

# Melatonin modulates the autophagic response in acute liver failure induced by the rabbit hemorrhagic disease virus

**Abstract:** Autophagy is an important survival pathway and participates in the host response to infection. Beneficial effects of melatonin have been previously reported in an animal model of acute liver failure (ALF) induced by the rabbit hemorrhagic disease virus (RHDV). This study was aimed to investigate whether melatonin protection against liver injury induced by the RHDV associates to modulation of autophagy. Rabbits were infected with  $2 \times 10^4$  hemagglutination units of a RHDV isolate and received 20 mg/kg melatonin at 0, 12, and 24 hr postinfection. RHDV induced autophagy, with increased expression of beclin-1, ubiquitin-like autophagy-related (Atg)5, Atg12, Atg16L1 and sequestosome 1 (p62/SQSTM1), protein 1 light chain 3 (LC3) staining, and conversion of LC3-I to autophagosome-associated LC3-II. These effects reached a maximum at 24 hr postinfection, in parallel to extensive colocalization of LC3 and lysosome-associated membrane protein (LAMP)-1. The autophagic response induced by RHDV infection was significantly inhibited by melatonin administration. Melatonin treatment also resulted in decreased immunoreactivity for RHDV viral VP60 antigen and a significantly reduction in RHDV VP60 mRNA levels, oxidized to reduced glutathione ratio (GSSG/GSH), caspase-3 activity, and immunoglobulin-heavy-chain-binding protein (BiP) and CCAAT/enhancer-binding protein homologous protein (CHOP) expression. Results indicate that, in addition to its antioxidant and antiapoptotic effects, and the suppression of ER stress, melatonin induces a decrease in autophagy associated with RHDV infection and inhibits RHDV RNA replication. Results obtained reveal novel molecular pathways accounting for the protective effect of melatonin in this animal model of ALF.

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**Key words:** acute liver failure, autophagy, melatonin, rabbit hemorrhagic disease

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Received December 20, 2013;  
Accepted January 31, 2014.

## Introduction

Acute liver failure (ALF) (sometimes referred to as fulminant hepatic failure) is a clinical syndrome resulting from rapid loss in hepatocyte function, typically associated with coagulopathy and encephalopathy in patients without pre-existing liver disease. Liver transplantation is the only therapy of proven benefit, but the rapidity of progression and the variable course of ALF limit its use [1]. As a result, reproducible experimental animal models resembling the clinical conditions are still needed to test new therapeutic approaches, and ALF presents many challenging opportunities in basic research. Most common models are based on surgical techniques or the use of hepatotoxic drugs and very few satisfactory viral models are available [2]. We have developed a viral model of ALF by means of the inoculation of rabbits with the virus of rabbit hemorrhagic disease (RHDV), which displays biochemical data, histological characteristics, and clinical features that resemble those in human ALF [3]. In rabbits infected with RHDV melatonin inhibits oxidative damage, apoptotic mechanisms and endoplasmic reticulum (ER) stress [4–7], which supports its potential therapeutic use in patients with ALF. A number of reports published in the last few

years indicate that properties of the indol as a potent antioxidant, modulator of apoptosis and positive regulator of immune functions contribute to melatonin's antiviral actions [8].

Autophagy is a catabolic process involving self-degradation of cellular components via the lysosomal pathway which acts as survival mechanism and can participate in the host response to infection [9, 10]. Autophagy is known to participate in both sensing oxidative stress and removing oxidatively damaged proteins and organelles [11], and plays a multifunctional role in host defense, by promoting pathogen clearance and modulating innate and adaptive immune responses [12]. The process begins with the engulfment of bulk amounts of cytoplasm by double membrane vesicles, termed autophagosomes, which ultimately fuse with lysosomes, generating an autolysosome in which the constituent is subsequently degraded [13]. At the molecular level the nucleation of the autophagosomal membrane is controlled by a molecular complex containing Bcl-2-interacting protein (beclin)-1, which allows the production of phosphatidylinositol 3-phosphate to occur [14]. Conjugation of ubiquitin-like autophagy-related (Atg)12 to Atg5 and formation of a complex with Atg16L1 is required in the elongation of the autophagosome

membrane. Autophagosome formation also requires the conversion of cytosolic-associated protein light chain 3 (LC3)-I to the membrane-bound LC3-II form. LC3 then binds to the adaptator protein p62 sequestosome 1 (p62/SQSTM1) which, in addition to its role in cell proliferation, inflammation or oxidative stress, facilitates the autophagic degradation of ubiquitinated protein aggregates in lysosomes [13, 15]. The interplay between autophagy and programmed cell death is complex, and recent data suggest the existence of a cross talk between autophagic and apoptotic pathways [16]. Some studies have demonstrated that autophagy may have an active contribution to cell death in virus infected cells [17]. However, there are also reports that autophagy can prolong survival of virus-infected cells by counteracting the apoptotic response [18, 19]. Given the well-known regulatory effect that melatonin has on apoptotic cell death, and the dual capabilities of autophagy to induce survival or death depending on cellular stress [20], melatonin modulation of autophagy is currently under consideration [21], and the regulatory role of the indol on the process of autophagy deserves further careful studies.

In this research, we analyzed whether melatonin administration modulates the autophagic response in RHDV-infected rabbits. Our findings provide evidence that reduction in liver damage by melatonin is associated with a lower RHDV RNA replication and a decrease in the incomplete autophagy induced by RHDV. Further elucidation of the role of autophagy and its relationship to oxidative stress and apoptosis in the pathogenesis of RHDV infection will help to a better knowledge of molecular mechanisms accounting for the protective effect of melatonin in this animal model of ALF.

## Materials and methods

### Virus and experimental model

Nine-week-old male New Zealand white rabbits were kept in the animal facility of the University of León with 12-hr light cycle at 21–22°C and 50% relative humidity. They were given a standard dry rabbit food and water ad libitum. Effects of melatonin were studied by sacrificing control rabbits and batches of infected animals at 18, 24, 30 and 36 hr post infection (hpi). Infection was induced by i.m. injection of  $2 \times 10^4$  hemagglutination units of an RHDV isolate [3, 4]. Melatonin was given (20 mg/kg body weight i.p.) at 0, 12 and 24 hpi; untreated animals received

4 mL of vehicle at 0, 12 and 24 hpi. Melatonin (Sigma, St Louis, MO, USA) was dissolved into absolute ethanol and further dilutions were made in saline. The final concentration of ethanol was 5%.

The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was specifically approved by the Ethics Committee of the University of León.

### Real-time RT-PCR

Total RNA was extracted from frozen rabbit liver using a Trizol reagent (Life Technologies, Madrid, Spain) and quantified using a Nano Drop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega, Madison, WI, USA). RNA integrity was confirmed by formaldehyde gel electrophoresis. Total RNA (1  $\mu$ g) was reverse transcribed as described [7] and mRNA was determined by real-time PCR analysis using SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and the appropriate primers (Table 1). Relative changes in gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method [22]. The cycle number at which the transcripts were detectable ( $C_t$ ) was normalized to the cycle number of  $\beta$ -Actin gene detection, referred to as  $\Delta C_t$ .

### Western blot analysis

For Western blot analysis, liver tissue (25 mg) was homogenized in 1 mL RIPA buffer containing protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH), maintaining temperature at 4°C throughout all procedures. Then the homogenate was incubated on ice for 30 min and finally the samples were centrifuged at 13,000 g for 30 min at 4°C. The supernatant fraction was stored at  $-80^\circ\text{C}$  in aliquots until use. Nuclear extracts were prepared from liver homogenates as described previously [23]. Briefly, 100 mg of liver from all animals was homogenized in  $5 \times 10^{-4}$  L of buffer A (0.01 M Hepes-KOH pH 7.9, 250 g/L glycerol, 0.420 M NaCl, 0.0015 M  $\text{MgCl}_2$ ,  $2 \times 10^{-4}$  M EDTA,  $5 \times 10^{-4}$  M DTT,  $2 \times 10^{-4}$  M PMSF) and a phosphatase inhibitor cocktail (Roche Diagnostics GmbH) to disrupt extracellular matrix and cellular membranes. Homogenates were centrifuged at 1000 g for 10 min at 4°C. The pellet was resuspended in

Table 1. Primers used in this study

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
Beclin-1	CATGCAATGGTGGCTTTCC	TCTCGCCCTTTCAACCTCTT
Atg5	CGTCCTGTGGCTGCAGATG	AAGGACACACTTCTTTGAGGAGATC
Atg12	TGCTGAAGGCTGTGGGAGAT	TGTTCCGCTCTACAGCCCATT
Atg16L1	CCACCAAACCGGCATGAG	CTTGCAGCTGGCTGTTCATT
p62/SQSTM1	AACAGAGGTGACCACCCTTCA	AGCACAGACTGGCTGGAAGTC
RHDV	TAGCCCAACAGAAGCACAAG	AAACAAGTCGTCAACCTCCC
BiP	ATTGACAATGGTGTCTTTCGAAGTC	CCCCGCCAGGTGAGT
CHOP	ATACATCACCACACTGAAAGCA	GCACTCGGCTGCCATCTC
$\beta$ -Actin	TGGCATCCTGACGCTCAA	TCGTCCCAGTTGGTCACGAT

$2.5 \times 10^{-4}$  L of buffer B (0.02 M NaCl Hepes- KOH pH 7.9, 250 g/L glycerol, 0.042 M NaCl,  $15 \times 10^{-4}$  M PMSF), homogenized, and incubated at 4°C for 30 min. Cellular debris was removed by centrifugation at 14,000 g for 15 min at 4°C. The supernatant fraction containing DNA binding proteins was recollected and stored at -80°C in aliquots until use. Protein concentration was measured by Bradford assay. Equal amounts of protein extracts (10–50 µg) were separated by 7–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred electrically to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 min at 37°C and probed overnight at 4°C with polyclonal anti-p62/SQSTM1, poly(ADP-ribose)polymerase-1 (PARP-1) and IRF7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-IRF7 (Cell Signaling Technology, Danvers, MA, USA) and LC3-I/II (Abcam, Cambridge, UK) antibodies at 1:200–1:1000 dilution with PBST containing 2.5% nonfat dry milk. Equal loading of protein was demonstrated by probing the membranes with a rabbit anti-β-Actin polyclonal antibody (1:2000; Sigma) and a rabbit anti-Lamin-B polyclonal antibody (1:200; Santa Cruz Biotechnology). After washing with TBST, the membranes were incubated for 1 hr at room temperature with secondary HRP conjugated antibody (1:5000; Dako, Glostrup, Denmark), and visualized using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden) [7]. The density of the specific bands was quantified with an imaging densitometer (Scion Image J Software 1.46a, Bethesda, MD, USA).

### Immunohistochemistry

Tissue samples were recovered, fixed in 10% buffered formalin and embedded in paraffin. Sections (4 µm) were dewaxed and hydrated through graded ethanol, cooked in 25 mM citrate buffer, pH 6.0, in a pressure cooker for 10 min, transferred into boiling deionized water and let to cool for 20 min. Tissue sections were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidase activity. The slides were incubated with rabbit anti-VP60 antibodies (Ingenasa, Madrid, Spain) overnight at 4°C. Subsequently, the sections were incubated for 30 min using the EnVision+ system and developed with a solution of 3-3-diaminobenzidine (DAB) (Vector Lab, Burlingame, CA, USA). The slides were stained with hematoxylin for 10 s and mounted. The specificity of the technique was evaluated by negative controls (omitting the incubation with the primary antibody and incubating it with nonimmune sera).

### Double immunofluorescence

For immunofluorescent double staining sections were dewaxed in xylene and rehydrated in graded ethanol to distilled water, do not allowing slides to dry at any time during this process. Heat mediated antigen was performed in a cooker filled with 1 mM EDTA (pH = 8). Sections were brought to a boil and then maintain at a sub-boiling temperature for 15 min. All subsequent incubations with

immunochemicals were performed in a humidified chamber. After unmasking and after blocking the nonspecific binding the sections were co-incubated with the LAMP-1 antibody (Santa Cruz Biotechnology) and LC3 (Abcam) (at 1:50 and 1:200 dilution, respectively) overnight at 4°C. After incubation with primary antibodies, samples were washed twice in PBS for 10 min at room temperature. Thereafter, the secondary antibodies donkey anti-rabbit conjugated with FITC and donkey anti-mouse conjugated with Dylight™549 (Jackson Immuno-Research, Baltimore, PA, USA) were applied for 2 hr at 21°C. After washing in TBS, the coverslips were mounted on Dako Cytomation Fluorescent Mounting Medium (Dako). In sections from each experimental group, the primary antibody was replaced by antibody diluent to assess for nonspecific binding of the secondary antibody [7]. The preparations were analyzed with an inverted fluorescent microscope (Nikon Eclipse Ti, Tokyo, Japan).

### Measurement of oxidized and reduced glutathione

Oxidized and reduced glutathione (GSSG and GSH, respectively) analysis was performed by the method of Hissin and Hilf [24], homogenizing 250 mg of tissue in 0.1 M sodium phosphate 5 mM EDTA buffer (pH 8.0) with 25% phosphoric acid at a proportion of 1:20.

### Caspase-3 activity

Lysates were prepared by homogenizing liver tissue in 0.25 mM sucrose, 1 mM EDTA, 10 mM Tris and a protease inhibitor cocktail (Roche Diagnostics GmbH). The lysates were then centrifuged at 14,000 g for 10 min at 4°C, and supernatants (50 µg protein) were incubated for 1 hr at 37°C in HEPES buffer containing 100 µM concentrations of the specific fluorogenic substrate 7-amino-4-methylcoumarin N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (DEVD-AMC). Cleavage of the caspase substrate was monitored using a spectrofluorimeter (Hitachi F-2000 fluorimeter, Hitachi LTD, Tokyo, Japan) at excitation/emission wavelengths of 380/460 nm. Activity was expressed as fluorescence units per milligram of protein per minute of incubation.

### Statistical analysis

Results are expressed as mean values ± standard error of the mean (S.E.M.). Data were compared by analysis of variance (ANOVA); when the analysis indicated the presence of a significant difference, the means were compared with the Newman-Keul's test. Significance was accepted when *P* was <0.05. Values were analyzed using the statistical package SPSS 19.0 (IBM Corporation, Armonk, NY, USA).

## Results

To determine the presence of the virus in the liver of rabbits infected with the RHDV viral VP60 antigen was examined by immunohistochemical techniques. Data obtained indicate a progressive increase in the extent of



labeling in RHDV-infected animals which reached a maximum at 36 hpi (Fig. 1A,B) in parallel to the increase in RHDV mRNA level (Fig. 1C). Melatonin treatment resulted in decreased immunoreactivity and significantly reduced RHDV mRNA expression (Fig. 1A–C), revealing an inhibitory role of the indol on RHDV replication. Viral replication associated to increased expression of the phosphorylated form of IRF7, a master regulator of type I-interferon-dependent innate immunity, and this effect was not modified by melatonin administration (Fig. 1D).

Autophagy activation by RHDV infection was confirmed by the significant increase of beclin-1 expression and the upregulation of components of the Atg12-Atg5-Atg16L1

complex at 18–24 hpi (Table 2). Another frequently used autophagy marker is LC3, which is converted from the cytosolic form LC3-I to the lipidated form LC3-II. Western blot data indicate that LC3-II expression increased markedly at 18–24 hpi, with a maximum rise of LC3-II/LC3-I ratio at 24 hpi, which suggests that RHDV could be blocking the autophagic process in liver cells at early postinfection periods (Fig. 2A). Effects on LC3 expression can reflect aggregated effects of induced autophagosome synthesis, decreased fusion with lysosomes and/or suppression of proteolytic turnover in the autophagolysosomes. Immunofluorescence analysis showed spots of green fluorescence which reached a maximum at 24 hpi, suggest-

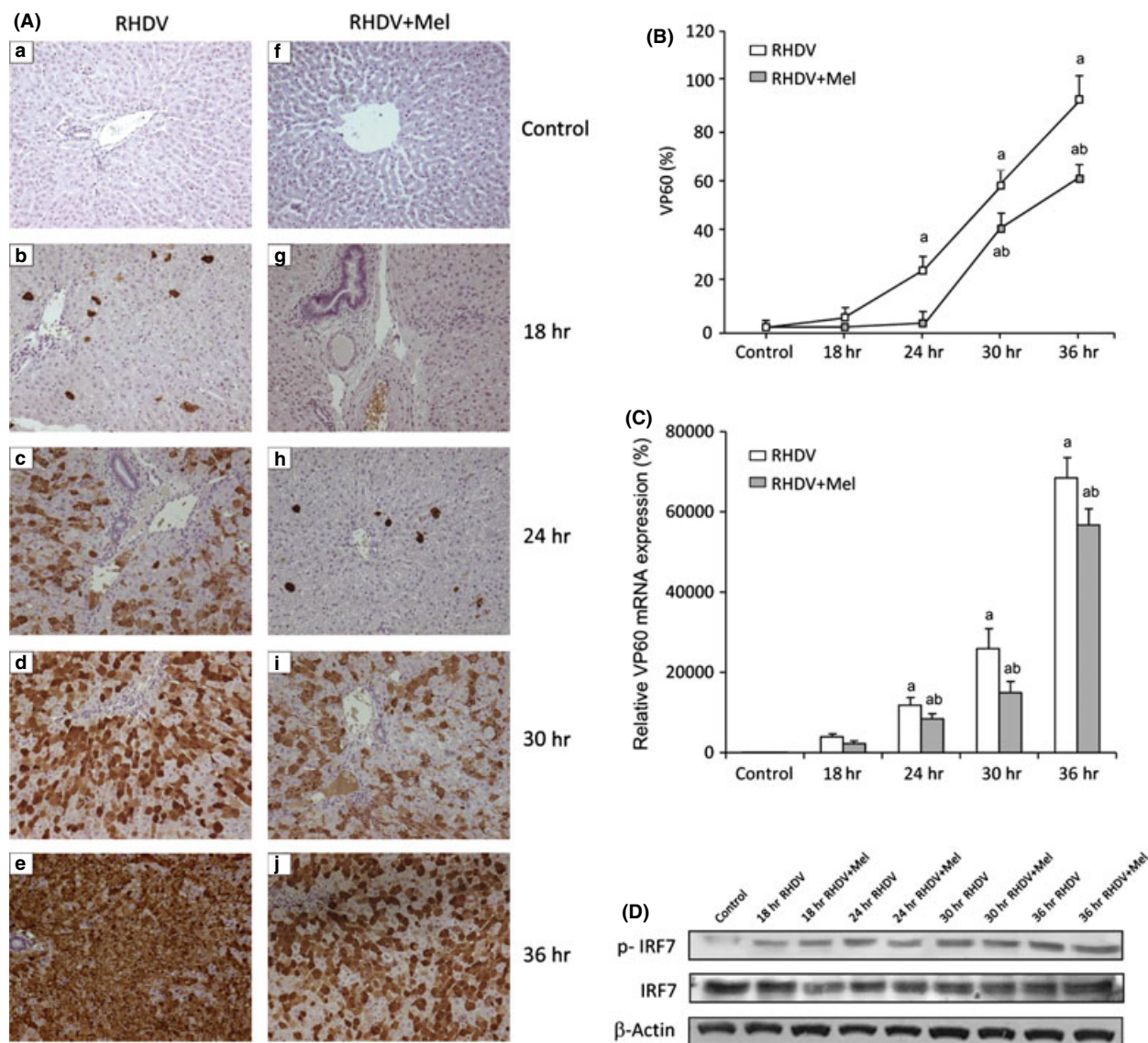


Fig. 1. Expression of the capsid protein VP60 and IRF7 in rabbit hemorrhagic disease virus (RHDV)-infected and melatonin-treated rabbits. (A,B) VP60 immunohistochemical labeling in hepatocytes: (a,f) Control, (b,g) 18 hpi; (c,h) 24 hpi; (d,i) 30 hpi; (e,j) 36 hpi. Paraffin-embedded sections were immunostained with a VP60 antibody. Original magnification: 200×. Data are presented as percentage change from the control group. Image analysis was performed using the ImageJ software v3.91 (<http://rsbweb.nih.gov/ij/>). (C) Levels of VP60 mRNA analyzed by real-time PCR assay and normalized against β-Actin. Values are expressed as means ± S.E.M. (n = 6). <sup>a</sup>P < 0.05, compared with Control, <sup>b</sup>P < 0.05, compared with RHDV, same period. (D) Western blot of IRF7. Proteins from liver extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands.

Table 2. Effect of rabbit hemorrhagic disease virus (RHDV) infection and melatonin on mRNA levels of genes related to autophagy and ER stress

	Control	RHDV18	RHDV18 + Mel	RHDV24	RHDV24 + Mel	RHDV30	RHDV30 + Mel	RHDV36	RHDV36 + Mel
Beclin-1	100 ± 8	222 ± 12 <sup>a</sup>	177 ± 16 <sup>a,b</sup>	234 ± 16 <sup>a</sup>	94 ± 7 <sup>b</sup>	143 ± 7 <sup>a</sup>	87 ± 7 <sup>b</sup>	87 ± 7	68 ± 7 <sup>a,b</sup>
Atg5	100 ± 4	170 ± 20 <sup>a</sup>	141 ± 6 <sup>a</sup>	149 ± 24 <sup>a</sup>	70 ± 8 <sup>a,b</sup>	129 ± 5 <sup>a</sup>	78 ± 5 <sup>a,b</sup>	95 ± 5	49 ± 5 <sup>a,b</sup>
Atg12	100 ± 12	193 ± 10 <sup>a</sup>	177 ± 9 <sup>a</sup>	135 ± 7 <sup>a</sup>	94 ± 8 <sup>b</sup>	89 ± 6	43 ± 7 <sup>a,b</sup>	91 ± 7	53 ± 7 <sup>a,b</sup>
Atg16L1	100 ± 5	177 ± 11 <sup>a</sup>	103 ± 12 <sup>b</sup>	114 ± 9	57 ± 9 <sup>a,b</sup>	86 ± 6	49 ± 9 <sup>a,b</sup>	80 ± 10	38 ± 9 <sup>a,b</sup>
p62/SQSTM1	100 ± 4	134 ± 7 <sup>a</sup>	92 ± 13 <sup>b</sup>	158 ± 12 <sup>a</sup>	108 ± 6 <sup>b</sup>	130 ± 10 <sup>a</sup>	75 ± 6 <sup>a,b</sup>	81 ± 4 <sup>a</sup>	53 ± 5 <sup>a,b</sup>
BiP	100 ± 6	264 ± 8 <sup>a</sup>	231 ± 9 <sup>a</sup>	302 ± 6 <sup>a</sup>	234 ± 7 <sup>a,b</sup>	216 ± 12 <sup>a</sup>	136 ± 15 <sup>a,b</sup>	129 ± 10 <sup>a</sup>	79 ± 9 <sup>a,b</sup>
CHOP	100 ± 7	389 ± 13 <sup>a</sup>	312 ± 24 <sup>a,b</sup>	672 ± 9 <sup>a</sup>	264 ± 4 <sup>a,b</sup>	244 ± 9 <sup>a</sup>	136 ± 18 <sup>a,b</sup>	114 ± 4	90 ± 7 <sup>b</sup>

Levels of mRNA were analyzed by real-time PCR assays. Data, normalized against  $\beta$ -Actin, are presented as percentage change from the control group. Values are expressed as means  $\pm$  S.E.M. (n = 6). <sup>a</sup> $P < 0.05$ , compared with Control, <sup>b</sup> $P < 0.05$  compared with RHDV, same period.

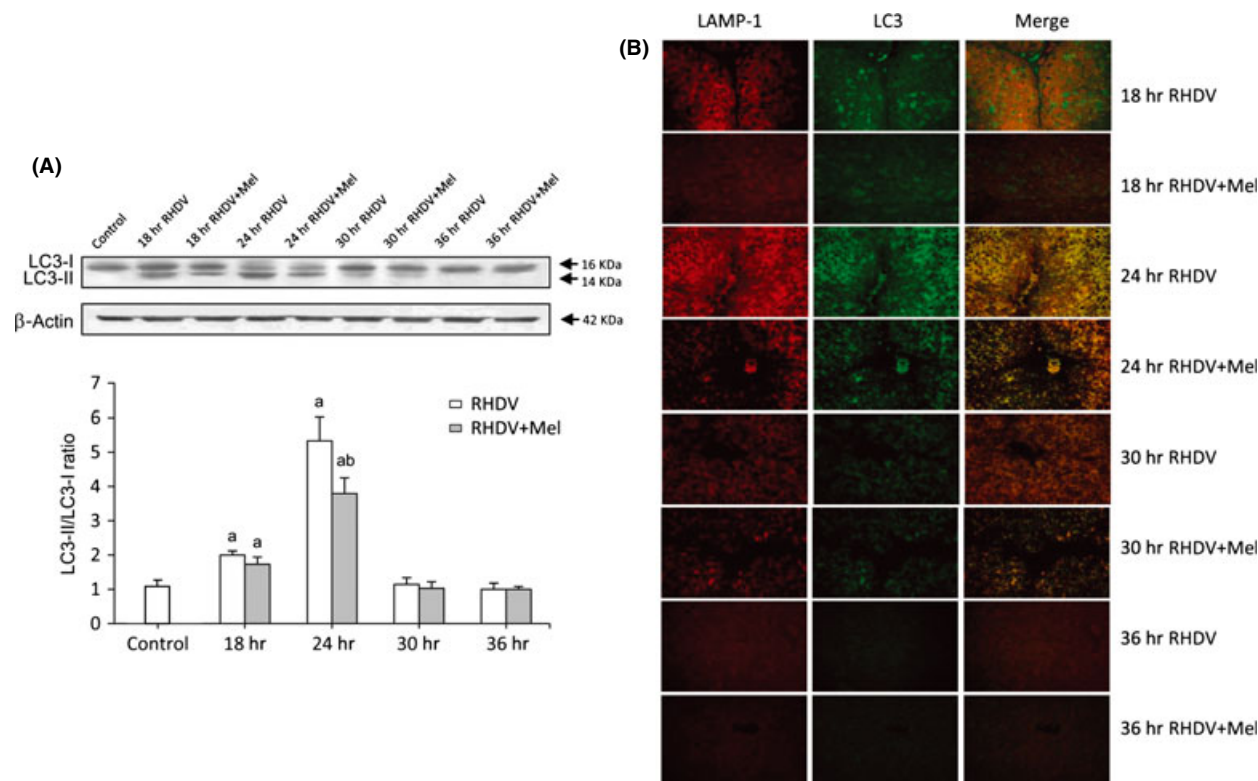
ing an elevated turnover of autophagosomes (Fig. 2B). At 18 hpi, there was little colocalization between LC3 and the lysosomal marker LAMP-1, but extensive overlap was observed at 24 hpi, presumably due to increased accumulation of autophagolysosomes. However, the parallel increase in p62/SQSTM1 mRNA levels (Table 2) may reflect a dysfunctional autophagy with impairment of the autophagic flux. The autophagic response in RHDV-infected rabbits was partially inhibited by melatonin treatment, as shown by the reduced LC3 immunofluorescent staining (Fig. 2B), the decrease in LC3-II protein expression (Fig. 2A), and the diminished mRNA expression of beclin-1, Atg5, Atg12 and Atg16L1 (Table 2).

Endoplasmic reticulum (ER) stress has been reported to induce the accumulation of autophagosomes, and autophagy may be an important part of normal ER function [25]. We have previously reported that melatonin treatment inhibits ER stress at 36 hpi in RHDV-infected rabbits [7]. During ER stress different transcription factors regulate the expression of ER chaperones that enhance the folding capacity of the ER, including CCAAT/enhancer-binding protein homologous protein (CHOP) and immunoglobulin-heavy-chain-binding protein (BiP/GRP78). As expected, mRNA levels for both chaperones increased following RHDV infection, with a maximum at 24 hpi, and this effect was significantly inhibited by melatonin administration (Table 2).

As previously reported [26], melatonin prevented the significant increase in the GSSG/GSH ratio observed in RHDV-infected rabbits (Fig. 3A). Data obtained also confirmed the already described effect of melatonin on apoptotic death induced by RHDV [6]; both activation of caspase-3, the common event initiated by multiple different stimuli that induces apoptosis, and proteolysis of PARP-1, a nuclear enzyme cleaved into a 85-kDA fragment by caspase-3 [27], increased significantly at 30 and 36 hpi, and these effects were significantly prevented by melatonin treatment (Fig. 3B,C).

## Discussion

Results from this study demonstrate that RHDV induced autophagosome and autophagolysosome formation, with increased expression of beclin-1, LC3-II/LC-I ratio and Atg5-Atg12-Atg16L1, and colocalization of LC3 and LAMP-1. The parallel increase in p62/SQSTM1 expression may reflect a dysfunctional autophagy similar to the previous reports following infection of liver cells by other RNA viruses [28, 29]. Data obtained also indicate that the RHDV-induced autophagic response was inhibited by melatonin administration. Mechanisms through which viruses induce autophagy remain unclear. One of the typical stress responses initiated by viral infection is ER stress, which down-regulates protein synthesis through the three branches of the unfolded protein response (UPR). During ER stress, different transcription factors regulate the expression of ER chaperones, such as CHOP and BiP, which enhance the folding capacity of the ER [7]. It is known that sensors of the UPR control signal-transduction pathways leading to the transcription of autophagic genes [30], and viruses capable of inducing



**Fig. 2.** Markers of autophagy in rabbit hemorrhagic disease virus (RHDV)-infected and melatonin-treated rabbits. (A) Western blot of LC3. Proteins from liver extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by  $\beta$ -Actin bands. The graph shows the LC3-II/LC3-I ratio. Values are expressed as means  $\pm$  S.E.M. (n = 6). <sup>a</sup> $P < 0.05$ , compared with Control, <sup>b</sup> $P < 0.05$ , compared with RHDV, same period. (B) Colocalization of LC3 and LAMP-1 by immunofluorescence analysis. In the left panels LAMP-1 positive staining (red fluorescence); in middle panels positive staining of LC3 (green fluorescence); right panels show the merge of two fluorescences.

ER stress have the potential to induce an autophagic response. Thus, previous studies have demonstrated that autophagy is associated to ER stress in HCV infection, and that persistent induction of ER stress and incomplete activation of autophagy play an important role in HCV pathogenesis [28, 31]. In addition, a newer hypothesis that is gaining support is that autophagic activities are sensitive to oxidative stress and to the generation of reactive species [11]. Indeed, a recent research has shown that oxidative stress triggers the accumulation of autophagic compartments without increasing the degradation of long-lived proteins in human neuroblastoma cells infected by herpes simplex virus type 1 infection [32]. Moreover, it has been reported that in MEF cells infected with the Chikungunya virus, independent induction of ER and oxidative stress pathways triggers autophagy through the inhibition of the mammalian target of rapamycin (mTOR) [18]. Therefore, the previously reported reduction of oxidative stress and ER stress by melatonin [7, 26], confirmed in this research through the decrease in CHOP and BiP expression and the increase in the GSSG/GSH ratio, could contribute to the inhibitory effect of the indol on the autophagic response induced by viral infection.

In our research, the autophagic activity induced by the RHDV declined in late periods of infection (30–36 hpi) in parallel to the increase in apoptosis. Different studies have

previously shown that virally induced autophagy is capable of preventing the early apoptotic death of cells [33], which suggests that autophagy might contribute to limit the cytopathic effect of viruses and the pathological consequences associated with cell death triggered by viral infection. This regulation of programmed cell death by autophagy could also be present in RHDV-infected hepatocytes. However, mechanisms responsible for the regulation of apoptosis by autophagy and for the overwhelming of the apoptotic response by the inhibitory effect of autophagy remain unknown. Melatonin is able to induce or inhibit autophagy based on cellular necessities and oxidative stress levels. There are reports that autophagy and survival are significantly enhanced in ischemic N2a cells treated with melatonin [34], and melatonin-induced autophagy protects against human prion protein-mediated neurotoxicity [35]. Moreover, melatonin induces autophagy and results in the effective removal of mutant-TGFBIP from granular corneal dystroph type 2 fibroblasts [36]. In contrast, melatonin is known to protect HeLa cells from rotenone-induced cell injury via inhibition of autophagy [37], to suppress cyclosporine A-induced autophagy in rat pituitary GH3 cells [38], to inhibit kainic acid-induced neurotoxicity in mouse hippocampus via inhibition of autophagy [39], and to attenuate methamphetamine-induced autophagy [40]. Consistent with studies which report parallel inhibition of autophagic processes and programmed



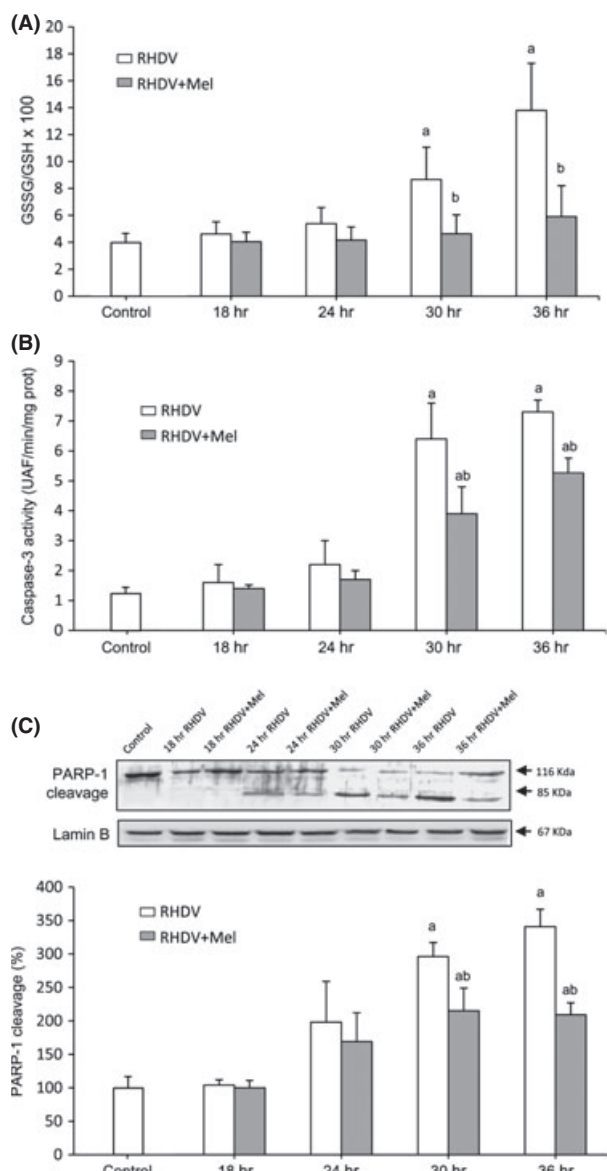


Fig. 3. Markers of oxidative stress and apoptosis in rabbit hemorrhagic disease virus (RHDV)-infected and melatonin-treated rabbits. (A) Oxidized to reduced glutathione ratio (GSSG/GSH) in hepatic tissue. (B) Liver activity of caspase-3. Samples were incubated with the specific fluorogenic substrate Ac-DEVD-AMC. (C) Western blot of PARP-1. Proteins from liver extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by Lamin B bands. The graph shows densitometric quantification. Values are expressed as means  $\pm$  S.E.M. (n = 6). <sup>a</sup>P < 0.05, compared with Control, <sup>b</sup>P < 0.05, compared with RHDV, same period.

cell death, in the present experiments both autophagy and apoptosis were significantly inhibited by melatonin. These effects of the indol could be the consequence of its antioxidant action, which may directly antagonize mechanisms contributing both to the intrinsic and extrinsic pathways of apoptosis [6], but also result in the inhibition of ER stress and, in turn, in diminished autophagic and apoptotic responses.

An additional interesting fact concerns the inhibition of RHDV induced by melatonin. The role of autophagy in relation to viral replication is dual and it may be beneficial to virus or host. Thus, although autophagy may be a cellular mechanism to clear viral infection, and its inhibition results in an increased replication and virulence of herpes simplex virus 1 (HSV1) or vesicular stomatitis virus (VSV), different viruses appear to use this response to enhance their own replication. It has been reported that the double membrane of the autophagosome supports poliovirus replication [41], the autophagic machinery is used for coronaviruses replication [42], HCV uses autophagy for the early protein translation [43], and Dengue virus activate autophagy to elevate viral replication [44]. Moreover, it is known that ER stress may play a positive role in HCV RNA replication through activation of autophagy, because siRNAs directed against sensors of the different branches of the UPR suppress HCV-induced lipidation of LC3, required for formation of autophagosomes, and reduce HCV RNA levels [28]. It has also been reported that inhibitors of X-box binding protein-1 (XBP-1), one of the ER stress mediators, inhibit in parallel HCV viral replication and autophagy in hepatoma cells [45]. Therefore, data obtained in this study could indicate that the decrease in RHDV RNA replication in melatonin-treated rabbits is a consequence, at least in part, of its negative effect on ER stress and on the autophagic response. Both HCV and Dengue virus are known to exploit the UPR-autophagy pathway to promote replication by suppressing innate antiviral immunity [46, 47], and a number of in vitro and in vivo studies have documented that melatonin plays a role in innate immunity [48]. However, in our experiments expression of the phosphorylated form of IRF7, a potent type I interferon inducer capable of modulating viral propagation in infected hepatocytes [49], increased in RHDV-infected rabbits and was not modified by melatonin. This suggests that RHDV is not able to abrogate type I interferon-dependent immune responses, and inhibition of viral replication by melatonin is probably unrelated to induction of innate immunity.

In conclusion, results from this study indicate that in addition to its anti-oxidant and anti-apoptotic effects, and the suppression of ER stress, melatonin induces a decrease in the autophagy associated with RHDV infection and inhibits RHDV RNA replication. Understanding the mechanisms behind the interplay of RHDV-induced autophagy with oxidative stress, ER stress and apoptosis may help to identify molecular pathways accounting for the protective effect of melatonin in this animal model of ALF.

### Acknowledgements

CIBERehd is funded by Instituto de Salud Carlos III, Spain.

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