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Complete List of Authors:	Frullini, Alessandro; Studio Medico Flebologico, Studio Medico Flebologico Da Pozzo, Eleonora; University of Pisa, Department of Pharmacy Felice, Francesca; University of Pisa, Department of Surgical, Medical and Molecular pathology and Critic area Burchielli, Silvia; CNR/Tuscany Region, Fondazione Toscana G. Monasterio Martini, Claudia; University of Pisa, Department of Pharmacy Di Stefano, Rossella; University of Pisa, Department of Surgical, Medical and Molecular pathology and Critic area
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Prevention of excessive Endothelin-1 release in sclerotherapy: *in-vitro* and *in-vivo* studies.

Alessandro Frullini[*], MD; Eleonora Da Pozzo[†], PharmB, ClinChem, PhD ; Francesca Felice[‡], ScB, PhD; Silvia Burchielli[§], ScB; Claudia Martini[†], PharmSc, Prof; Rossella Di Stefano[‡], MD.

[*]Studio Medico Flebologico, Figline Valdarno- Florence, Italy; [†]Department of Pharmacy, University of Pisa, Italy; [‡]Department of Surgical, Medical and Molecular pathology and Critic area, University of Pisa, Italy; [§]Fondazione Toscana G. Monasterio, Pisa, Italy.

Corresponding authors: Eleonora Da Pozzo, Department of Pharmacy, University of Pisa, 6, via Bonanno, 56126 Pisa, Italy, eleonora.dapozzo@farm.unipi.it; Alessandro Frullini, loc Viesca al borro 104, 50066 Reggello, Florence-Italy, info@venevaricose.it.

Running head: ET-1 release in sclerotherapy

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ABSTRACT

Background: The foam sclerotherapy technique has become one of the most commonly used treatments for superficial venous insufficiency. Despite excellent results, few visual/neurological disturbances have been recently reported; their pathogenesis is still debated but a correlation with Endothelin-1 release from the treated vein has been proposed.

Objective: The purpose of this work was to evaluate the Endothelin-1 release following sclerotherapy and to investigate the effects of the anti-endothelin drug Aminaphtone.

Methods&Materials: As in-vitro sclerotherapy model, an endothelial cell culture, mimicking vascular endothelium, was pre-treated with Aminaphtone and exposed to detergents. Cell survival and Endothelin-1 release were measured. In in-vivo experiments, forty-five rats, fed with different Aminaphtone-rich diets, were subjected to sclerotherapy and the systemic Endothelin-1 was measured.

Results: Aminaphtone cell exposure caused a statistically significant reduction in Endothelin-1 release, both before and after in-vitro sclerotherapy. Rats fed with Aminaphtone showed a trend towards reduced mortality and a significant decrease of Endothelin-1 release after sclerotherapy.

Conclusions: This is the first study in which an anti-endothelin agent was able to cause a significant reduction of Endothelin-1 release during sclerotherapy. Although clinical studies are required, these findings might advocate the use of anti-endothelin agents in prophylaxis of neurological or visual disturbances after sclerotherapy.

INTRODUCTION

Foam Sclerotherapy (FS) has truly revolutionized the treatment of Chronic Venous Insufficiency allowing saphenous insufficiency to be treated with excellent results at short and medium follow up. Longer follow up is even better for other ablation techniques but, due to the minimal invasiveness of FS, this approach is becoming one of the most widely used worldwide (1,2,3,4,5). Another possible approach involves Liquid Sclerotherapy (LS), which has been practiced for a long period of time and is still the most commonly used treatment for minor varicosities (6,7).

The safety of sclerotherapy is well known and despite the large number of treatments per year, very few complications are reported (8,9). One of the most concerning side effects is the rapid onset of neurological and visual disturbances (10,11,12). Jia reported a 1.4% incidence of visual and neurological disturbances in a meta-analysis of 69 studies on more than 9000 patients (13). Some papers reported visual disturbances after FS of up to 14% (14,15,16,17). Conversely, a study by Raymond-Martimbeau reported an incidence of 0.21% in transient events (18). Notwithstanding the benign outcome of the vast majority of occurrences, few cases of more severe complications have been reported (19,20,21,22,23).

The disturbances are more frequent in patients with a history of migraine with aura, as some researchers believe that these are true migraine episodes (24,25,26). Actually, the role of micro-bubbles and their possible migration through a patent foramen ovale (PFO) in the left circulation and subsequent embolization are the classical explanation for visual and neurological disturbances after FS (27). To emphasize this point, the presence of air has been documented in the cerebral circulation after FS (28,29). This theory has been recently criticized by our group (30,31). In fact, even at a lower percentage, visual and neurological symptoms have been reported after LS where air is not used. It is also very difficult to postulate micro-embolization only in the cerebral district or in the retina (e.g. the largest concentration of bubbles should be in the aortic root but coronary symptoms are not reported as complications of FS). For these reasons, it is more reasonable that other factors contributed to cause the visual and neurological symptoms after sclerotherapy. In

particular, in a previous study we demonstrated a significant increase in Endothelin-1 (ET-1) blood levels in rats treated with the sclerosant drugs polidocanol (POL) and sodium tetradecyl sulfate (30). Moreover, in a small group of 11 patients, ET-1 blood levels were measured before and after sclerotherapy, demonstrating similar results (31). Recently, our group introduced the administration of Aminaphtone (AMNA) associated to sclerotherapy. AMNA is a well-known capillary-protector molecule with an anti-Endothelin effect; it down-regulated the ET-1 production on ECV304 cells (32), a permanent cell line exhibiting both endothelial and epithelial characteristics (33).

Based on these preliminary findings, the initial aim of the study was to investigate the release of ET-1 using an *in-vitro* model consisting of cultured Human Umbilical Vein Endothelial Cells (HUVEC), exposed to a sclerosant solution, in the presence or absence of AMNA pre-treatment. We also investigated whether a daily AMNA-rich diet was able to exert positive effects in rats subjected to foam sclerotherapy.

METHODS

Materials.

Endothelial basal medium and supplements (EBM-2 Bullet kit) were purchased from Lonza (Walkersville, MD,US). Protease inhibitors cocktail (1:800) and sodium ortovanadate (0.2 mM) were purchased from Sigma-Aldrich (StLouis, MO,US). Polidocanol was obtained from Kreussler Pharma (Weisbaden,Germany). Aminaphtone powder was supplied by Laboratori Baldacci SpA (Pisa,Italy). All other materials or chemicals were obtained through standard commercial sources.

Cell Cultures.

HUVECs derived from umbilical veins of three different fresh cords as previously reported (34). Cells were cultured in endothelial basal medium supplemented with VEGF, basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, ascorbic acid, hydrocortisone and 5% fetal bovine serum. HUVEC phenotype was confirmed by their cobblestone morphology and by the expression of von Willebrand factor. Cells from early passages were plated on gelatin-coated 96 well plates and treated at sub-confluence.

Cell survival in the presence of AMNA.

HUVEC were plated in 24-well multiplates (12,000 cells/cm^2), and maintained at 37°C and 5% CO_2. These culture conditions ensured in two days the well almost complete cell coverage (90% confluence), thus resembling an endothelial surface (35). 24 hours after seeding, the cell medium was replaced with fresh medium containing DMSO (control) or 6 μg/ml of AMNA dissolved in DMSO; 6 μg/ml dose corresponds to the peak plasma concentration observed after oral administration of AMNA 75 mg, a dosage currently used in therapy (36). At 0.5, 6, 24 and 72 hours of drug treatments, the living cell number was evaluated using a semi-automatic cell counter (Scepter 2.0, Millipore).

***In-vitro* model of sclerotherapy**.

HUVEC were plated in a 96-well plate (12,000 cells/cm^2) and maintained in fresh medium at 37°C and 5% CO_2 for 48 hours to mimic the endothelial surface. Then, the culture medium was replaced with fresh medium contained protease inhibitors and sodium orthovanadate (Supplemented Fresh Medium, SFM), and polidocanol diluted in medium at different concentrations (0.005-1 %), for 5 seconds (the lowest possible exposure time). Then, the medium was collected and cells were washed with warm phosphate-buffered saline (PBS). After 1 hour of cell recovery in SFM, the viability was determined by a tetrazolium salt based assay (Promega CellTiter 96® AQueous Cell Proliferation Assay), according to the manufacturers' instructions. The production of formazan in the cell culture was measured by absorbance at 490 nm, using a microplate reader (Victor Wallac 2, Perkin Elmer, CA, US). The obtained data were analyzed and reported as an EC_{50} curve using Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA 92037 USA).

The viable cells were also counted from photos taken by an inverted microscope (Hund, Wetzlar, Germany) equipped with a digital camera (Nikon, Sesto Fiorentino, Italy). Five fields (10x magnification) were captured for each sample. For each image, the total number of cells was quantified using the image analysis software ImageJ (public domain, Image Processing and Analysis in Java, National Institutes of Health).

ET-1 level measurement.

HUVEC were seeded in 96 well-plates (12,000 cells/cm^2). After 24 hours, the cell medium was replaced with fresh medium containing DMSO (control) or 6 µg/ml of AMNA, and drug treatments were continued for different times (0.5, 3, 6, 12 and 72 hours). At the end of the exposure times, the treated cell media were collected and the evaluation of the free ET-1 released by the cells was performed through an immunoenzymatic assay (Endothelin-1 ELISA kit, Enzo Life Science).

Furthermore, the measurement of ET-1 released by the cells was performed following the *in-vitro* sclerotherapy, as above described. Briefly, at the end of the above exposure times, the DMSO or AMNA treated cell media were replaced with SFM containing POL at 0.050% or 0.50%. After 5 seconds, the medium was collected from each well and the amount of ET-1 was evaluated.

Animals.

Adult male Wistar rats, with an average weight of 300 g, were fed a standard diet for two weeks to acclimatize. All procedures were approved and performed under the guidelines of the Directive 2010/63/EU of the European Parliament and of the Council, and the Guide for the Care and Use of Laboratory Animals by the local Ethics Committee.

***In vivo* sclerotherapy model.**

Forty-five Wistar rats were divided into three groups, containing 15 rats. Different concentrations of AMNA were orally administered in the diet to Wistar rats for 15 days: Group I (GI) received a standard diet (control), Group II (GII) received 30 mg/kg/day AMNA and Group III (GIII) received 150 mg/kg/day AMNA. After two weeks, all rats underwent FS. The animals were anesthetized with Zolazepam/Tiletamine (40 mg/kg) and Xilazina (5 mg/kg). After exposure of the left femoral vein at the groin and cannulation, we injected 0.2 mL Polidocanol 1% foam produced with the Tessari method (ratio 1:3) (10) into the femoral vein. 1 mL of blood was withdrawn from the aorta, before sclerosant injection (t0), and after five minutes (t5). All blood samples were centrifuged and plasma stocked at -20°C until ET-1 dosage. ET-1 was measured using the immunoenzymatic assay.

Statistics.

Statistical analyses were performed using the Graph-Pad Prism 5 software (GraphPad Software, Inc., La Jolla, CA 92037 USA). The analyses of *in-vitro* and *in-vivo* data were done using the One-Way Anova analysis of variance followed by Bonferroni's Multiple Comparison test. A p value of <0.05 was considered statistically significant. All data, derived from three independent experiments, were presented as mean ± SD.

RESULTS

Effects of AMNA on Cell Viability

The treatment with AMNA 6 µg/ml did not significantly affect cell viability. The viable cell count in the AMNA treated sample is similar to that of control for each exposure time (data not shown), confirming that AMNA 6 µg/ml is very safe for endothelial cells.

***In-vitro* sclerotherapy model**.

To perform the *in-vitro* sclerotherapy model, we determined the effect of polidocanol on cell survival. HUVEC survival after polidocanol treatment showed a concentration-dependent modality. As shown in figure 1 (panel A), after administration of the sclerosant drug at low concentrations (0.005-0.025%), the amount of proliferating HUVECs did not change with respect to the control. At higher concentrations (0.05-1 %), there was a rapid reduction in cell viability. The EC_{50} curve calculation reported a value of 0.0489% for the half-maximal effective concentration of polidocanol after 5 seconds of exposure time. This concentration was used as the reference dose for the following experiments.

-Figure 1-

The microscopy analysis (figure 1, panel B, C and D) showed similar results: after treatment with 0.05% and 0.5% polidocanol, the viable cells resulted to be 44.36% and 2.25% respectively.

ET-1 cell release measurement before and after *in–vitro* sclerotherapy.

Before performing *in-vitro* sclerotherapy, we assessed the amount of ET-1 collected in the medium after AMNA exposure that represent the ET-1 released by the cells. Our baseline values are in accordance with previous literature (37). As reported in figure 2, the exposure of HUVECs to AMNA resulted in the reduction of free ET-1 levels. This reduction is statistically significant after 6 ($p<0.01$) and 12 hours ($p<0.05$), with respect to control.

-Figure 2-

A small amount of ET-1 normally accumulates in vesicles inside the cell (38). The detergent polidocanol is able to destroy cells by disrupting cellular and organelle membranes (39), thus the *in-vitro* sclerotherapy model was used to evaluate the amount of ET-1 accumulates in the cells. We supplemented medium with protease inhibitors in order to limit the protein degradation related to lysing enzyme release. The figure 3 showed the ET-1 levels measured in the medium immediately after POL treatment; such ET-1 represent the intracellular Endothelin, which is released following cell and vesicular membrane lysis. The concentrations of intracellular ET-1 were reduced in cells pre-treated with AMNA for 6 hours ($p<0.001$), with respect to control. Notably, the values of ET-1 found after the administration of polidocanol 0.05% (the concentration causing the 50% of cell lyses) (figure 3, panel A) were about the half of those found after treatment with polidocanol 0.5% (the concentration causing the 100% of the cell lyses) (figure 3, panel B), confirming our previous data.

-Figure 3-

Prior studies on endothelial cells have suggested the storage of ET-1 in storing granules (39), but to the best of our knowledge, no *in-vitro* studies have quantified the ratio between the intracellular stored ET-1 and the constitutively secreted ET-1. Anyway, our finding about intracellular and secreted ET-1 are in agreement with a previous study, reporting that only comparatively low amounts of vesicular ET-1 could be recovered from large quantities of endothelial cell homogenates (40).

Protective effects of oral pre-medication with AMNA *in-vivo.*

The rat animal model was used to test ET-1 release after sclerotherapy in previous studies (30). Here, out of 15 rats per group, 6 rats in GI (normal diet), 2 rats in GII (diet with 30 mg/kg/day AMNA) and 3 rats in GIII group (diet with 150 mg/kg/day AMNA) died within five minutes since sclerosant injection (t5) (Figure 4, panel A). These findings showed that pre-treatment with oral AMNA reduced immediate rat mortality (40% in controls, 13.3% in GII and 20% in GIII) after sclerotherapy, nevertheless the data failed to reach significance (Fisher's exact p value = 0.21).

Regarding ET-1 levels, figure 4 (panel B) shows ET-1 production after AMNA premedication in rats treated with 1% polidocanol. No difference was observed at t0 between groups, except for a trend toward increase in the levels of ET-1 in GIII group. Five minutes (t5) after the sclerosant injection, GII rats exhibited a significant reduction in ET-1 plasma levels compared with rats receiving a normal diet (GI) or a diet highly AMNA-enriched (GIII) ($p<0.05$ vs GI and $p<0.05$ vs GIII, respectively). Surprisingly, the GIII animals, receiving the highest amount of AMNA in diet, had a slight ET-1 increase at t0 and a lesser ET-1 plasma level reduction than the GII rats; this finding could be of interest for further and more in-deep investigations.

-Figure 4-

DISCUSSION

Sclerotherapy is a safe and very common treatment for venous insufficiency, used in both minor cosmetic conditions (i.e. telangiectasia or reticular veins) or in larger veins, such as truncal disease involving the great or the short saphenous veins. Sclerotherapy is also very commonly used for the treatment of recurrent varicose veins or for vascular malformations (2, 4, 5, 6).

Despite the enthusiastic use of foam sclerotherapy and the proven safety of this procedure, the slight number of reports of transient visual and neurological disturbances require more investigation (13). Indeed, the European guidelines for sclerotherapy in chronic venous disorders recommend caution in the use of FS with patients with a history of migraine attacks associated with visual and neurological disturbances (41). However, the same adverse events have been also reported with

liquid sclerotherapy and this is in conflict with the explication postulating the bubbles micro-embolization as cause of the adverse effects (16). We have demonstrated the ET-1 release after sclerotherapy, both with liquid and foamed agents, in rats and in patients (30,31). Thus, based on these findings, we have hypothesized that after sclerotherapy, the released ET-1 reaches the right heart chambers and, in the absence of a PFO, spreads into the pulmonary circulation, where a large number of ET receptors are present and are able to bind the blood ET-1. Moreover, ET-1 has a very short half-life and the passage through the pulmonary circulation actually delays the arrival of ET-1 to the left chambers of the heart. In contrast, patients with PFO might have a faster passage of ET-1 rich blood to the left heart, and a lower amount of ET-1 could be sequestered by pulmonary ET receptors. This hypothesis could explain the higher frequency of sclerotherapy-induced transient neurological and visual disturbances in patients with PFO (15).

Up to now, we postulate five variables that could affect the chance of developing such complications: 1) higher amount of ET-1 release due to larger volumes of sclerosants or basal augmented release from the endothelium; 2) presence of a PFO with fast passage of ET-1 rich blood into the left ventricle; 3) incomplete vein spasm immediately after injection causing a prolonged release of ET-1 from the irritated endothelium; 4) patient variability (e.g. migraine patients); 5) concomitant use of drugs with anti-endothelin action. The combination of these five variables could explain why some patients show side effects and other do not.

In conclusion, this study confirmed that HUVEC produces and releases high levels of ET-1, and that the pre-treatment with AMNA reduces both released and intracellular levels. These data agree with the previously demonstrated inhibitory effect of AMNA on ET-1 production, in the cell line ECV 304, probably due to an interference with the pre-pro-ET-1 gene transcription (32). Significant functional and phenotypic differences reported in the literature between HUVEC and ECV304 (42, 43, 44) suggest that ECV304 derived from bladder carcinoma cell line T24 (45, 46), rather than from vein endothelial. In this light, here, for the first time, the influence of AMNA on ET-1 levels was directly evaluated on primary human endothelial cells. A cellular model is not strictly

comparable with a living system and its multiple variables, but our goal was to investigate the relationship between AMNA and ET-1 release. In this study, we demonstrated that a single administration of AMNA reduces in 6 hours the levels of secreted and cellular stored ET-1. It is plausible that reduced levels of ET-1 could be maintained subjecting the cells to recurrent administrations of AMNA. In this manner, AMNA could be considered a preventive drug; when given prior sclerotherapy, it could be reduced ET-1 release levels during sclerotherapy.

Establishing the drug efficacy in a laboratory setting and translating that experience into a therapy is a difficult and complex task, but the significant reduction of ET-1 levels by AMNA pre-treatment, and the excellent safety profile of this drug, suggest that a similar approach on sclerotherapy patients could be useful. However, our work is in its early stages and clinical studies are required before supporting the systematic use of anti-endothelin agents in prophylaxis of disturbances after sclerotherapy.

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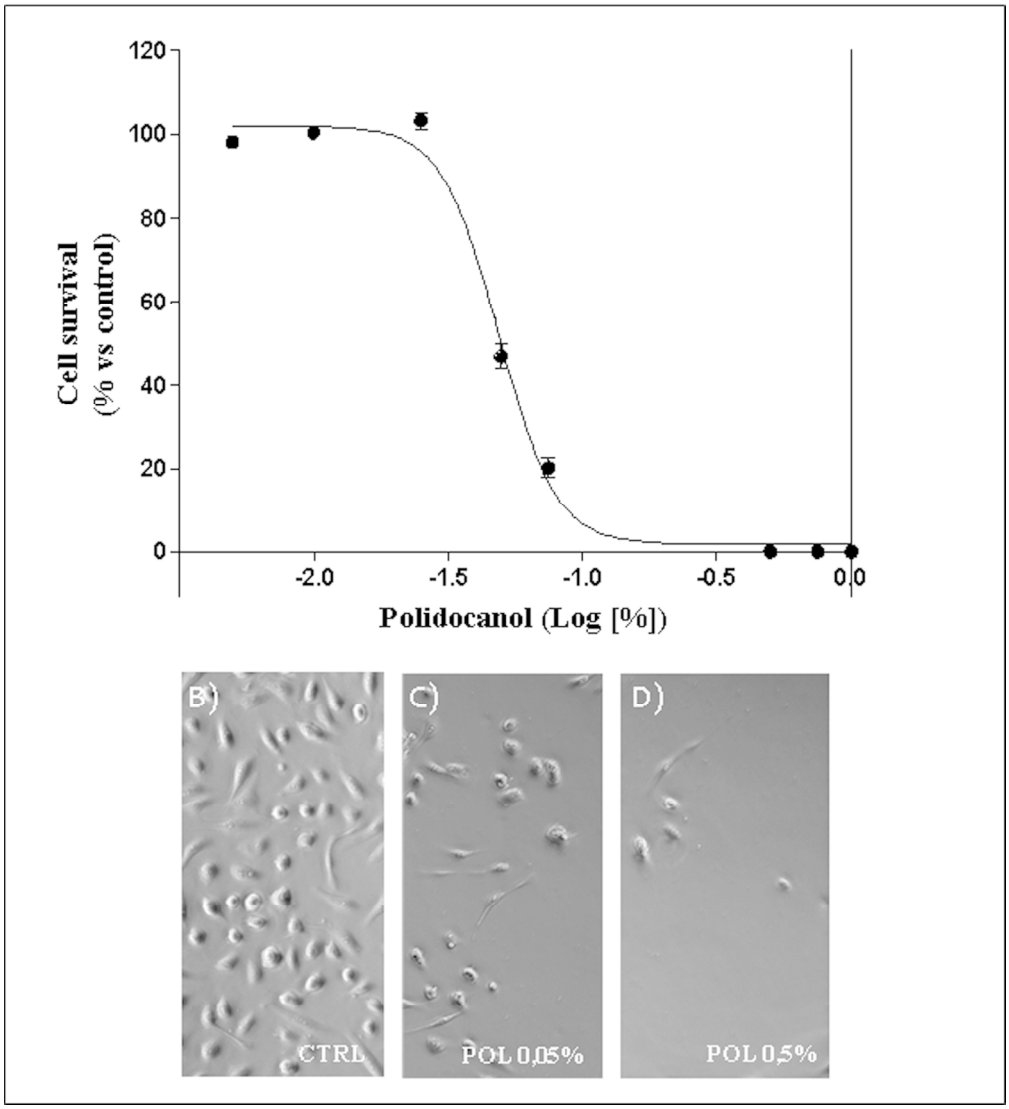
Figure Legends

Figure 1: ***In-vitro*** **sclerotherapy model**. (**A**): HUVEC survival after polidocanol exposure, as shown by EC_{50} curve; (**B,C,D**): representative pictures of HUVECs control (B), HUVECs after polidocanol exposure (C: 0.05% and D: 0.5%) (10x magnification). Data are expressed as mean ± SD.

Figure 2: **ET-1 cell release.** Levels of ET-1 assessed in the medium from HUVEC, following DMSO (white bars) or AMNA (grey bars) cell exposure, at different incubation times. Data are expressed as ET-1 (pg/ml) mean values ± SD. **p<0.01 AMNA 6h vs DMSO 6h; *p<0.05 AMNA 12h vs DMSO 12h.

Figure 3: **ET-1 cell release after *in-vitro* sclerotherapy**. (**A**): levels of intracellular ET-1 in cells pre-exposed to DMSO or AMNA for different incubation times, and then exposed to 0.05% POL; (**B**): levels of intracellular ET-1 in cells pre-exposed to DMSO or AMNA, for different incubation times, and then exposed to 0.5% POL. Data are expressed as ET-1 (pg/ml) mean values ± SD. ***p<0.001 AMNA 6h vs DMSO 6h.

Figure 4: ***In-vivo*** **sclerotherapy model**. (**A**): Rate of animal survival after sclerosant injection in rats treated without AMNA (GI), 30 mg/kg/day AMNA (GII) and 150 mg/kg/day AMNA (GIII); (**B**): ET-1 plasma levels before sclerosant injection (t0), and after five minutes (t5), in control rats and in rats treated with AMNA-enriched diet. Data are expressed as mean ± SD *p<0.05 t5 GII vs t5 GI.



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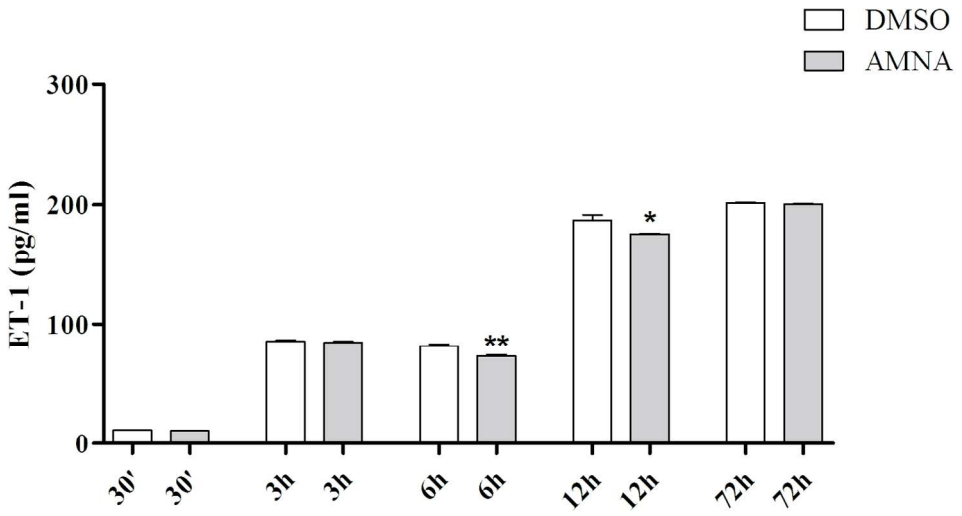
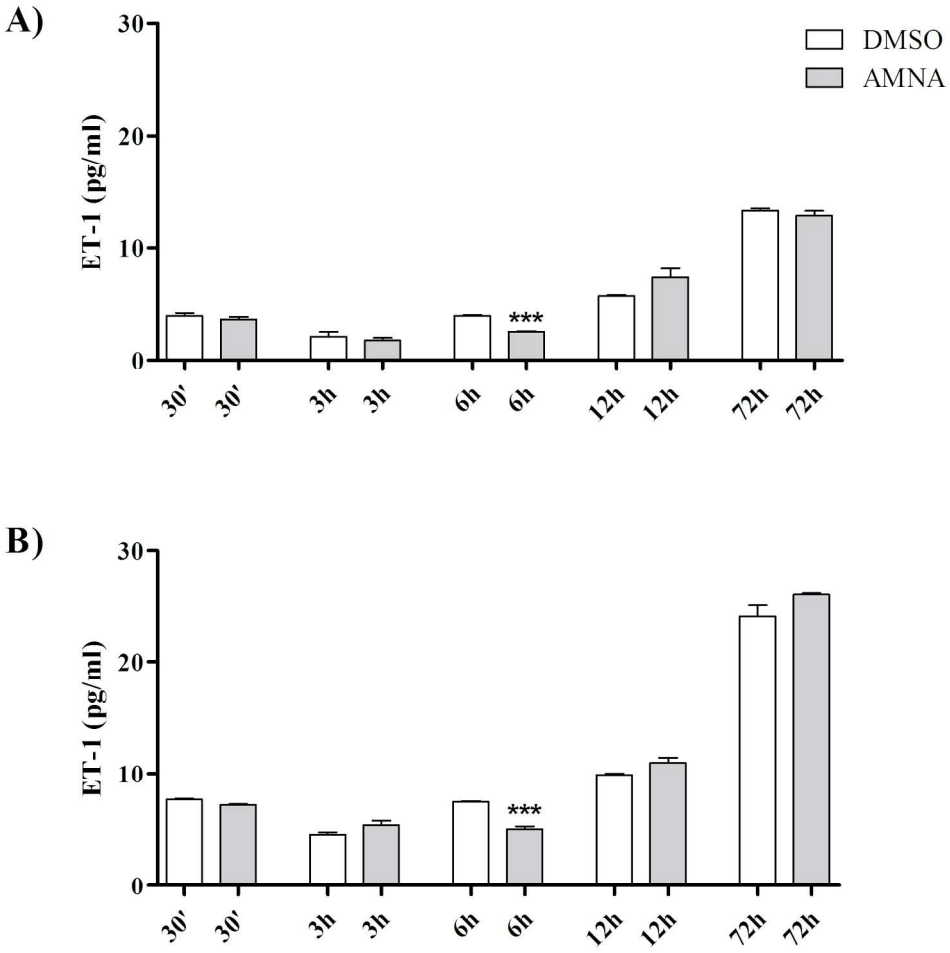
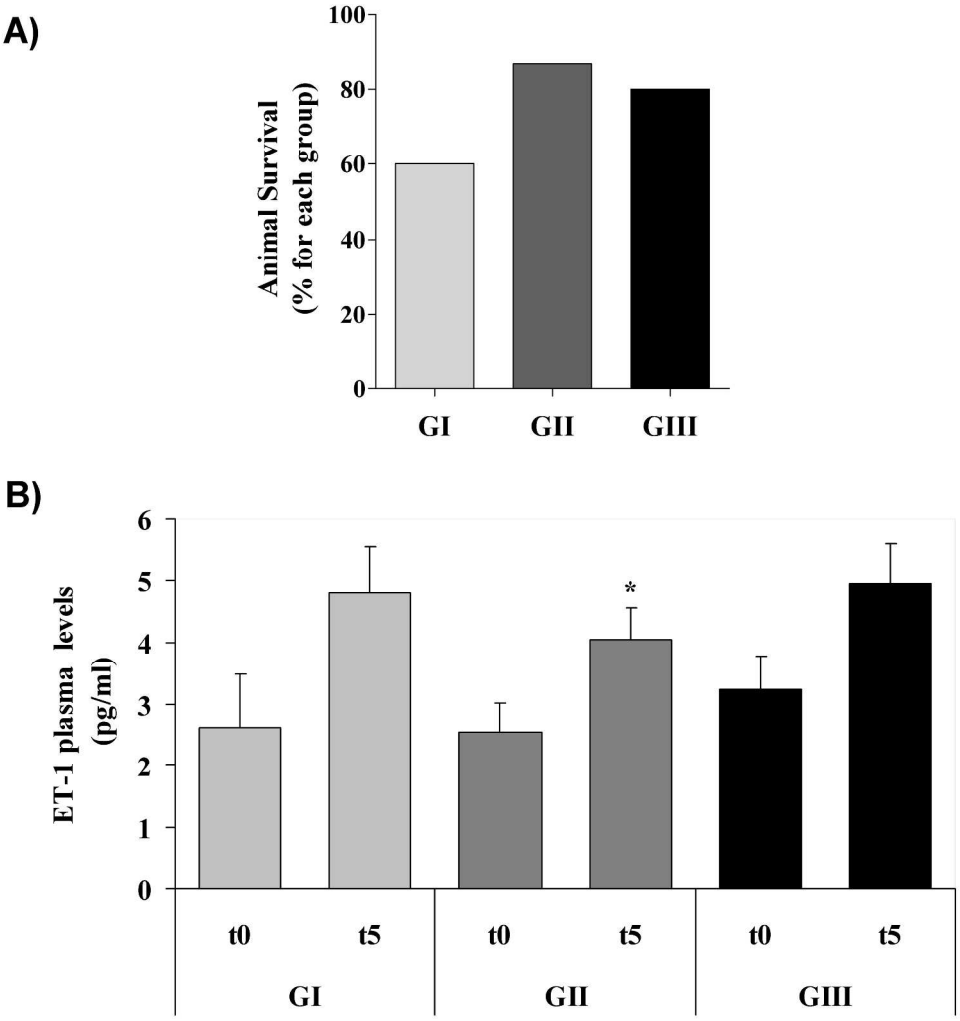


141x77mm (300 x 300 DPI)



189x194mm (300 x 300 DPI)



155x172mm (600 x 600 DPI)