ROS production, and additionally they have membranotropic properties, we decided to investigate whether they affect LMP. Specific methods to identify lysosomal membrane permeabilization (LMP) in cells and tissues have been described in the literature. In our study the LMP was assessed based on relocation of enzymes from lysosomal fraction (pellet) to supernatant of lysosomal fraction of liver homogenate. However, of all the analyzed lysosomal enzymes, only the β-glucuronidase activity increased in the supernatant; 24 and 48 h after the dose of 1 mg (Fig. 1B). In contrast, a significant increase in the supernatant was noted in the case of arginine and leucine aminopeptidases, especially 24 and 48 h after the dose of 1 mg, but this does not result from lysosomal membrane lability. Arginine aminopeptidase (aminopeptidase B) and leucine aminopeptidase are cytosolic exopeptidases and the cytosol is the physiological site of their activity. They provide the final events in the journey of polypeptides through a cell (Foulon et al., 1999; Matsui et al., 2006). A time-dependent increase in the activity of both aminopeptidases in cytosol indicates a decline in extralysosomal proteolysis, which can be a result of both blockage of protein synthesis and/or proteasomal proteolysis by mistletoe toxin. It is known that a temporary block in protein synthesis by inhibitors leads to an arrest in ubiquitin-mediated proteasomal degradation (Shenkman et al., 2007). It is an important mechanism that may prevent depletion of essential short-lived proteins during the translation arrest (Shenkman et al., 2007). For a long time, the proteasome and lysosome systems were regarded as independent, non-cooperating degradative pathways. Currently, it is believed that there is a link between them (Korolchuk et al., 2009). It has been proven that many proteins are substrates of both degradative systems, and in certain conditions proteasomal substrates can be digested by lysosomes, and *vice versa* - the impairment of the one system is compensated by activating the other (Korolchuk et al., 2009).

The role of autophagy in tumour development, progression and cancer therapy is enormously complex (Marinković et al., 2018). Recent studies have shown that the modulation of the autophagy process is a promising therapeutic strategy for the enhancement of anticancer treatments (Marinković et al., 2018). However, autophagy may function as both a tumour suppressor and tumour promoter. In hypoxic tumour regions, in nutrient and oxygen deprivation conditions, upregulated autophagy is essential for tumour cell survival (White, 2015).

Due to autophagy, tumour cells are resistant to extremely stressful conditions. Most anticancer drugs, as well as ionizing radiation, affect autophagy. Usually, these treatments increase autophagy in tumour cells (some drugs induce autophagosomes formation and autophagic flux, thus truly increase autophagy, whereas other agents increase autophagosomes accumulation but not increase autophagic flux – thus they block autophagy by inhibiting fusion with the primary lysosome) (Thorburn et al., 2014). Sometimes autophagy protects tumour cells against cancer therapy, and sometimes it is required for the therapy to kill the cancer cell (Thorburn et al., 2014). It is probable that the anticancer properties of the mistletoe extracts, to a certain extent, are also due to the modulation of autophagy processes.

In conclusion, our study shows a different spectrum of effects of treatment with Iscador P, which seem to focus on perturbations in intracellular degradation/autophagy and changes in the morphology of mitochondria and rough endoplasmic reticulum. It seems that these changes are largely caused by the inhibition of protein synthesis by mistletoe lectins and viscotoxins acting at high concentration, but other components of pine mistletoe extract, such as alkaloids, flavonoids, phenylpropanoids, peptides, amino acids, several enzymes, phytosterols, saccharides, polyalcohols and minerals may also contribute to these effects.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.04.044.

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