Viscum Album L. Influence on the Antioxidant Enzymes Activity in Ehrlich Tumor Cells *In Vivo*

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Bulletin UASVM Veterinary Medicine 71 (1) / 2014, 198-203
Print ISSN 1843-5270; Electronic ISSN 1843-5378

Abstract

Viscum album L., (Santalaceae) is a semi-parasitic plant that grows on various trees. It is widely used in cancer treatment. The present study assesses the influence of oxidative stress in mistletoe induced tumor cytotoxicity in tumor cells. Ehrlich ascites carcinoma (EAC) was induced by injecting 10^6 cells/animal, ip, (in day 0), in Swiss female mice. Mistletoe ethanolic extract was injected in dose of 50 mg/kg b.w, i.p., three times before (days -6, -3, -1) and after (1, 3, 6) EAC inoculation. 14 days after EAC inoculation, the mice were euthanized for estimation of tumor proliferation, hematological parameters, the antioxidant status in plasma and tumor cells. In EAC bearing mice, *Viscum album* ethanolic extract exhibited a significant antiproliferative effect, as compared with untreated group, reflected in reduced EAC cell concentration, increased percentage of non-viable cells and low plasma antioxidant activity. These changes were found only in pre-treated groups, while post-treated groups showed no significant differences. The antiproliferative effect was significantly correlated with decreased activity of plasma antioxidant enzymes. In pre-treated groups, antioxidant enzymes activity of the EAC tumor cells experienced important changes, in correlation to cytotoxic effect, whereas less significant variations in post-treated group were found. Even if the underlying mechanisms are still to be ascertained, *Viscum album* alcoholic extract proved, a significant, selective antitumor effect, without obvious harmful effect on mice.

Keywords

Viscum album extract, Swiss mice, oxidative stress, plasma, tumor cells

INTRODUCTION

Viscum album L. (Santalaceae) (VA), commonly known as European Mistletoe, is a hemi-parasitic plant that grows on various trees. VA has a long tradition as medicinal plant, being used in traditional medicine as a popular remedy for hypertension, vascular disease, epilepsy, arthritis and rheumatism for centuries. Nowadays, mistletoe extracts are widely used as complementary therapy for cancer patients (Ostermann *et al.*, 2009).

Recently, many *in vitro* and *in vivo* experimental studies investigate the antitumor properties of VA extracts (Khil *et al.*, 2007, Cebovic *et al.*, 2008). Various clinical reports also revealed that VA extracts can improve the life quality and increase the survival time in different types of cancer (Ostermann *et al.*, 2009). The mistletoe's antitumor effect is based on two distinct bioactivities: direct cytotoxic effect and the increase of the cell mediated immunity. The lectins are responsible for direct toxicity against tumor cells (Khil *et al.*,

2007), while the oligosaccharides enhance the natural killer cells efficiency (Lee *et al*., 2009). In addition, the antitumor human cytotoxic T lymphocytes are selectively activated by mistletoe compounds (Tabiasco *et al*., 2002).

Increasing evidence suggest the involvement of reactive oxygen species (ROS) in VA anticancer effects, even though the mechanisms are still unknown and sometimes controversial. Lectin mediated cytotoxicity is a possible mechanism for the cytotoxicity of ROS. VA agglutinins increase the production of ROS in Hep3B tumor cells. ROS production might be related to the decrease in mitochondrial energy, a key event in regulation of apoptosis (Kim *et al*., 2004). On the other hand, mistletoe lectins prove significant antioxidant effects in non malignant cell line LLC-PK1, and they directly prevent the synthesis of free radicals, thus avoiding the lipid peroxidation, preserving the cell viability (Kim *et al*., 2010).

The effects of mistletoe extracts on tumor implanted mice are even more intricate. Antiproliferative effect on Ehrlich ascites carcinoma (EAC) is stronger, if the therapy is instituted previously ascites establishment, while simultaneous or post therapy was far less effective. Mistletoe extracts have no influence on antioxidant enzymes or lipid peroxidation, in healthy animals, but they reduce the oxidative stress markers in EAC inoculated mice (Cebovic *et al*., 2008). In general, these observations suggest that the cytotoxic mechanism of the *Viscum album* extract on the EAC development may involve the induction of oxidative stress in the EAC cells without significantly affecting the antioxidant status of other tissues.

The present study assesses the influence of oxidative stress in mistletoe tumor cytotoxicity by comparative evaluation of oxidative stress markers with proliferation parameters and inflammatory systemic response. In order to assess whether the increased oxidative stress in tumor cells is induced by the direct toxicity or is mainly mediated throughout immune response, the mistletoe extract was administrated both previously and after tumor inoculation. Previous administration enhances the immune defense mechanisms, while the therapy that follows the ascites inoculation exerts mainly a direct cytotoxic/cytostatic effect.

MATERIALS AND METHODS

Plant materials:

The *Viscum album* (plant raised from the apple tree – *Malus domestica* Bork) was harvested from Cluj area (Transylvania, Romania), in November – December 2009. The vegetal products were dried and grounded into a fine powder. Preparation of tinctures*: the VA tincture was prepared according to European Pharmacopoeias, method 2a, by cold extraction (maceration). 100 g of fresh VA plant was* kneaded to a paste consistency *(70% moisture). The plant material was then mixed with 70g 90% vol. ethanol. The plant-ethanol mixture was macerated for 10 days, with periodic mixing, and then pressed and filtered. The extraction ratio was 1:1. The final tincture had 8.5% dry residue.*

Before inoculation, alcoholic solution was maintained in a rotary evaporator 40°C, until 3/4 of the content evaporates, than filled with sterile saline solution up to 0.5ml/animal. The aqueous solution was immediately administrated i.p. in order to prevent the bacterial and fungal contamination. The control group received 0.5ml alcohol 70°, i.p., previously evaporated, similarly to plant extract method.

EXPERIMENTAL DESIGN

Animal care and experimental procedures followed requirements of the *Guide for the Care and Use of Laboratory Animals* (Department of Health Education, and Welfare, National Institute of Health, 1996). The animal tests and experiments were allowed by the Bioethical Board of the Faculty of Veterinary Medicine Cluj-Napoca. The animals were caged in polycarbonate cages, at controlled temperature of 21-22ºC, humidity (40-60%) and 12/12h light/dark cycle. Standard lab chow, provided by National Institute for Research and Development Cantacuzino Bucharest, and water were freely available. The experiment was carried out on 40 white Swiss female mice, 30.12±3.38g body weight. Animals were divided into five equal experimental groups of 8 mice, under the following treatments: group I - control receiving only placebo, as described above, group II - mice receiving the *Viscum album extract* alone, 50mg/kg b.w. i.p., in the 1^{st} , 3^{rd} and 6^{th} day, group III - mice with implanted with EAC cells, 10^6 ascitic cells each, in the day 0, group IV - mice pre-treated with VA extract 50 mg /kg b.w. i.p., 6^{th}, 3^{rd} and 1^{st} day previously EAC implantation and group V –

mice treated with the same dose of VA in the 1st, 3rd and 6th day, after EAC implantation. 14 days after EAC implantation, blood was collected from the retro orbitary sinus under anesthesia and the euthanasia was induced by prolonged narcosis. Blood samples were immediately centrifuged at 4°C, then plasma was frozen at -20°C and then kept at deep freezer.

Abdominal ascitic fluid was measured with a syringe, and then transferred in phosphate buffer solution (4°C, pH 7.4). The samples were subjected to repeated centrifugations at 4500 rpm for 5 min, then at 12000 rpm for 3 min, at 4°C to obtain dense cell suspension, which was kept in deep freezer until use. The cell viability was assessed by Tripan blue staining (0.4% in PBS), the viable tumor cell concentration was counted in a Burker camera (dilution 1:10). Body weight was measured at the beginning and at the end of experiment. Blood hematology was investigated with Abacus Junior Vet, automatic analyzer Diatron Messtechnik, Budapest, Hungary.

Antioxidant enzyme measurement assay

The activity of several antioxidative enzymes was determined both in EAC cells and plasma samples. The activity of catalase (CAT), xanthine oxidase (XOD), and peroxidase (Px) were determined using Xanthine Oxidase Assay Kit, Catalase Assay Kit, and respectively, Hydrogen Peroxide Assay (BioVision, USA), according to the manufacturer specifications. The results were measured by METERTECH Spectrophotometer SP-830 Plus.

Statistical analysis

All data are reported as the mean ± SEM. Statistical analyses were performed by one-way analysis of variance ANOVA, followed by post hoc Tukey's range test procedure, for pair-wise comparisons. Pearson's correlation was used in order to asses the correlation between normally distributed variables, interpretation was done according to Colton scale. Statistical significance was at $p<0.05$ (95% confidence interval). Statistical analysis and figures were obtained using GraphPad Prism version 5.0. for Windows, GraphPad Software, San Diego California USA.

RESULTS AND DISCUSSIONS

EAC volume, concentration of EAC cells and cell viability

The EAC volume and the difference in body weight remained unchanged in all treated groups as compared with the untreated EAC control group. However, significant decrease in EAC cell concentration and viability were found in pre-treated EAC mice, but not in post-treated ones. Therefore, the present data suggest that pre-treatment interferes more significantly with the establishment of ascites, than post-treatment (Tab. 1).

The EAC development was responsible for significant increase in white blood cells (WBCs) count (Tab. 1). Pearson correlation test, also, proved a good correlation between WBCs and variation in body weight ($r=0.51$, $p<0.05$), and,

Tab. 1 Effects of the Viscum album extract on the variation on body weight, WBCs count, ascites volume, EAC cell concentration and EAC cell viability

	WBCs (10^3/µl)	Dif b.w.(%)	EAC vol (ml)	Cell conc. (10^3/µl)	non viable cells (%)
Control	6.85±0.74	-7.19±2.32	-	-	-
Viscum album	6.80±0.76	-10.06±0.96	-	-	-
EAC	21.98±5.19 [a]	34.12±5.21 [a]	8.44±1.8	73.75±26.65	6.64±2.74
VA + EAC	23.39±2.66	34.51±7.05	9.44±0.91	22.00±1.95[b]	14.96±5.17 [b]
EAC + VA	55.70±19.54 [b]	46.68±6.88	9.43±0.96	77.67±29.66	0.17±0.00

Ehrlich Ascites Carcinoma inoculated mice (EAC), Ehrlich Ascites Carcinoma inoculated mice previously treated with mistletoe extract (V + EAC) and Ehrlich Ascites Carcinoma inoculated mice treated with mistletoe extract after inoculation (EAC + V).
Data are expressed as mean ± S.E.M for 8 mice per group.
[a]= $p<0.05$ as compared to Control group, [b] = $p<0.05$ as compared to EAC group

EAC volume (r=0.68, p<0.05), but not with EAC cell concentration; therefore elevated WBCs count may be assumed to EAC development.

However, post-treatment increased much more the WBCs, while pre-treatment showed no effect. VA extract, in EAC free mice, had no effect on the WBCs count. Our previous studies showed that the mistletoe extract did not influence the peritoneal fluid cytology either (data not shown).

Activities of antioxidant enzymes

Antioxidant enzymes (CAT, XOD, Px) were examined in the blood samples of all experimental groups (Fig. 1).

In EAC free mice, VA therapy induced no changes in antioxidant enzymes activity. Furthermore, the establishment of EAC was associated with elevated enzymatic antioxidant activity; post-treatment with VA had no influence, while pre-treatment provided some protection. All antioxidant enzymes were significantly decreased as compared with EAC untreated group, although they remained higher than control. In fact, the enzyme profile followed the same trend like the tumor growth, the intensity of antioxidant activity was highly positive correlated with the EAC development (variation of the body weight and EAC volume) (CAT/b.w. dif %, r=0.86 p<0.001, XOD/b.w. dif %, r=0.85 p<0.001, and respectively Px/b.w. dif %, r=0.87 p<0.001). The high positive correlation was, also, present among the antioxidant enzymes.

The activity of the antioxidant enzymes (XOD, CAT, Px) in EAC cells is shown in Fig. 2.

The antioxidant enzyme activity was significantly different between untreated EAC group and the other two groups treated with the *Viscum album* extract. In pre-treated VA tumor cells, CAT activity was significantly lower, and XOD activity increased in a significant manner, whereas post-treatment had no influence on these two enzymes. The single elevated enzyme in both VA groups was the Px, however it had elevated activity in pre-treated tumor cells as compared with post-treated ones. Changes were correlated with the reduction of tumor cells concentration in ascites fluid; an acceptable correlation (0.25<r<0.5) between antioxidant enzyme activity (CAT, XOD, Px) and EAC cell concentration was found. The only positive correlation was between plasma CAT and tumor cell CAT (r=0.82, p<0.001), the other two enzymes showed negative correlation between plasma activity and activity in EAC cells, XOD (r= -0.7, p<0.001) and Px (r= -0.5, p<0.05).

Previous *in vivo* and *in vitro* studies proved that *Viscum album* extracts had certain antitumor

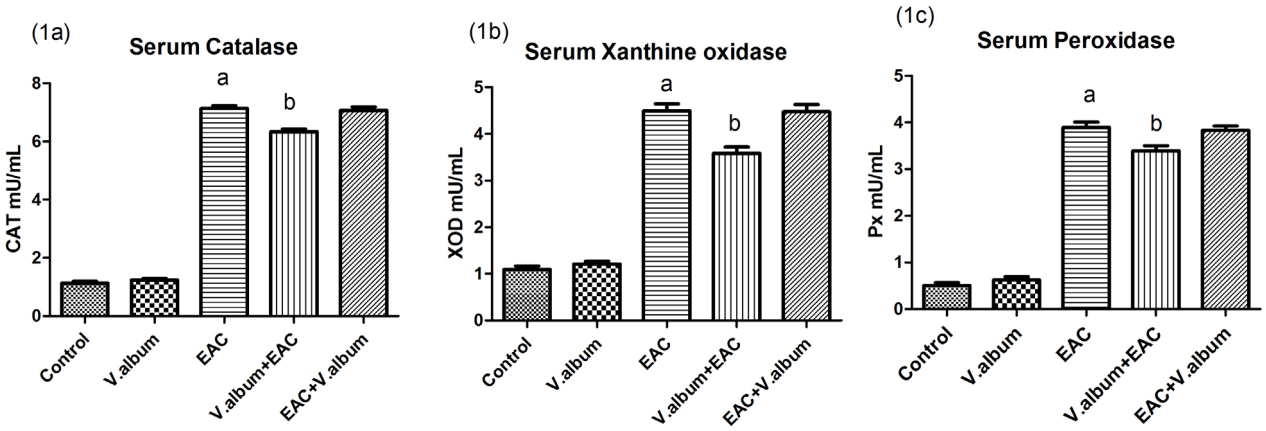

Fig. 1. Effects of *Viscum album* extracts on plasma levels activities of antioxidant enzymes (mU/mL)
1a) catalase (CAT), 1b) xanthine oxidase (XOD), and 1c) peroxidase (Px)
Control mice (Control), mistletoe extract inoculated mice (V album), Ehrlich Ascites Carcinoma inoculated mice (EAC), Ehrlich Ascites Carcinoma inoculated mice previously treated with mistletoe extract (V album + EAC) and Ehrlich Ascites Carcinoma inoculated mice treated with mistletoe extract after inoculation (EAC + V album).
Data are expressed as mean ± S.E.M for 8 mice per group.
[a]= p<0.05 as compared to C group, [b] = p<0.05 as compared to EAC group

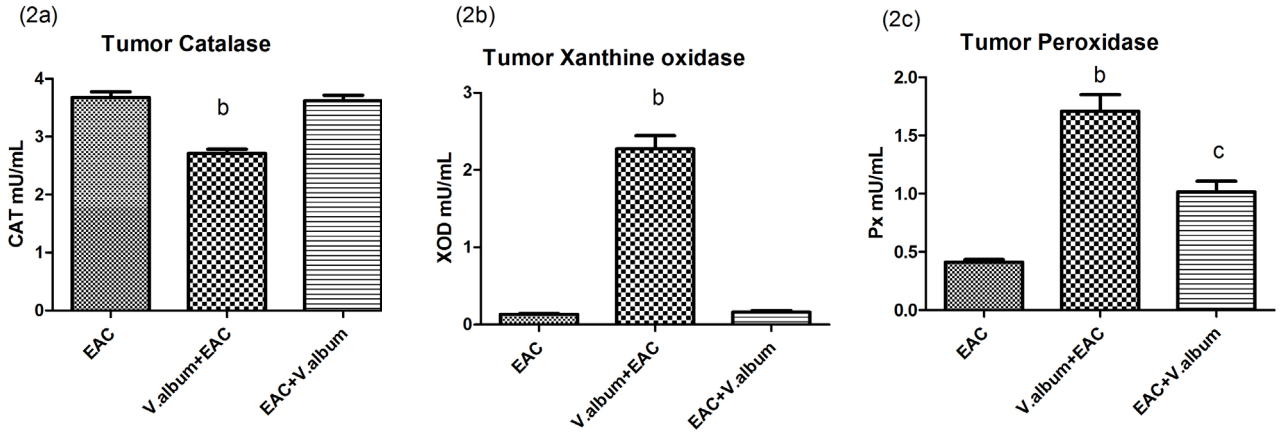

Fig. 2. Effect of *Viscum album* extracts on tumor levels activities of antioxidant enzymes (mU/mL)
a) catalase (CAT), b) xanthine oxidase (XOD), and c) peroxidase (Px)
Ehrlich Ascites Carcinoma inoculated mice (EAC), Ehrlich Ascites Carcinoma inoculated mice previously treated with mistletoe extract (V album + EAC) and Ehrlich Ascites Carcinoma inoculated mice treated with mistletoe extract after inoculation (EAC + V album).
Data are expressed as mean ± S.E.M for 8 mice per group.
[2] = $p<0.05$ as compared to EAC group

effects. Antitumor mechanism is still to be establish, but two distinct activities represent the most accepted hypothesis, firstly throughout the enhancement of the immune response via activation of NK cells and T lymphocytes, secondly by their direct cytotoxicity against tumor cells (Tabiasco *et al*., 2002, Khil *et al*., 2007).

The mistletoe cytotoxicity is based mainly on the toxic polypeptides with low molecular mass known as viscotoxins. Their mechanism is largely unknown, but recent evidence involves the reactive oxygen species (ROS). Oxidative stress plays a critical role in loss of mitochondrial membrane potential induced by *Viscum album* viscotoxins; antioxidants (such as N-acetyl-L-cysteine and cyclosporine A) prevent the loss of mitochondrial membrane potential and cell death. A possible mechanism for generation of ROS is the activation of c-Jun NH_2-terminal kinase (JNK), which was previously known as a downstream target ROS (Kim *et al*., 2004). Interestingly, *Viscum album* extracts act differently on normal cells. In non malignant cell line LLC-PK1, mistletoe lectins down regulated the synthesis of NO, O_2^- and OH^- radicals (induced by sodium nitropruside and pyrogallol), and, consequently, prevented the lipid peroxidation, and improved cell viability (Kim *et al*., 2010).

Ehrlich ascites carcinoma (EAC) is a widely used animal model, antitumor efficacy of various plant extracts representing one of its main application. Anticancer effect is quantified by attenuation of EAC-induced weight gain (Pai *et al*., 2012), decreasing in ascites volume and in viable cell count (Bhattacharya *et al*., 2011, Hossain *et al*., 2012). Reduction of the cancer systemic effects are other commonly used application of EAC in phytotherapy research; plant extracts improved the hematological parameters (Pai *et al*., 2012), oxidative stress markers in plasma (Bhattacharya *et al*., 2011) and liver (Pai *et al*., 2012, Hossain *et al*., 2012).

However, only few studies investigated the influence of antioxidant systems in antitumor effect of *Viscum album* extracts *in vivo*; one study evaluated the effect of mistletoe extracts on tumor implanted mice (simultaneously in females and males). Similar to our study, pre-treatment proves to interfere with the establishment of the ascites tumor growth more significantly than when it is administered simultaneously, or after EAC inoculation. The parameters evaluated in study were the EAC volume, cell concentration, and percentage of non-viable cells. From our point of view, the most suggestive parameter was EAC volume that reaches the lowest amounts in pretreated groups, both males and females, results confirmed by our findings. The other two parameters showed significant differences between males and females, the most obvious

in the percentage of non-viable cells, but the authors offered no clue for this finding (Cebovic *et al*., 2008). Interestingly, therapy with N-acetyl-L-cysteine (an antioxidant) provided a similar protective effect by reducing the EAC volume and cell concentration, but it had no effect on the cell viability (Cebovic *et al*., 2008).

In the available papers, the authors intended to explain the effects of *Viscum album* extract by direct action on tumor cells, although, from our point of view, this approach is accurate only in the case of *in vitro* models. *In vivo*, the therapeutic activity of VA active compounds is based on the direct activity against tumor cells, but also on the enhancement of immune response.

Our findings showed a clear correlation between the EAC development and plasma activity of the antioxidant enzymes, whether the increased oxidative stress was a direct result of tumor growth, or an effect of enhanced inflammatory status is to be ascertain. A significant correlation among total WBCs count, EAC volume and EAC cell concentration was also found, but the correlation between WBCs and activity of antioxidant enzymes was less obvious.

Pre-treatment with *Viscum album* extract down regulated the plasma oxidative stress, but no effect in post-treatment was found. Hence, it is more likely to exert an indirect effect, by reducing the ascites development, than a direct antioxidant activity. Mistletoe therapy was responsible for increased oxidative stress in EAC tumor cells followed by a reduction of tumor cells concentration, but in pre-treated group only, whereas the post treated group revealed less important changes in the enzyme activity. The oxidative changes were correlated with cell concentration in ascitic fluid. These findings suggest a significant ROS mediated antitumor effect, but not directly against the ascitic tumor cells. In our opinion, a specific immune cytotoxic response is likely to be involved. This supposition explains why pre-treatment was much effective, but the present study provided no prove to support the hypothesis. However, other studies suggested that mistletoe lectin enhances the immune system through modulation of lymphocytes, NK cells, and macrophages (Kim *et al*., 2009).

CONCLUSIONS

Viscum album L. alcoholic extract proved a significant, selective, antitumor effect, being able to reduce the EAC cell concentration and viability, in correlation with increased oxidative stress in tumor cells. In plasma, mistletoe extract provided also an antioxidant effect. All parameters in study revealed that the therapy prior to EAC inoculation was far more effective than the post therapy; therefore mistletoe extract seems more likely to wield an indirect immune mediated effect than a direct cytotoxicity on tumor cells.

Acknowledgments. Research and publications costs have been supported by POSDRU/89/1.5/S/62371.

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