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RESEARCH ARTICLE

Alrp, a survival factor that controls the apoptotic process of regenerating liver after partial hepatectomy in rats

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Abstract

Augmenter of Liver Regeneration (Alrp) enhances, through unknown mechanism/s, hepatocyte proliferation only when administered to partially hepatectomized (PH) rats. Liver resection, besides stimulating hepatocyte proliferation, induces reactive oxygen species (ROS), triggering apoptosis. To clarify the role of Alrp in the process of liver regeneration, hepatocyte proliferation, apoptosis, ROS-induced parameters and morphological findings of regenerating liver were studied from PH rats Alrp-treated for 72 h after the surgery. The same parameters, evaluated on regenerating liver from albumin-treated PH rats, were used as control. The results demonstrated that Alrp administration induces the anti-apoptotic gene expression, inhibits hepatocyte apoptosis and reduces ROS-induced cell damage. These and similar data from *in vitro* studies and the presence of 'Alrp homologous proteins' in viruses as well as in mammals (i) allow to hypothesize that Alrp activity/ies may not be exclusive for regenerating liver and (ii) suggest the use of Alrp in the treatment of oxidative stress-related diseases.

Keywords: *Liver regeneration, oxidative stress, hepatocyte survival, mitochondrial protection*

Abbreviations: *Alrp, Augmenter of Liver Regeneration protein; rAlrp, recombinant Alrp; Erv1, Essential for respiration and vegetative growth; HPS, high power fields; PBS, phosphate buffer saline; BSA, Bovine serum albumin; ROS, Reactive Oxygen Species.*

Introduction

Augmenter of Liver Regeneration (Alrp), identified by us, purified to homogenicity, sequenced and determined in its physical and chemical properties [1,2], was initially named Hepatic Stimulatory Substance (HSS) and characterized as an hepatic growth factor [3].

In rats, Alrp-mRNA is present in almost all tissues in relatively small amounts; large quantities have been found in testis, muscle, the nervous system and liver [2], suggesting that Alrp may play a fundamental role in the function of these organs. Recently we demonstrated that Alrp is present in human myocytes of healthy subjects [4].

Alrp sequences have been identified in all organisms [5] ranging from viruses [6] to insects [7], mammals [4,8,9] and even in photosynthetic organisms [10]. Alrp has been demonstrated to be structurally and functionally homologous to Erv1, a protein, present in *Saccharomyces cerevisiae*, essential

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for mitochondrial biogenesis and yeast viability [11]. Heterologous gene complementation studies in yeast have confirmed the functional similarities between Alrp and Erv1 [12].

Although different in their molecular weight and cytoplasmic localization [8,13,14], all 'Alrp homologous proteins' contain a well-conserved amino acid domain at the C-terminus part responsible for their sulphydryl oxidase enzymatic activity [10,15].

It is known that recombinant Alrp (rAlrp) administration, (i) in partially hepatectomized rats, increases cell proliferation [1,3] and ameliorates cellular tropism via 'augmenting' cell volume [16] and (ii) in intact rats, induces mitochondrial Transcription Factor A (mtTFA) expression, a mitochondrial DNA regulatory factor, determining mtDNA transcription increase, ATP production and oxygen consumption [17].

Recently, in different experimental models of cell proliferation or apoptosis, it has been documented that Alrp acts as an anti-apoptotic/cell survival factor [18–22]. So far no data are available on Alrp anti-apoptotic activity in the animal model of cell proliferation triggered by 70% liver resection.

The aim of the present study was to evaluate the anti-apoptotic/cell survival capacity/ies of Alrp on regenerating liver after 70% partial hepatectomy (PH). Recombinant Alrp was administered to PH rats (500 ng/every 8 h for a period of 72 h after PH), starting immediately after surgery. Albumin-treated PH rats (500 ng/every 8 h for a period of 72 h after PH) were used as control. The animals were sacrificed at different times after surgery and hepatocyte apoptosis and proliferation, PH-induced oxidative stress parameters (clusterin expression, lipid peroxidation, protein carbonylation) and morphological findings (histology and electron microscopy) were evaluated on liver tissue (Table I).

Experimental procedures

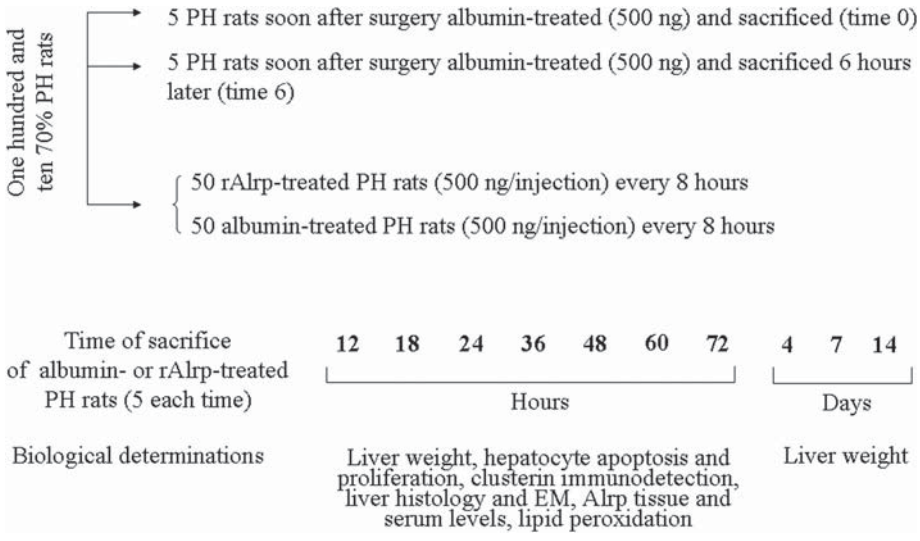
Reagents

Recombinant Alrp (rAlrp), expressed in transfected COS-1 cells and monoclonal antibody against rAlrp were produced and donated by the Laboratory of Biochemistry and Molecular Biology, University of Georgia, Athens, GA. As other investigators, we used a 15 kDa MW recombinant isoform of Alrp, which contains the well conserved, biologically active, C-terminal part [17,23–26]. Albumin and other chemical reagents were purchased, if not specifically reported, by Sigma-Aldrich (Milan, Italy). The polyclonal antibody against Alrp (a gift by Dr T. Lisowsky) had already been used for specific identification of Alrp [4,8,13,18]. To raise the Alrp antibody a purified hexahistidinyl-tagged carboxyl-terminal fragment of Alrp (residues 81–205) was used [8,13]. The antibody against rat thioredoxin was purchased from Imcocorp (Stockholm, Sweden).

Animals

One hundred and ten F344 male rats, weighing 180–200 g, purchased from Charles River (Milan, Italy), were used. The animals, housed in a light (7 am–7 pm) and temperature (21 ± 1°C) controlled room, could eat and drink *ad libitum*. They were cared for according to the standard procedures indicated in the 'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health (NIH 80–23). Seventy per cent partial hepatectomy was performed according to Higgins and Anderson [27], under deep ether anaesthesia. As reported in Table I, 10 PH rats were albumin-treated, (i.p., 500 ng/rat at the time of PH), five were sacrificed immediately after surgery ('time 0' group) and five 6 h later ('time 6' group). The remaining 100 PH rats were divided into two groups of

Table I. Protocol of PH rats treatment and biological evaluations.



50 animals each: one group was treated with multiple (every 8 h) rAlrp injections (rAlrp-treated PH rats, 500 ng/injection, i.p.), starting soon after the surgery and sacrificed, five each time, at 12, 18, 24, 36, 48, 60, 72 h and at 4, 7 and 14 days; the other 50 PH rats, later on called 'albumin-treated PH rats', were treated with multiple (every 8 h) albumin injections (500 ng/injection, i.p.) and sacrificed following the same protocol as for rAlrp-treated PH rats. The dosage of rAlrp administered to PH rats is similar to that already used in other, *in vivo*, experiments [17,23,24]. In the rats sacrificed from 0–72 h, liver tissue and blood samples were taken to evaluate: (i) serum and tissue Alrp level, (ii) hepatocyte proliferation, (iii) hepatocyte apoptosis, (iv) pro- (Bax and Caspase 3) and anti-apoptotic (Bcl-2) gene expression, (v) tissue clusterin expression, (vi) lipid peroxidation, (vii) protein carbonylation, (viii) histological and (viiii) electron microscopy evaluation; in all animals, at the time of sacrifice, the weight of the regenerating liver was measured.

Biological determinations

Alrp serum and liver tissue determinations

Alrp serum evaluation

A standard enzyme-linked immunoassorbent assay (ELISA) procedure was used to evaluate Alrp serum levels. Briefly, high binding capacity ELISA 96-well plates were coated with a mouse monoclonal antibody to rAlrp. Wells were washed three times and incubated with the test samples or standard rAlrp. After rewashing and incubation with rabbit anti-rAlrp polyclonal antibody, the wells were incubated with anti-rabbit IgG conjugated to HRP. The colour was allowed to develop and the optical density of each well was measured on an ELISA plate reader (ELx 800, BioTex Instrument, Inc., Winooski, VT 05404, USA).

Western blot analysis

For liver cytosol preparation, tissues samples were homogenized in lysis buffer (100 mM Tris HCl pH 7.5, 300 mM NaCl, 4 mM EDTA, 0.1% SDS, 1% IGEPAL, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate) containing 1 × protease inhibitors (Complete^{TM}; Roche Applied Science, Milan, Italy) and centrifuged at 18 000 x g for 30' at 4°C. Protein concentration was evaluated by Bradford method (Bio-Rad Laboratories srl, Milan, Italy).

Aliquots of 20 µg of total proteins of each sample were loaded on 8% sodium dodecyl sulphate-polyacrylamide gels (Bio-Rad Laboratories srl). Proteins were then transferred onto a nitrocellulose membrane probed with primary antibody specific for Alrp and thioredoxin (Imcocorp, Stockholm, Sweden). The primary antibodies were detected using alkaline-phosphatase-conjugated secondary antibodies (Bio-Rad Laboratories srl) and the immunocomplexes revealed with a chemiluminescence system (Pierce Biotechnology, Thermo Scientific, Rockford, IL).

Hepatocyte proliferation and apoptosis

Bromo-deoxyuridine (BrdU) incorporation

Two hours before killing, the animals were injected, i.p., with 120 mg/kg b.w. of BrdU (Sigma, St Louis, MO). BrdU incorporation was detected with a monoclonal anti-BrdU antibody (DAKO Corporation, Carpinteria, CA). Hepatocyte proliferation was assessed in the lobular area of the entire liver where the number of labelled and unlabelled nuclei was evaluated. A minimum of 1000 hepatocytes per liver lobe was counted by two different operators.

Hepatocyte apoptosis (TUNEL)

Liver sections of 3 µm thickness were used to detect DNA fragments of apoptotic cells by the Terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labelling (TUNEL) method (Enzo Kit, Life Sciences, NY). Apoptotic hepatocytes were determined by two different operators, checking apoptotic signals in 20 high-power fields (HPS, 400 × magnification).

Apoptotic gene mRNA determination

Total RNA extraction, RNA preparation and first-strand cDNA synthesis. Total RNA was extracted from liver tissue by using the Rneasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using random hexamers and the TaqMan Reverse Transcription Reagents (Applied Biosystems, Monza, Italy) with 3.125 U/µl of MultiScribe Reverse Transcriptase in a final volume of 50 µl.

MULTI-PCR for apoptotic genes mRNA determination. The Multiplex RT-PCR APO2B (Eppendorf, Milan, Italy) commercial kit was used. Optimal amounts of mixed primers, specific for the Open Reading Frame (ORF) region of the studied apoptotic genes (Casp-3, Bcl-2, Bax, Bcl-XL, Bcl-XS) are present in the kit. Primers for GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) served as a control for a housekeeping gene. Ten microlitres of each sample obtained by PCR were then electrophoresed on agarose gel (4%) in the presence of ethidium bromide and photographed with a Polaroid camera by using UV trans-illuminator (Sigma). The densitometric analysis of the signal of each amplified gene was evaluated by the Optilab System 2.0 (Graftek Srl, Pavia, Italy). The data were reported as a ratio between the densitometric value of each apoptotic gene and that of GAPDH.

Oxidative stress parameters

Lipid peroxidation

Cytosol preparation. One hundred milligrams of rat liver were pottered with 800 µl of RIPA buffer (Tris HCl, NaCl, EDTA, SDS, IGEPAL, Na deoxycholate), containing protease inhibitors (Complete^{TM}; Roche

Applied Science, Milan, Italy) and anti-phosphatases (sodium orthovanadate), according to a standard protocol as previously reported by us [18]. The homogenate was centrifuged for 20' at 18 000 x g at 4°C. Protein concentration was evaluated using the Bradford assay following the manufacturer's instructions (Bio-Rad Laboratories, Segrate-Milan, Italy).

Thiobarbituric acid reactants. The amount of aldehydic products generated by lipid peroxidation was quantified by the thiobarbituric acid (TBA) reaction according to the method described in the literature [28]. Briefly, 500 µl of cytosol from liver tissue were mixed with 500 µl of a 0.2% solution of trichloroacetic acid (TCA) and centrifuged for 10' at 10,000 x g at room temperature. The supernatant was then mixed with a solution of 20% TCA added with 0.5% TBA. The solution was heated at 95°C for 30', cooled in an ice bath and centrifuged for 10' at 10 000 x g at room temperature. The absorbance of the supernatant was read at 532 nm and the value detracted to that measured at 600 nm, which represents the non-specific absorption. The extinction coefficient used to calculate the TBA reactans (TBARS) concentration, expressed in term of µmol of malondialdehyde (MDA)/g of proteins, was 155 mM/cm. Three different determinations were performed on each liver tissue sample and the data expressed as mean ± SD.

Protein carbonylation

Protein carbonylation was determined by measuring the content of carbonyl groups assayed using the dinitrophenylhydrazine according to Levine et al. [29]. Briefly, 300 µl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl were added to 200 µl of cytosol sample (corresponding to 0.5 mg of protein) and incubated for 1 h at room temperature in the dark. Control samples were incubated in 300 µl of 2 M HCl under the same conditions. Samples were added with 500 µl of 20% TCA and centrifuged at 4°C for 15 min at 25,000 x g. The precipitates were washed three times with 1 mL ethanol-ethyl acetate (1:1) to remove the free DNPH and lipid contaminants. After drying, precipitates from DNPH-treated samples were dissolved in 600 µl 6 M guanidine hydrochloride in sodium phosphate buffer 20 mM, pH 2.3. The carbonyl contents were calculated by reading at 370 nm using an absorption coefficient of 22 $mM^{-1}cm^{-1}$ against pellets derived from the 2.5 mol/L HCl-treated samples.

Clusterin expression

Immunofluorescence detection. Five-micrometre-thick tissue slides were deparaffinized in xylene for 1 h and rehydrated in decreasing concentrations of ethyl alcohol in water and finally in PBS. Antigen retrieval was performed by microwaves, incubating the slides in buffer citrate (pH 6.0) three times for 3 min each at 750 W. After washing in PBS, endogenous peroxidase was quenched with H_2O_2 for 10 min. Non-specific sites were blocked by incubating the slides with a blocking solution (FCS 10% + BSA 1% in PBS) for 1 h. The slides were then incubated with the primary rabbit anti-clusterin antibody (sc-8354, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:100, overnight at 4°C. The following day, after three washes in PBS, the slides were incubated, at room temperature, for 2 h with a secondary anti-rabbit Alexa 488 (Invitrogen, Molecular Probes, Eugene, OR) antibody diluted 1:200, and after three washes in PBS, with TO-PRO-3-iodide (Invitrogen, Molecular Probes) diluted 1:7000 in PBS for 20 min at room temperature. Finally the slides were mounted with an anti-fading mounting, covered with a coverslip and observed with a confocal microscope (Leica TCS SP2, Leica Microsystems Srl, Milan, Italy). To verify the specificity of the immunoreaction, appropriate controls were done incubating tissue slides with only the secondary Ab or using the pre-immune rabbit serum as primary Ab. We repeated the experiments three times. The green colour identifies the clusterin immunodetection and the blue colour the nuclei.

Western blot analysis. Fifty micrograms of total cytosolic proteins were separated by 4–12% SDS-PAGE (Invitrogen, Milan, Italy) and transferred onto nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad Laboratories srl). The membranes were blocked with 3% non-fat dry milk in TBS (15 mM Tris-HCL pH 7.5, 0.5 M NaCl) for 2 h at room temperature and then incubated overnight at 4°C with a primary antibody against clusterin (sc-8354, Santa Cruz Biotechnology, Inc.) diluted 1:100 and β-actin (sc-1616, Santa Cruz Biotechnology, Inc.) in TTBS (15 mM Tris-HCL pH 7.5, 0.5 M NaCl, 0.05% Tween 20). The following day, after washing the membranes three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 2 h at room temperature, then washed three times with TBST. Detection of immunocomplexes was performed with a chemiluminescence system (Pierce Biotechnology, Thermo Scientific, Rockford, IL), the values were normalized to β-actin expression and the level of protein expression determined using Molecular Image Chemidoc XRS+ with imageLab Software (Bio-Rad).

Liver tissue analysis

H&E histological examinations

The hepatic tissue sections from rats were stained with haematoxylin and eosin (H&E) and then examined under an optical microscope. All hepatic tissues

were observed and each sample was evaluated by two different pathologists (200 × and 400 × magnifications were observed).

Electron microscopy

Liver tissue sections, from albumin- and rAlrp-treated PH rats, were fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l PBS at pH 7.4 for 5 h at 4°C. Then the sections were post-fixed in 1% OsO_4 in PBS for 30 min at 4°C, washed in several changes of PBS, dehydrated in graded alcohols and embedded in Epon-Araldite (TAAB; Reading, UK). Semithin sections (1 μm thick) were heat-stained with toluidine blue borate [30]. Ultra-thin sections for EM were mounted on formvar-coated nickel grids and stained routinely with uranyl acetate and lead citrate [31]. The grids were observed under a Morgagni 268 electron microscope (FEI; Hillsboro, OR).

Autophagic vesicles were semi-quantitatively evaluated on liver tissue samples from two albumin- and two rAlrp-treated PH rats, randomly individuated, at each time point of the protocol reported in Table I. Five tissue sections were prepared for each animal and a grid for each one analysed. In each grid the presence of autophagic vesicles was determined observing, randomly, two different fields, each containing, on average, 10 cells. The results have been expressed as the number of autophagic vesicles/100 cells.

Liver tissue recovery

Liver weight from all PH rats was determined at the time of their sacrifice until the 14th day after the surgery, carefully removing all non-hepatic tissues. The data reported at each time point are the mean ± SD of the liver weight of all animals sacrificed at the same time point.

Statistical analysis

The results obtained are expressed as mean ± standard deviation ($M \pm SD$). Statistical comparison among groups was determined using analysis of variance test (ANOVA). Where indicated, individual comparisons were performed using Student's *t*-test. Statistical significance was ascribed to the data when $p < 0.05$.

Results

Alrp serum and liver tissue determination

Alrp serum levels determined by ELISA

Figure 1 reports Alrp serum levels determined in albumin- (1A) and rAlrp-treated (1B) PH rats sacrificed at different times after surgery following the protocol reported in Table I. In albumin-treated PH rats, compared to 'time 0' value, a significant ($^*p < 0.01$) increase of Alrp serum level was soon registered at 6 h after PH; its maximal expression (6-times higher than the 'time 0' value) was reached by the 18th h, subsequently declining to the 'time 0' level for the rest of the period of observation. A similar Alrp serum profile was registered in 70% PH rats which had not received albumin (personal data not reported).

Alrp serum profile of rAlrp-treated PH rats (Figure 1B) revealed, compared to the 'time 0' value, a significant ($^*p < 0.001$) and stable increase of Alrp for all the period of the treatment.

Alrp serum and tissue levels determined by Western blot analysis

Figure 2 reports Alrp expression determined, by Western blot analysis, on serum and liver cytosol from albumin-treated PH rats sacrificed at different times after surgery. In the liver tissue two different Alrp isoforms were identified, one of 23 and one of 21 kD; only the 21 kD isoform was present in the serum (Figure 2A). In the liver cytosol, low levels of both molecules were detected at 'time 0' and no dramatic changes of their expression were registered

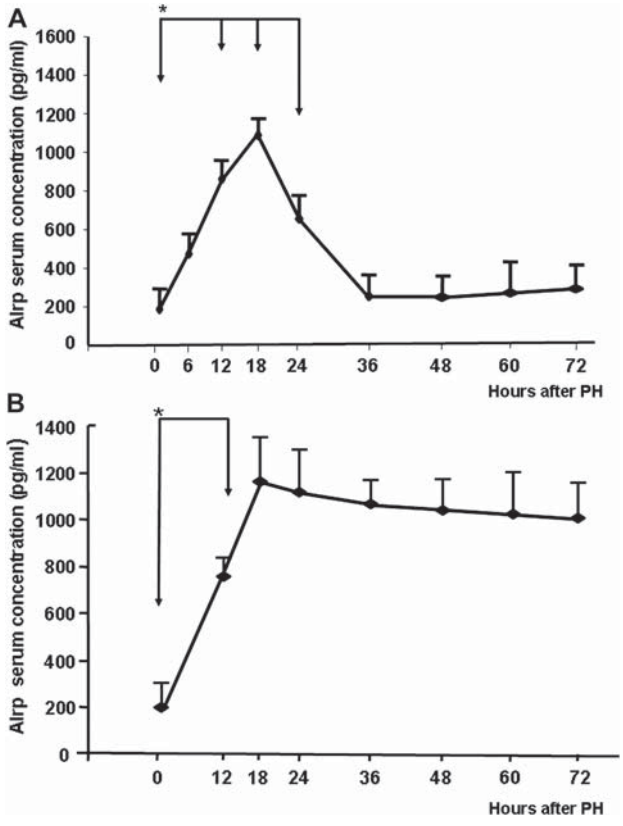


Figure 1. Alrp serum levels of albumin- and rAlrp-treated PH rats determined by ELISA. In albumin-treated PH rats (A), a constant Alrp serum increase ($^*p < 0.01$) starting immediately after surgery, is evident. The maximal expression was registered by the 18th h, then slowly degrading to the pre-surgery value. In rAlrp-treated PH rats (B), rAlrp administration determined a constant and stable increase of Alrp, statistically significant ($^*p < 0.001$) compared to 'time 0' value, since the 12th h after surgery. The data reported are expressed as mean ± SD and were obtained from three different measurements.

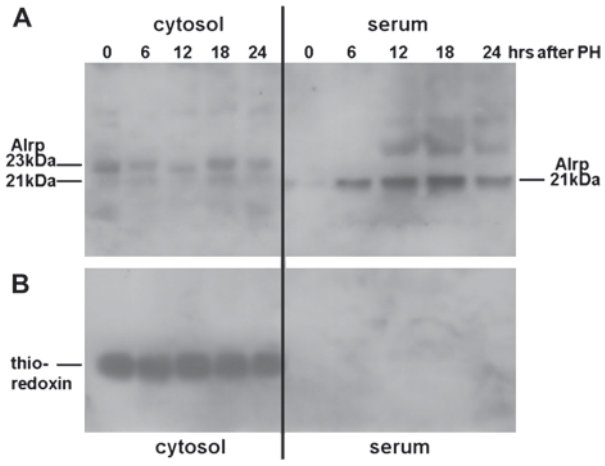


Figure 2. Alrp determination, by WB analysis, in serum and liver cytosol from albumin-treated PH rats. Western blot analysis, performed in cytosol and serum from albumin-treated PH rats is reported. Two different Alrp isoforms were identified in rat serum and in liver tissue: a 21 kDa and a 23 kDa (A), of which only the 21 kDa significantly increases after PH, in particular in the serum, reaching a maximal level by the 18th h. (B) Any presence of thioredoxin into the serum was detected indicating no substantial unspecific release of proteins from damaged cells.

during all the period of the observation. The panel for the 21 kDa isoform in the serum had a different pattern: at 'time 0' it was poorly expressed but, during the regenerative process, it constantly increased, reaching the nadir by the 18th h after surgery and then diminished by the 24th h. The immunodetection of thioredoxin on the same nitrocellulose filter used for Alrp identification revealed a total absence of this cytosolic protein in the serum samples (Figure 2B), indicating the lack of significant cell damage induced by surgery and demonstrating that the 21 kDa Alrp isoform detected in the rat serum was newly synthesized and actively released.

Hepatocyte proliferation and apoptosis

Figure 3 reports hepatocyte proliferation, determined by BrdU incorporation (3A) and hepatocyte apoptosis, determined by TUNEL assay (3B).

BrdU incorporation

Hepatocyte proliferation in albumin-treated PH rats shows a profile of labelled hepatocytes (LI) typical of the process of liver regeneration after 70% PH (24th h LI = 22.0 ± 3.5; 36th h LI = 21 ± 2.4; 48th h LI = 13 ± 3.5, 72nd h LI = 14.2 ± 2.7); all data were statistically different ($p < 0.001$) compared to 'time 0' value (LI = 3.2 ± 1.9). rAlrp administration induced a further increase of hepatocyte proliferation, statistically different when compared to albumin-treated PH rats values, at the 24th h (rAlrp LI = 29.0 ± 4.2; $p < 0.05$ vs 'time 24 h' albumin-treated value) and at the 36th h (rAlrp LI = 27.0 ± 3.8; $p < 0.05$ vs 'time 36 h' albumin-treated value). The data obtained by the 48th h (rAlrp LI = 17.5 ± 3.9) and by the 72nd h (rAlrp LI = 17.0 ± 3.8) after the surgery were higher, compared to albumin-treated PH rats values, but were not statistically different.

Apoptosis evaluation (TUNEL)

Figure 3B reports the hepatocyte apoptosis evaluated during the time of the observation. In albumin-treated PH rats, comparing to 'time 0' (0.7 ± 1.1 apoptotic hepatocytes/20HPS), a statistically significant increase of the apoptotic process was detected in all time points (24th h: 7.5 ± 1.2 apoptotic hepatocytes/20 HPS; $p < 0.001$ vs 'time 0'; 36th h: 4.3 ± 0.5 apoptotic hepatocytes/20HPS; $p < 0.001$ vs 'time 0'; 48th h: 4.1 ± 0.8 apoptotic hepatocytes/20HPS;

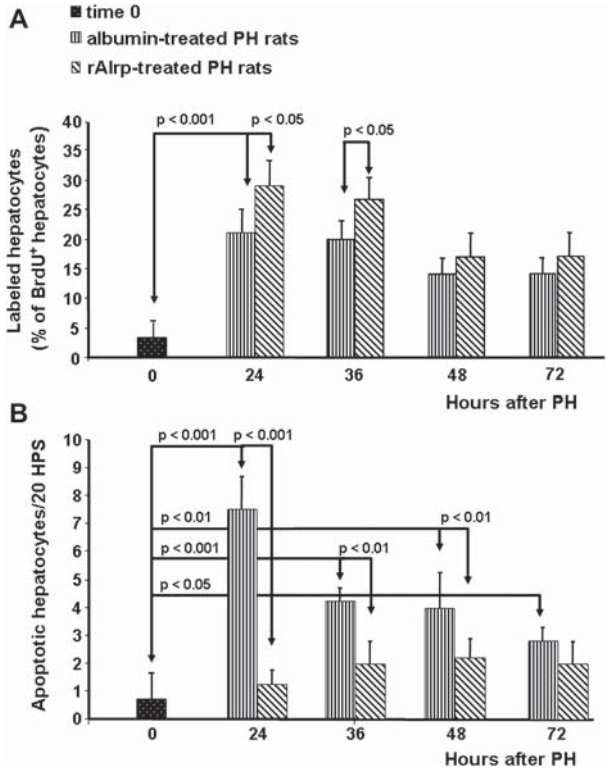


Figure 3. Hepatocyte proliferation (A) and apoptosis (B) in liver samples from albumin- and rAlrp-treated PH rats. (A) The percentage of BrdU-positive hepatocytes are reported,. An increase of hepatocyte proliferation, in albumin-treated PH rats, was registered, compared to 'time 0'. rAlrp administration induced a further increase of hepatocyte proliferation, statistically different, compared to albumin-treated PH rats values, at the 24th and 36th h after the surgery. (B) The number of apoptotic hepatocyte/20 HPS is reported. In albumin-treated PH rats, a statistically significant increase of the apoptotic hepatocytes was detected in all time points of the observation, as compared to 'time 0'. Instead, in rAlrp-treated PH rats the PH-induced apoptotic process was consistently reduced at any time point of the observation. Indeed a significantly lower level of apoptotic hepatocytes was observed, as compared to albumin-treated PH rats, throughout the entire period of observation. The data, $M \pm SD$, are from three separate evaluations provided by two different pathologists, observing 20 high power fields each time.

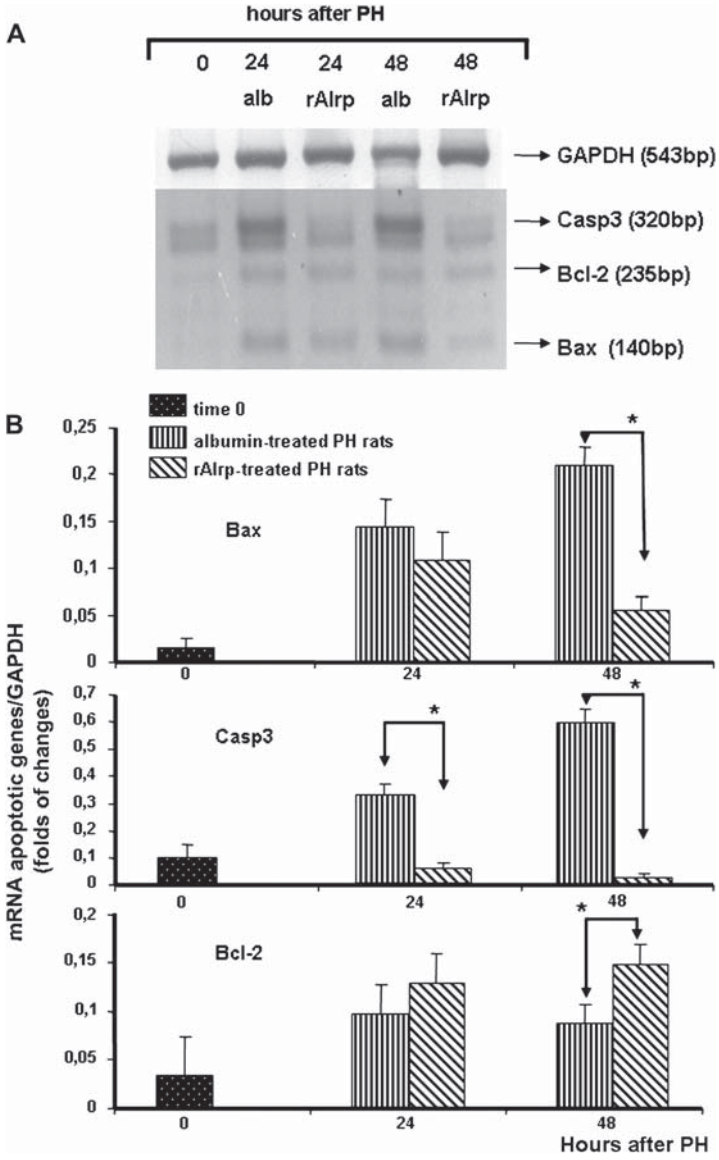


Figure 4. mRNA apoptotic gene expression in albumin- and rAlrp-treated PH rats. (A) The apoptotic gene mRNA expressions are reported. A down-regulation of pro-apoptotic genes (Casp-3 and Bax) has been detected, at the 24th and 48th h after PH, in rAlrp-treated PH rats in comparison to albumin-treated PH rats. An up-regulation of the anti-apoptotic gene (Bcl-2) mRNA expression was, on the contrary, evident in rAlrp-treated PH rats, compared to albumin-treated PH rats, during the entire period of observation. The densitometric analysis of each amplified gene product (B) showed a statistically significant reduction ($^{*}p < 0.001$) at the 48th h time point for Bax and at the 24th and 48th h time points for Casp-3. A similar significant difference was evidenced evaluating the densitometric expression of Bcl-2 mRNA product in liver tissue from rAlrp-treated PH rats as compared to albumin-treated PH rats ($^{*}p < 0.001$) by the 48th h after surgery. The listed data, expressed as mean ± SD are from three different measurements.

$p < 0.01$ vs 'time 0', and 72nd h (2.9 ± 0.4 apoptotic hepatocytes/20HPS; $p < 0.05$ vs 'time 0') after the surgery.

In rAlrp-treated PH rats an inhibition of the apoptotic process was observed at any time point. Indeed, significantly lower numbers of apoptotic hepatocytes were observed, compared to albumin-treated PH rats, by the 24th h (1.2 ± 0.7 apoptotic hepatocytes/20HPS; $p < 0.001$ vs 'time 24 h' value of albumin-treated PH rats), the 36th h (2.0 ± 0.8 apoptotic hepatocytes/20HPS; $p < 0.01$ vs 'time 36' value of albumin-treated PH rats) and the 48th h (2.2 ± 0.7 apoptotic hepatocytes/20HPS; $p < 0.01$ vs 'time 48' value of albumin-treated PH); a reduced level of hepatocyte apoptosis was detected by the 72nd h (2.0 ± 0.9 apoptotic hepatocytes/20HPS) after surgery, but not statistically different compared to 'time 72' value of the albumin-treated PH rats.

Apoptotic genes expression

Figure 4 reports mRNA expression of apoptotic genes (Bcl-2, Bax, Casp-3) evaluated in liver tissue samples from albumin- and rAlrp-treated PH rats.

A decrease of mRNA expression of the pro-apoptotic genes (Bax and Casp-3) was found in rAlrp-treated PH rats in comparison to albumin-treated PH rats (Figure 4A).

The densitometric analysis of Bax mRNA expression confirmed a reduction in the rAlrp-treated animals, statistically different ($^{*}p < 0.001$), in comparison to albumin-treated PH rats, at the 48th h after surgery (Figure 4B).

A similar picture emerged by the densitometric analysis of Casp-3 mRNA expression. A statistically significant ($^{*}p < 0.001$ vs albumin-treated PH rats) decrease was registered both at the 24th and 48th h after surgery in rAlrp-treated PH rats.

Concerning the anti-apoptotic gene expression, an up-regulation of Bcl-2 mRNA was recorded at the 24th and 48th h after PH in rAlrp-treated rats in comparison to albumin-treated PH rats (Figure 4A). The densitometric analysis of Bcl-2 mRNA signal revealed an increase in rAlrp-treated PH rats, statistically different ($^{*}p < 0.001$) in comparison to albumin-treated PH rats, at the 48th h (Figure 4B).

No statistically significant differences between the two groups of PH rats were detected for Bcl-XS and Bcl-XL mRNA expression at any time point of the observation (data not reported).

Oxidative stress parameters

Figures 5–7 report the evaluation of the oxidative stress-related parameters (lipid peroxidation, protein carbonylation and clusterin) in liver tissue from albumin- and rAlrp-treated PH rats.

Lipid peroxidation

Figure 5A reports MDA levels determined in liver tissue samples from the two groups of PH rats at different times after the surgery. In liver tissue from albumin-treated PH rats, a gradual, constant and statistically significant ($^{*}p < 0.01$) increase of lipid peroxidation, in comparison to 'time 0' (MDA µmol/g proteins: 26.0 ± 4.2), was registered during the regenerative process (MDA, µmol/g proteins; 24th h: 42.7 ± 7.5; 48th h: 44.0 ± 7.0; 72nd h: 51.3 ± 8.2). A different profile was detected in liver tissue from rAlrp-treated PH rats: compared to albumin-treated PH rats, a lower level of MDA was detected at the 24th h (MDA, µmol/gr proteins: 39.2 ± 5.1), at the 48th h (MDA, µmol/g proteins: 29.5 ± 5.3; $^{\wedge}p < 0.05$) and the 72nd h (MDA, µmol/g proteins: 28.4 ± 5.7; $^{*}p < 0.01$) after the surgery.

Protein carbonylation

Figure 5B reports the protein carbonylation, determined by carbonyl group levels, in liver tissue from albumin- and rAlrp-treated PH rats sacrificed at different times after surgery. In albumin-treated PH rats an increase of carbonylic groups, compared to 'time 0' value (C = O groups; 1.75 ± 0.27 nmol/mg proteins) was registered at the 24th h (C = O groups; 2.33 ± 0.25 nmol/mg proteins, $^{*}p < 0.01$), 48th h (C = O groups; 2.25 ± 0.23 nmol/mg proteins) and 72nd h (C = O groups; 1.98 ± 0.25 nmol/mg proteins). In liver tissue from rAlrp-treated PH rats, protein carbonylation was lower, compared to albumin-treated PH rats, at the 24th h (C = O groups; 2.084 ± 0.23 nmol/mg proteins), the 48th h (C = O groups; 1.74 ± 0.25 nmol/mg proteins; $^{\wedge}\ p < 0.05$ compared to albumin-treated PH rat value at the 48th h) and 72nd h (C = O groups; 1.74 ± 0.24). No statistical differences were observed comparing the rAlrp-treated PH rats values with the 'time 0' C = O value.

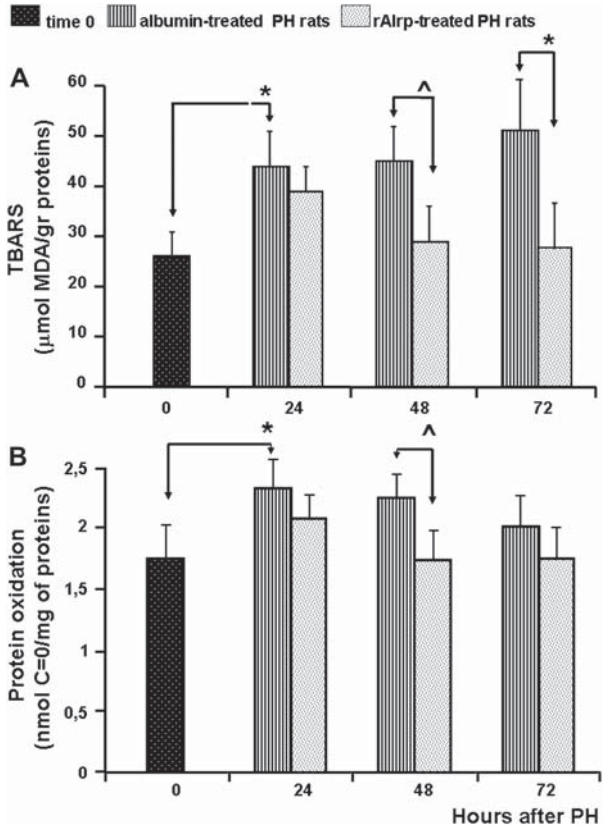


Figure 5. Lipid peroxidation and protein carbonylation. (A) Lipid peroxidation, determined by malondialdehyde levels (MDA). In albumin-treated PH rats, compared to 'time 0' value, a gradual, constant and statistically significant ($^{*}p < 0.01$) increase of lipid peroxidation was registered. In rAlrp-treated PH rats, MDA levels do not change dramatically, compared to 'time 0' value, and result statistically reduced from albumin-treated PH rat value both at the 48th h ($^{\wedge}p < 0.05$) and the 72nd h ($^{*}p < 0.01$) after the surgery. The data, mean ± SD are of three different analyses including each time all the animals of the respective groups. (B) Protein carbonylation expression (nmol C = O/mg of proteins). A statistically significant increase of C = O groups was registered in albumin-treated PH rats after the surgery. rAlrp treatment seems to prevent protein oxidation, keeping low the C = O group levels, which, starting from the 48th h, becomes significantly reduced ($^{\wedge}p < 0.05$), compared to albumin-treated PH and reaching the 'time 0' value.

Clusterin immunodetections

Immunofluorescence analysis

Figures 6A–C report the clusterin immunodetection determined by confocal microscopy. A cytosolic clusterin localization was evidenced in all the liver

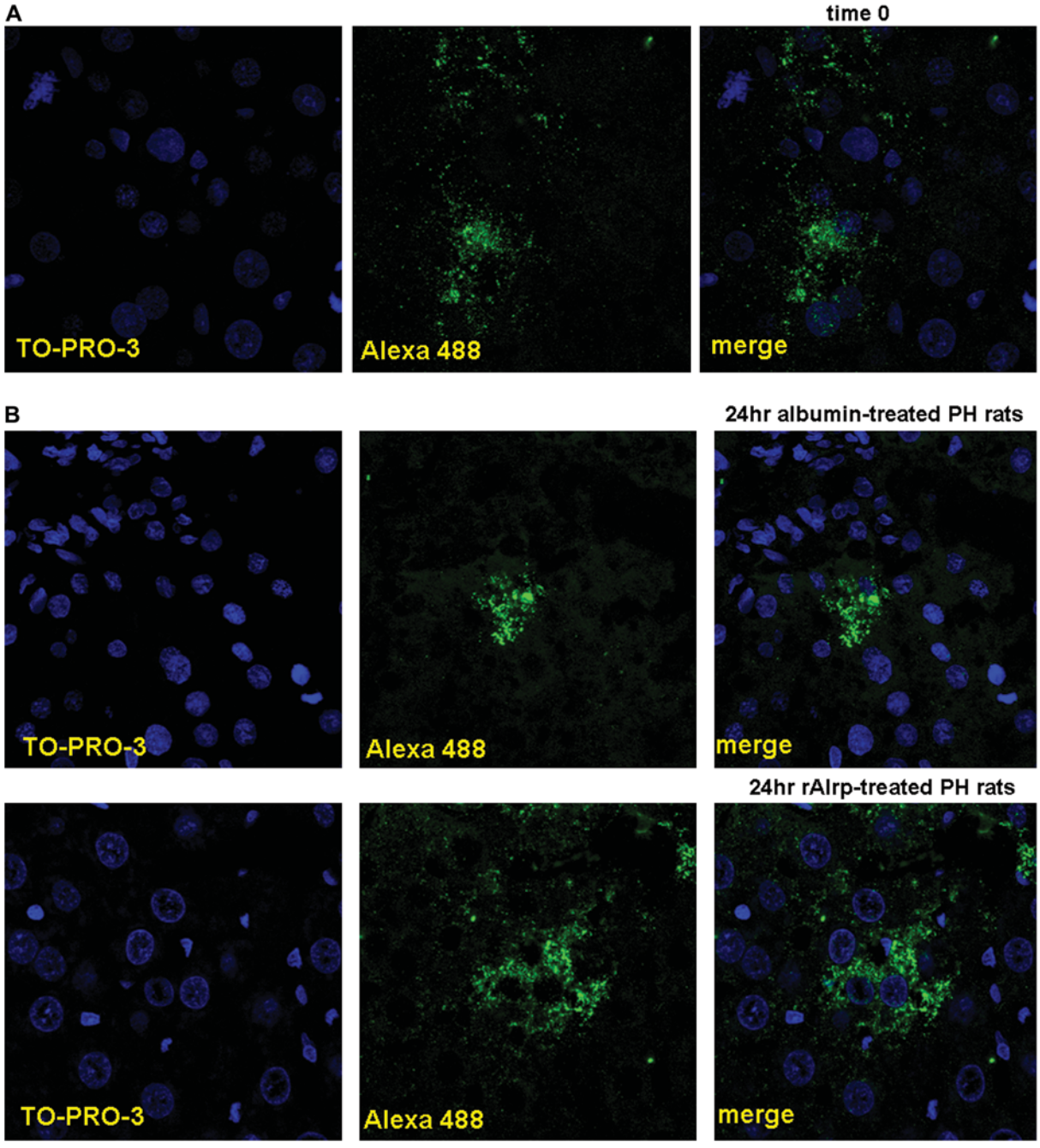


Figure 6. Liver tissue clusterin immunofluorescence detection. Confocal immunodetection identified the presence of clusterin in the cytosol, both in albumin- and rAlrp-treated PH rats. A low expression of the protein was detected in 'time 0' liver tissue (A), which similarly increases, by the 24th h after surgery, in liver tissue from both group of PH rats (B). By the 48th h, while in albumin-treated PH rats it gets back to the 'time 0' value, in rAlrp-treated rats it remains high (C). For each determination the nuclear identification, evidenced by TO-PRO-3, the specific clusterin immunodetection, evidenced by Alexa 488, and the 'merge' images are reported.

tissue samples analysed [32]. In the 'time 0' liver tissue sample, clusterin expression was low (6A), but it increases, by the 24th h, equally in albumin- and in rAlrp-treated PH rats (6B). By the 48th h, a different clusterin expression was registered in the liver tissue from the two animal groups. In liver tissue from albumin-treated PH rats it returned to the 'time 0' level, whereas in liver tissue from rAlrp-treated PH rats it remained highly expressed (6C). For each determination the nuclear identification, evidenced by TO-PRO-3, the specific clusterin immunodetection, evidenced by Alexa 488, and the 'merge' are reported.

Immunoblot analysis

Figure 7A reports the immunoblot analysis of clusterin expression in liver tissue from 'time 0' rats and from albumin- and rAlrp-treated PH rats at the 48th h after surgery. We focalized our study on this period of the regenerative process because at this

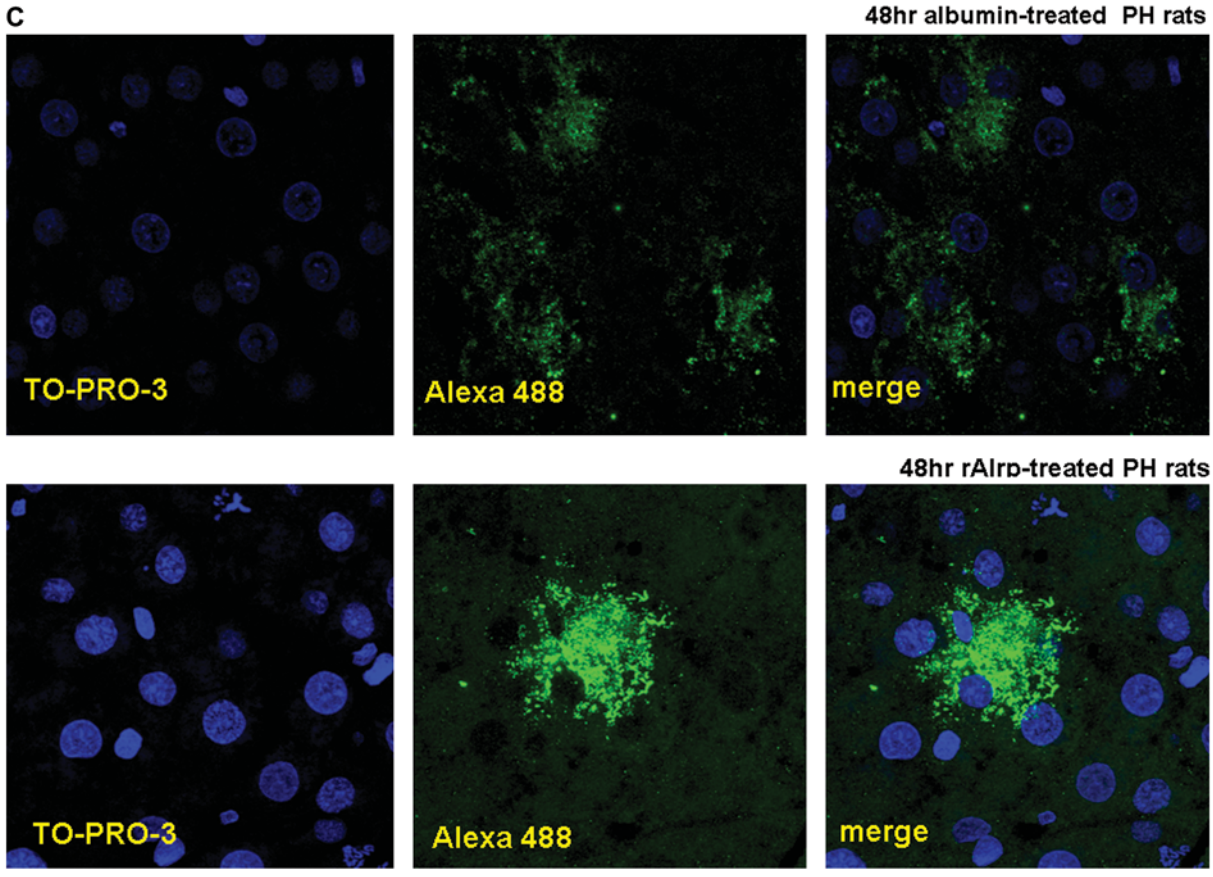


Figure 6. *(Continued)*

time different levels of clusterin were detected, among the two groups of rats, by the immunofluorescence analysis (Figure 6C). No difference was evidenced, by the 48th h, in clusterin level of liver tissue from albumin-treated PH rats, compared to 'time 0' value; instead a clear increase of clusterin expression was detected in liver tissue from the 48 h rAlrp-treated PH rats, compared to liver tissue from 'time 0' and from 48 h albumin-treated PH rats. Protein normalization by β-actin expression revealed a statistically significant ($p = 4.2 \times 10^{-4}$) clusterin increase in rAlrp-PH rats, in respect to albumin-treated PH rats (Figure 7B). The data represent the results from three different analyses.

Liver tissue analysis

H&E findings

Figures 8A–C report the H&E findings of liver sections from albumin- and rAlrp-treated PH rats sacrificed at the 24th (Figure 8A), 48th (Figure 8B) and 72nd (Figure 8C) h after the surgery. Diffuse and severe hepatocyte ballooning, light nuclear activation with multiple nucleoli, focal cytoplasmic eosinophilia, along moderate vascular congestion, were observed in liver tissues from albumin-treated PH rats at 24 h after the surgery (Figure 8A); in contrast, in rAlrp-treated PH rats the liver tissue exhibited a moderate hepatocyte ballooning in the zone 1 and 2, a well conserved centrolobular zone 3 with well identified hepatic sinusoids, absence of vascular congestion and light cytoplasmic eosinophilia (Figure 8A). By the 48th h (Figure 8B), in liver tissue from albumin-treated PH rats, diffuse hepatocyte necrosis and apoptosis, swelling, diffuse and severe fatty and vacuolar degeneration, hepatocyte ballooning, vascular congestion along with multiple haemorrhagic infiltrates, destruction of the hepatic lobule structure were detected; in contrast, in rAlrp-treated PH rats well conserved hepatic sinusoids in zone 3, along with rare apoptotic signals, few light nuclei and diffuse steatosis were detected (Figure 8B). Figure 8C reports the histological finds observed by the 72nd h after PH. A micro-vesicular steatosis and vascular congestion along with diffuse hepatocyte apoptosis, hepatocyte ballooning and moderate nuclear activation were observed in liver tissue sections from albumin-treated PH rats; in contrast, rAlrp-treatment seems to better preserve hepatocyte from ballooning, necrosis/apoptosis and induces reduced vesicular congestion. In both liver tissue samples the presence of mitotic figures were observed, particularly numerous in rAlrp-treated PH rats, confirming the proliferative stimulus of Alrp reported in the literature [1,3] and in the present manuscript (Figure 3A). In Table II a semi-quantitative evaluation of the histological findings is reported. For

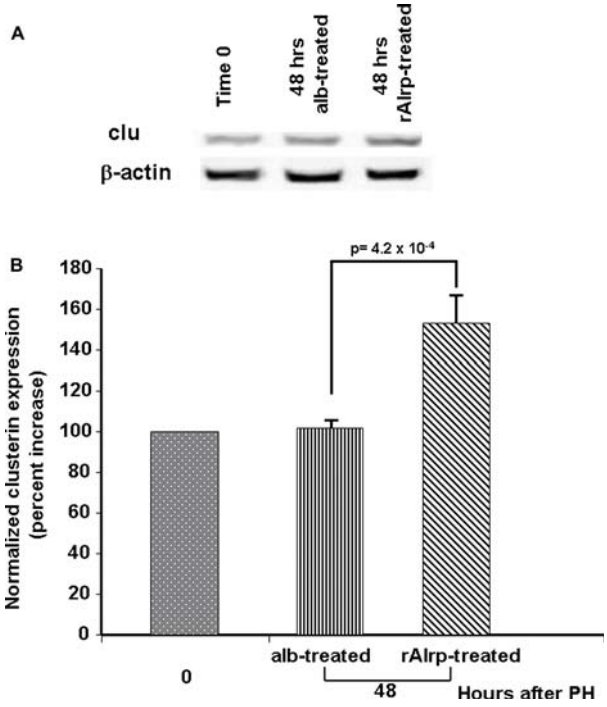


Figure 7. Liver cytosol clusterin immunoblot analysis. No differences on clusterin expression in liver cytosol from 48 h albumin-treated PH rats and 'time 0' rats were evidenced; instead a higher clusterin level, compared both to 'time 0' and to albumin-treated PH rat, was detected in liver tissue from rAlrp-treated PH rats by the 48th h (A). Protein normalization by β-actin expression on the same nitrocellulose filter revealed an increase of clusterin of more than 50% compared to albumin-treated PH rats (B). The data, expressed as mean ± SD, represent the results from three separate analyses.

each time point, a 200 × and a 400 × magnification images are reported. Normal histological features of liver tissue were observed at 'time 0' liver tissue samples.

Electron microscopy observations

Electron microscopy analyses on liver tissue from albumin- or rAlrp-treated PH rats, at different times after surgery (24, 48 and 72 h), were performed. Normal cellular structure, by the 24th h after PH, was observed in liver tissue from both groups of rats (data not reported). Figures 9A and B report the EM observations evidenced by the 48th and 72nd h in both groups of rats. In albumin-treated PH rats (Figure 9A) a progressive cellular hydropic swelling, a presence of lipid drops and a perinuclear steatotic degeneration (48th h, 3500 × magnification), numerous autophagosomes containing lysosomes, with internal cytoplasmic residues, and degenerated cytoplasmic organelles (72nd h, 4400 × magnification) were evidenced, suggesting that degenerated organelles in these cells have been eliminated by autophagy (15.6 ± 3.1 vesicles/100 cells). In the insert on Figure 9A, a higher magnification (11 000 ×) of an autophagosome is shown. Figure 9B reports the EM findings observed in rAlrp-treated PH rats at the 48th and 72nd h after the surgery. Normal cellular structure, significantly less vacuolar degeneration (48th h, 11 000 × magnification), cells with no particular cytoplasmic alterations, intact and well conserved organelles, presence of rare autophagic structured lysosomes (3.2 ± 1.1 vesicles/100 cells) (72nd h, 5600 × magnifications) were evidenced.

Liver weight recovery

In Figure 10 the liver weight recovery of albumin- and rAlrp-treated PH rats, at different times after surgery, is reported. The sequential percentage recovery of liver mass of rAlrp-treated PH rats seems to precede liver mass recovery of albumin-treated PH rats. A first difference, between the two groups of animals, was observed by the 36th h after surgery, becoming statistically significant ($^{*}p < 0.05$) by the 48th and 72nd h, maintaining the difference by the 4th day and slowly declining to the same value by the 7th and 14th day, time when the liver mass has been almost totally recovered and the optimal liver weight/body weight ratio reached.

Discussion

Several years ago we identified a protein, named Augmenter of Liver Regeneration [1], unable to initiate

Table II. Histological findings on liver tissue from albumin- and rAlrp-treated PH rats.

	Markers						
Liver tissue sample	ballooning hepatocyte	necrosis-apoptosis	vascular congestion	haemorrhagic infiltrates	nuclear activation	sinusoids identification	steatosis
'T0' time	no	no	no	no	no	yes	no
24 PH albumin	+++	+	+	–/+	++	yes	no
24 PH rAlrp	+	–/+	–	–	–	yes	no
48 PH albumin	+++	+	++	+–/+	+	yes	+
48 PH rAlrp	+	–/+	–/+	–	–/+	yes	++
72 PH albumin	+++	+	+	+	+	yes	+
72 PH rAlrp	+	–/+	–/+	–	–/+	yes	–/+

+ = presence of the parameter considered in less than 30% of the tissue section observed at 200 × magnification; ++ = presence of the parameter considered in 30–60% of the tissue section observed at 200 × magnification; +++ = presence of the parameter considered in 60–90% of the tissue section observed at 200 × magnification.

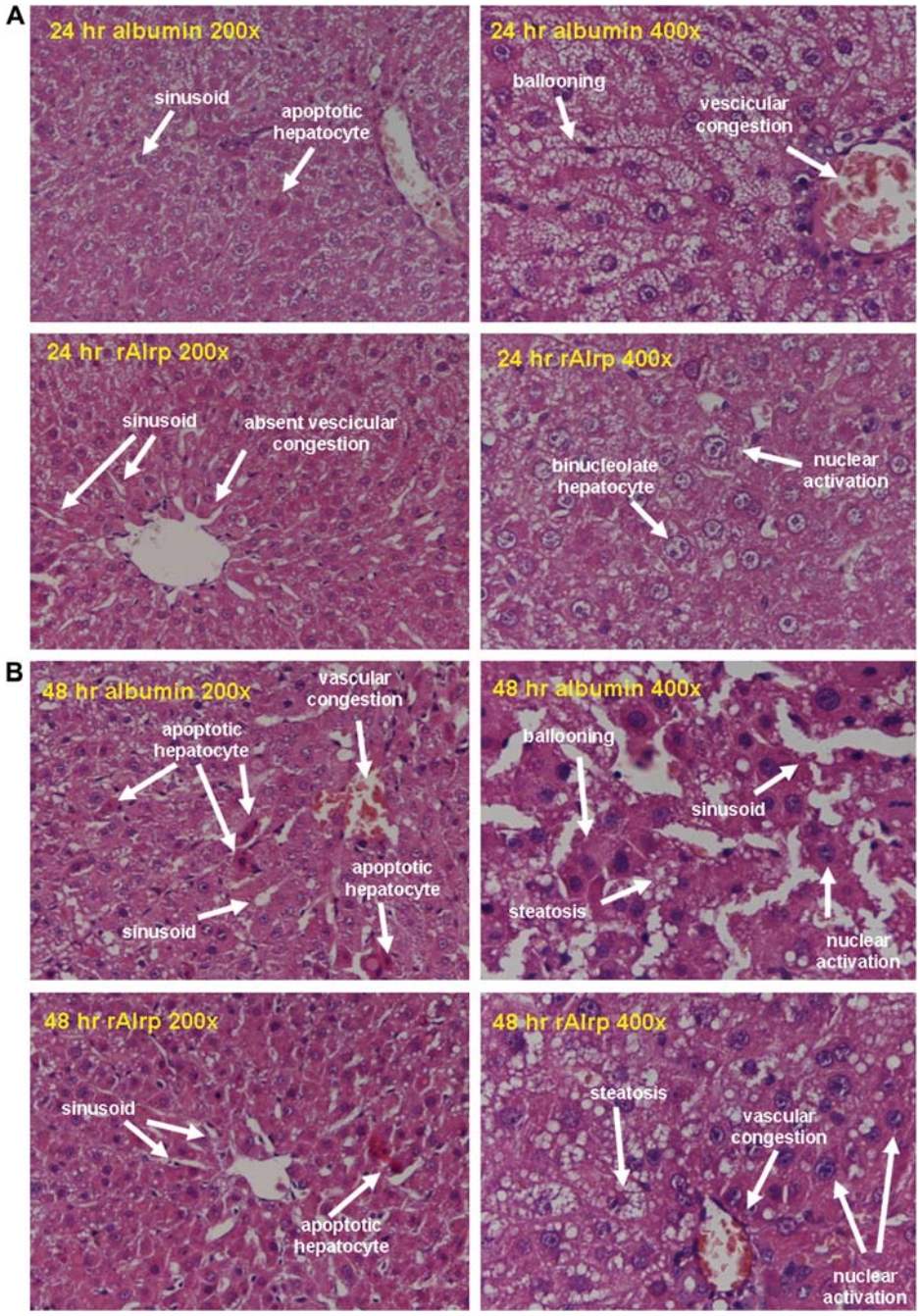


Figure 8. H&E analysis of liver tissue from albumin- and rAlrp-treated PH rats sacrificed at different times after the surgery. Representative photomicrographs of haematoxylin-eosin (H&E) staining of liver sections from albumin- and rAlrp-treated PH rats are reported. Diffuse and severe hepatocyte ballooning, light nuclear activation with multiple nucleoli and focal cytoplasmic eosinophilia were observed in liver tissues from control PH rats at 24 h after surgery; in contrast, in rAlrp-treated PH rat the liver tissue exhibited a moderate hepatocyte ballooning in zone 1 and 2, a well conserved centrolobular zone 3 (A; see arrows). By the 48th h, in liver tissue from albumin-treated PH rats, diffuse hepatocyte necrosis and apoptosis, swelling, diffuse and severe fatty and vacuolar degeneration, hepatocyte ballooning and destruction of the hepatic lobule structure were detected; in contrast, in rAlrp-treated PH rats, rare apoptotic signals and few light nuclei were detected (B; see arrows). By the 72nd h a marked microvescicular steatosis and vascular congestion were observed in albumin-treated PH rats; rAlrp-treatment resulted in a reduced vescicular congestion and apoptotic hepatocytes and a consistent number of mitotic figures (C; see arrows). For each time point, a 200 × and a 400 × image is reported. The slides were separately observed by two different pathologists.

cell proliferation as a primary mitogen, both *in vivo* and *in vitro*, but able to 'augment' hepatocyte proliferation only when administrated to experimental animals in which the regenerative process has already been primed (40% PH rats) [1,3,16].

A growing number of data now attribute to Alrp the functional role of an anti-apoptotic/cell survival factor. We demonstrated that Alrp, when present in the culture medium of human-derived neuroblastoma cells induced to apoptosis by H_2O_2, lowers the apoptotic

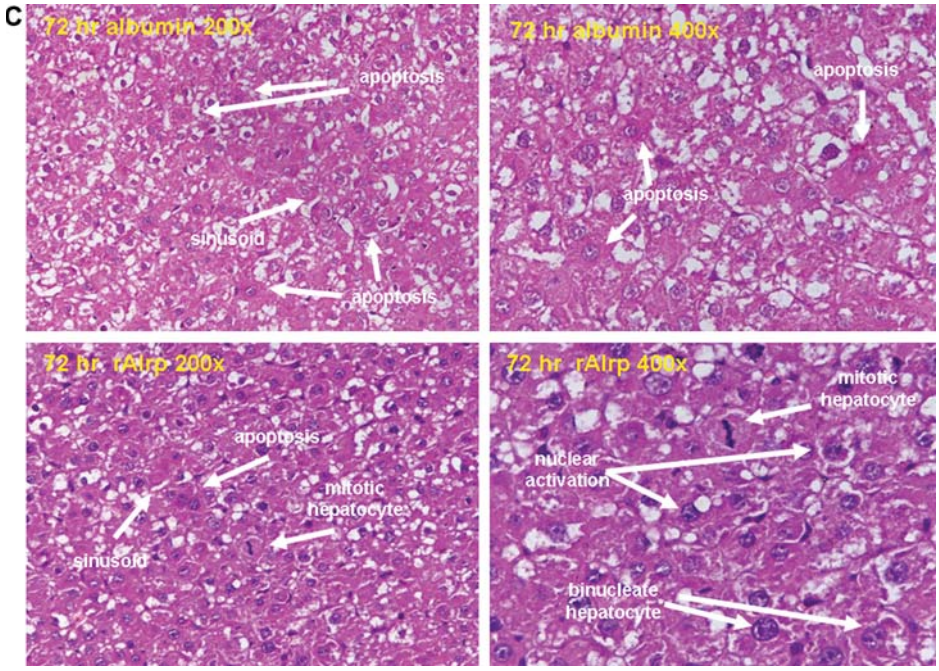


Figure 8. *(Continued)*

cell number, ameliorates mitochondria membrane integrity, reduces cytochrome *c* mitochondrial release, thus supporting cell survival and preventing the apoptotic process [18]. Similar results have been reported in rat 'primary' hepatocytes transfected with antisense oligonucleotide for Alrp mRNA [19] and in hepatoma cells [20]. Furthermore, Todd et al. [14] reported a pro-survival role of Alrp in the maintenance of murine embryonic stem cell pluripotency, preserving the structural and functional integrity of mitochondria, Liao et al. [21] demonstrated, in the renal ischemia/reperfusion experimental model of male Sprague-Dawley rats, an improvement in renal functions, a reduced extent of kidney injury, an enhancement of renal tubular cell regeneration and a reduction of tubular injury after rAlrp administration; the authors conclude that the effect of Alrp is associated to the enhancement of renal tubular cell regeneration. Recently a protective, anti-apoptotic, effect of Alrp for human hepatocytes in culture has been reported [22]. To our knowledge, no data refer a protective role of Alrp in the apoptotic processes consequent to the loss of hepatic tissue (70% PH).

In the present paper, for the first time, we refer the data obtained in 70% PH rats administrated with rAlrp for a period of 72 h after surgery. We chose this experimental animal model of cell proliferation for different reasons: first of all, it has been demonstrated that Alrp mRNA, normally expressed in the intact rat liver, even minimally, dramatically increases in the regenerating liver, by the 12th h after PH, [33,34]; secondly, liver mass recovery after PH is a physiological process characterized, at the beginning, by the stimulation of the proliferative spur of hepatocytes and by the induction of anti-apoptotic mechanisms (*early phase*) [35–37] followed by the up-regulation of pro-apoptotic stimuli to prevent uncontrolled tissue overgrowth (*late phase*) [37,38].

Our data demonstrate the presence of two different Alrp isoforms in rat serum and/or liver tissue: a 21 kDa and a 23 kDa (Figure 2A), of which only the 21 kDa isoform significantly increases after PH, in particular in the serum, reaching a maximal level by the 18th h, suggesting that this is the Alrp isoform active in the process of liver regeneration after PH.

Furthermore, maintaining high Alrp serum levels in rAlrp-treated PH rats (Figure 1B), a drastic decrease of hepatocyte apoptosis (Figure 3B) was determined, for most of the time of observation, compared to albumin-treated PH rats, along with a significant increase of anti-apoptotic gene expression (Figure 4).

Many reports in the literature refer, even if separately, to Alrp expression increase [33,34,39] and apoptotic processes inhibition [37,38,40], during the *early phase* of the regenerative process after PH. So far, no one has considered these two events to be strictly related. By the data herein reported, it is then possible that, also during the 'physiological' recovery of the liver mass after PH, Alrp operates as an anti-apoptotic/cell survival factor, as it seems to be widely accepted [18–22].

Moreover, rAlrp administration seems to be important also for clusterin induction, one of the few secreted chaperons that carry out its 'quality control' check outside of cells [41], normally present in liver tissue from non-hepatectomized rats [42]. We showed that, after PH, clusterin expression increases

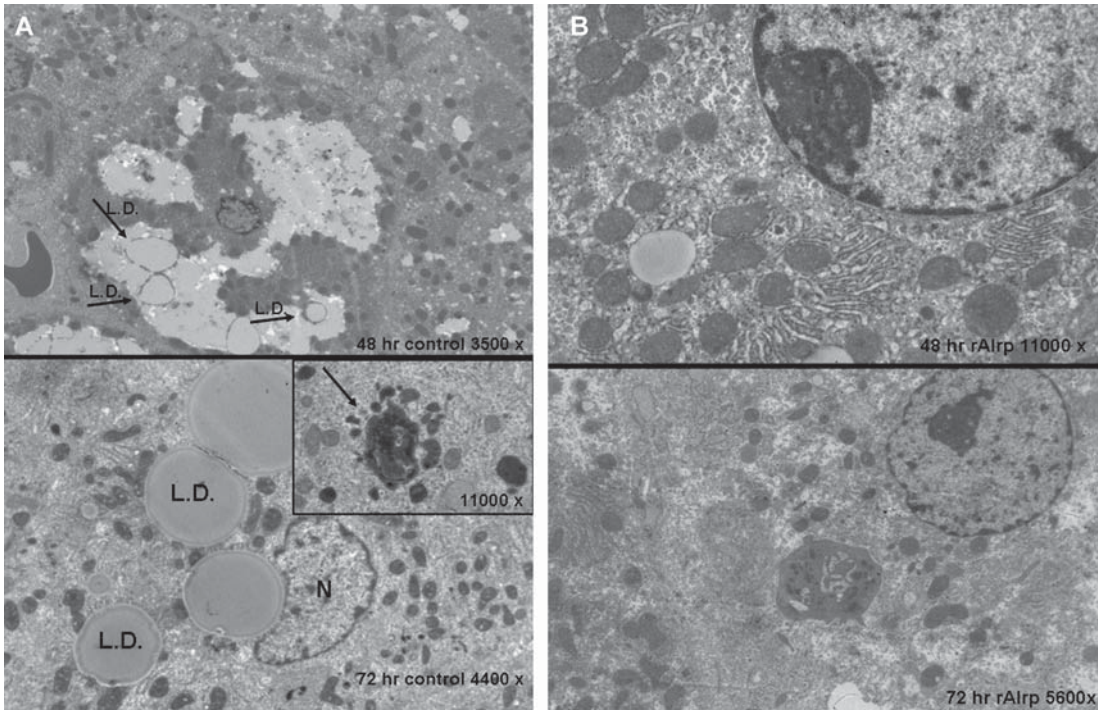


Figure 9. Electron microscopy analysis of liver tissue from albumin- and rAlrp-treated PH rats sacrificed at different times after surgery. Representative photomicrographs by EM of liver tissue from albumin- and rAlrp-treated PH rats performed at different times after surgery. Normal cellular structure by the 24th h after PH was observed in liver tissue from both groups of rats (data not reported). (A) and (B) The observations evidenced by the 48th and 72nd h. In albumin-treated PH rats progressive cellular hydropic swelling, presence of lipid drops (L.D., arrows) and perinuclear steatotic degeneration (A, 48th h, 3500 × magnifications), numerous autophagosomes containing lysosomes, with internal cytoplasmic residues and degenerated cytoplasmic organelles (A, 72nd h, 4400 × magnifications) were evidenced. In the insert, at a higher magnification (11 000 ×), an autophagosome is shown. In rAlrp-treated PH rats, normal cellular structure, significantly less vacuolar degeneration (B, 48th h, 11 000 × magnifications), cells with no particular cytoplasmic alterations, intact and well conserved organelles, presence of rare autophagic structured lysosomes (B, 72nd h, 5600 × magnifications) were evidenced. Liver tissue sections were observed, separately, by two different pathologists.

in albumin-treated PH rats by the 24th h, returning to the 'time 0' value by the 48th h (Figures 6 and 7); in rAlrp-treated PH rats an increased clusterin expression was evidenced both at 24th and 48th h (Figures 6 and 7). These data indicate that the rAlrp treatment is able to sustain clusterin expression, controlling, together with the other anti-oxidative agents, such as catalases and peroxidases, the oxidative stress induced by the PH. It is well known, in fact, that as a consequence of liver mass loss, indifferently determined (viruses, drugs, alcohol or resection), ROS, lipid peroxidation (LPO) and protein carbonylation increase [43–54], determining cell death via apoptosis. The decrease of LPO and carbonyl oxidation, herein reported in rAlrp-treated PH rats (Figure 4), seems to be the beneficial effect of both Alrp high expression and clusterin induction; to the same coordinate events could also be related the tissue preservation observed by H&E (Figure 8) and EM (Figure 9). In fact infrequent signals of hepatocyte ballooning, vascular congestion, destruction of lobule structure, necrosis and apoptosis were distinctly observed in liver tissue from rAlrp-treated PH rats, in contrast to liver tissue from albumin-treated PH rats (Figure 8 and Table II). Similarly, EM analysis (Figure 9) showed rare or absent signals of cellular swelling and apoptosis, on the contrary to liver tissue from albumin-treated PH rats. All these effects determined a different progressive rate of liver mass recovery between the two groups of rats, significantly evident during the first days of the regenerative process and slowly disappearing, by the 7th and the 14th day, when an equal and optimal liver weight/body weight ratio was achieved (Figure 10).

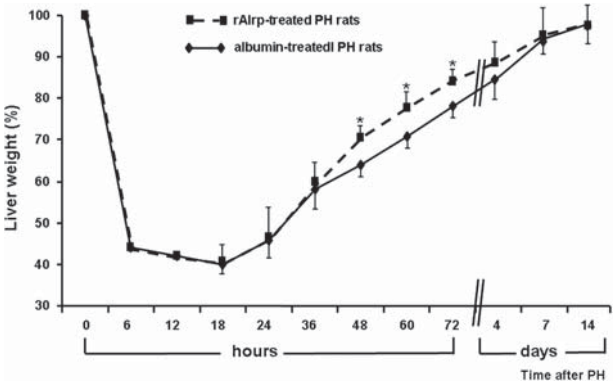


Figure 10. Liver weight. The average percentage of recovery of normal liver weight in albumin- and rAlrp-treated PH rats at different time points after surgery showed a faster and statistically significant recovery in rAlrp-treated PH rats, compared to albumin-treated PH rats. The data are expressed as $M \pm$ SD.

In summary in this report we demonstrated, also *in vivo*, the biological properties of Alrp as a cell protective/anti-apoptotic factor and as a stimulatory substance of the anti-oxidative agents. Both these effects are crucial for cell survival and, prospectively, this protein could become suitable for pharmacological uses in the management of apoptosis- and/or oxidative stress-related diseases.

The presence of Alrp in all the evolutionary steps, from viruses to mammals, indicates this factor as fundamental in the regulation of cell proliferation and ROS-dependent apoptosis, elucidating, in part, the vain attempt, so far, to obtain an Alrp-deficient mouse strain (personal data).

Declaration of interest

This work was supported by grant, Prin prot. n° 2004061897 from the Ministry of the University and Research (MiUR) of Italy. Patent Protocol n° PCT/IB2007/001208. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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