



Melatonin modulates mitophagy, innate immunity and circadian clocks in a model of viral-induced fulminant hepatic failure

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Abstract

The haemorrhagic disease virus (RHDV) is a non-cultivable virus that promotes in rabbits an acute disease which accomplishes many characteristics of an animal model of fulminant hepatic failure (FHF). Beneficial effects of melatonin have been reported in RHDV-infected rabbits. This study investigated whether protection against viral-derived liver injury by melatonin is associated with modulation of mitophagy, innate immunity and clock signalling. Rabbits were experimentally infected with 2×10^4 haemagglutination units of a RHDV isolate and killed at 18, 24 and 30 hours after infection (hpi). Melatonin (20 mg/kg body weight ip) was administered at 0, 12 and 24 hpi. RHDV infection induced mitophagy, with the presence of a high number of mitophagosomes in hepatocytes and increased expression of mitophagy genes. Greater expression of main innate immune intermediaries and inflammasome components was also found in livers with RHDV-induced FHF. Both mitophagy and innate immunity activation was significantly hindered by melatonin. FHF induction also elicited an early dysregulation in clock signalling, and melatonin was able to prevent such circadian disruption. Our study discloses novel molecular routes contributing to RHDV-induced damage progression and supports the potential of melatonin as a promising therapeutic option in human FHF.

KEYWORDS

circadian clocks, fulminant hepatic failure, innate immune response, melatonin, mitophagy, rabbit haemorrhagic disease virus

Javier González-Gallego and María Jesús Tuñón share senior authorship.

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1 | INTRODUCTION

Fulminant hepatic failure (FHF) is a life-threatening clinical pathology, induced by drugs, toxins and viral hepatitis, with a complex set of mechanisms responsible for the impairment of liver function.¹⁻³ In the absence of suitable animal models to better study causes and treatments of liver damage, specially of viral origin, our group developed an FHF model by rabbit haemorrhagic disease virus (RHDV) infection which complies many requirements of human FHF and reproduces the most representative histologic and biochemical parameters and clinical symptoms of the human disease.⁴⁻⁸ The RHDV virus is a member of the viral family Caliciviridae, which has no possibility to be cultured, and RHDV infection results in a mortality rate higher than 90% within 2-3 days because of the massive liver damage in adult rabbits,⁹ supplying a valuable platform to evaluate cell-damaging mechanisms in severe human FHF, as well as the therapeutic potential of hepatoprotective therapies in this condition. Thus, we have reported by using this model the involvement of different processes, including endoplasmic reticulum stress, apoptosis, oxidative stress and autophagy, in liver injury progression.^{8,10-14} However, given that severe FHF is still one of the most challenging health problems in clinical medicine,⁴ a better knowledge of molecular mechanisms contributing to hepatic damage is still required.

Mitochondria homeostasis is crucial for regulating cellular response against FHF-induced damage, where disruption of mitophagy, an autophagic process of degradation of damaged mitochondria and mitochondrial biogenesis generates loss of mitochondrial quality control.¹⁵⁻¹⁷ Moreover, an increasing number of studies highlight the major role of mitochondria as key platform of intracellular signalling, modulating the innate immune and inflammatory responses mainly by mitophagy.^{17,18} Aside from mediating host defence against virus replication, the innate immune response may also contribute to FHF pathogenesis, since dysregulated immunity by excessive activation leads to clinical complications derived from liver injury.^{17,19} Despite the well-established crosstalk between mitophagy and immune response, few investigations have accurately described the role of both processes during acute hepatic damage in a suitable FHF model.^{16,20} In recent years, modulation of mitochondria homeostasis by mitophagy has been tightly associated with circadian clock machinery.²¹ Some circadian clock regulators, such as brain and muscle arnt-like protein 1 (BMAL1) and, to a greater extent, nuclear receptor subfamily 1 group D member 1 (REV-ERB α), have demonstrated to directly control mitophagy and mitochondrial biogenesis.^{22,23} Moreover, overactivation of innate immune response derived from viral cell damage could be a consequence of dysregulation on the circadian rhythms.^{1,24} In summary, growing evidence places mitochondria as a central scaffold in the crosstalk between mitophagy, innate immunity and inflammasome activation, and its modulation by clock machinery arouses a growing interest.^{1,15}

Melatonin is a product of the pineal gland which has proved to regulate a high number of molecular mechanisms, including endoplasmic reticulum stress, apoptosis and autophagy, involved in the progression of hepatic damage.^{1,15,25-33} In rabbits infected with RHDV, melatonin reduces liver injury by a combination of antioxidant,

anti-inflammatory and anti-apoptotic effects, being also able to modulate sphingolipid metabolism.^{5,10,13,34} Beneficial effects of melatonin on liver diseases such as fibrosis and hepatocarcinoma are closely related to the biological clock machinery,^{28,30} and recent studies found a likely REV-ERB α implication on inflammasome activation and mitochondria homeostasis during severe hepatic damage.^{16,23,35}

However, there is still no study in which melatonin impact on these mechanisms has been evaluated. Therefore, in the present work we analysed in RHDV-infected rabbits the viral-derived alterations on mitophagy and innate immune response, along with the modulation of the circadian clock signalling, and the melatonin effects on these processes. Data obtained reveal new molecular mechanisms accounting for the protective effect of the indole in this viral animal model of FHF and support the potential of melatonin as an antiviral agent.

2 | MATERIALS AND METHODS

2.1 | Virus and experimental model

New Zealand white rabbits (9 weeks of age) were kept at 21-22°C and 50% relative humidity with a 12-hour light cycle, water ad libitum and standard dry food for rabbits. Viral infection was performed by intramuscular injection of 2×10^4 haemagglutination units of an RHDV isolate.³⁶ Melatonin was administered (20 mg/kg body weight ip) at 0, 12 and 24 hour after infection (hpi). Effects of melatonin were studied in liver tissue by killing a rabbit control group ($n = 6$) and batches of infected animals at 18, 24 and 30 hpi ($n = 6$ each). Period times were selected in accordance with previous studies demonstrating the rapid evolution of the RHDV infection.⁹ The conduct of this study rigorously followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the NIH, and was specifically approved by the Ethics Committee of the University of León.

2.2 | Biochemical analysis

Blood samples from rabbits were collected in heparin tubes from the marginal ear vein at 18, 24 and 30 hpi, for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Samples were analysed in the Instrumental Techniques Laboratory of the University of León using standard techniques.

2.3 | Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from frozen rabbit liver with TRIzol reagent (Life Technologies; Madrid, Spain) and quantified employing a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Purification of RNA was performed by incubating RNA with RQ1 RNase-free DNase (Promega; Madison, WI, USA), and formaldehyde gel electrophoresis was carried out to

evaluate RNA integrity. As previously described,¹⁰ total RNA was reverse-transcribed and analysed by qRT-PCR with SYBR Green I Master (Roche Diagnostics GmbH; Mannheim, Germany) and the corresponding primers (Table 1). Relative changes in gene expression were determined employing the $2^{-\Delta\Delta Ct}$ method.³⁷

2.4 | Western blot analysis

Liver tissue (25 mg) was homogenized in 1 mL RIPA buffer completed with protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH). The homogenate was incubated 30 minutes on ice and centrifuged 30 minutes at 13 000 g and 4°C. Bicinchoninic acid assay (BCA) was performed to measure protein concentration, separating equal protein amounts (30 µg) by 7%-12% sodium

dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene difluoride membranes (Millipore; Bedford, MA, USA), blocked for 30 minutes at 37°C with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) and incubated overnight at 4°C with polyclonal anti NLR family pyrin domain-containing 3 (NLRP3), granzyme (GZMA) (Santa Cruz Biotechnology; CA, USA), phosphorylated signal transducer and activator of transcription 6 (p-STAT6), STAT6 (Cell Signaling Technology; Danvers, MA, USA), PTEN-induced putative kinase protein 1 (PINK1), E3 ubiquitin-protein ligase parkin (PARKIN), mitofusin (MFN1), voltage-dependent anion-selective channel protein 1 (VDAC), BNIP3-like protein (BNIP3L), BCL2/adenovirus E1B 19 kD protein-interacting protein 3 (BNIP3), FUN14 domain-containing protein 1 (FUNDC1), TIR-domain-containing adapter-inducing interferon-beta (TRIF) and phosphorylated interferon regulatory factor 3

TABLE 1 Primers used in qRT-PCR analysis

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
ASC	ATCAAGGCCATTCTGAGAAGAT	CGGTGCTGGTCCACGAA
β -ACTIN	TGGCATCCTGACGCTCAA	TCGTCCCAGTTGGTCACGAT
BMAL1	GGCGCCGGGATAAGATG	GCACGTGGGAACCAGAGAA
BNIP3	TGACACGTGAATCCAGTCAACA	GCTGATTTCCCATCCACATCA
BNIP3L	GTACGATCGTGTACCTGCAA	CCACGGGTTCTACTTTGTAGAGT
CASP1	CACAGACTACGCAGGACAACCA	GCGGGCAAAGCTTGAGACT
CLOCK	AGGAACAAGGTTTGACGTTTT	GTAACCGTCAGGTGCGTGAA
CRY1	GAGGCAAGCCGTTTGAATATTG	AGACCTCGGTATCGTAAAGCT
FUNDC1	GCTGGTGTGCAGGATTTTTGT	CCACCACCCACTGCAGTTG
GZMA	CCCTAAAATGGGAGAGGATGTG	TCCCCACCCCTGCAACT
IFN- α	GGGATGCAACCCTCTAGATTC	ACAGGCTTGACAGCCACTCA
IL1 β	CTCCTGCCAACCTACAACAA	TCCAGAGCCACAACGACTGA
MAVS	CATGGGCAATGTGTGATGCT	GGCCTATCCCAGCGCTTT
NKG2D	AAGGGACTGTAGTTTCACAAAGCA	GCTCCATAGAATCCCGTTCA
NLRP3	TGTCTACGTCCAGCTTTTG	AGCCAGAGTCTGCGAATGTT
PARKIN	TCCCAGTGGAGGTCGATTCT	CTTAGCAACCGCTCCTTGA
PER1	CAGTTGTGGAGAAATTTGGGTTT	TCTATGTTTCGGAGGGTTGAGAA
PER2	GGTCAGCGTCGGGAAGAA	GTCATGCGGAAGGGATGGTA
PINK1	GGTCACGCACAGAAAATCCA	GGAAAACCCGGATGATGTTG
PRF1	CACATATGGTGTGAGCTGCAACCT	AGCATATGGCACGGTAGAGGAA
REV-ERB α	AGCTGCCCGAGGCTGAT	CTCTGCTTTGCATGGGAAGAG
RIG-I	GGATAATGGCAGGTGCAGAGA	TTTTGGGCCAGTTTCTCTTGT
STING	GGCCCCGGAGTTTCAGA	TTCCCTCGGCAGGTTTCT
TLR3	TGTCCCTTTGGATGCTGTGTAC	CAGTCGGCTACTTCTGTCTCA
TLR4	GTATCGCCTTCTCAGCAGGAA	GCCGCCCCAGGACAGT

Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; BMAL1, brain and muscle arnt-like protein 1; BNIP3, BCL2/adenovirus E1B 19kDa protein-interacting protein 3; BNIP3L, BNIP3-like protein; CASP1, caspase-1; CLOCK, circadian locomotor output cycles protein kaput; CRY, cryptochrome; FUNDC1, FUN14 domain-containing protein 1; GZMA, Granzyme; IFN- α , interferon- α ; IL1 β , interleukin 1 β ; MAVS, mitochondrial antiviral signalling; NKG2D, natural killer group 2D; NLRP3, NLR family pyrin domain-containing 3; PARKIN, E3 ubiquitin-protein ligase parkin; PER, period; PINK1, PTEN-induced putative kinase protein 1; PRF1, Perforin; REV-ERB α , nuclear receptor subfamily 1 group D member 1; RIG-I, retinoic acid-inducible gene 1; STING, stimulator of interferon genes; TLR: Toll-like receptor.

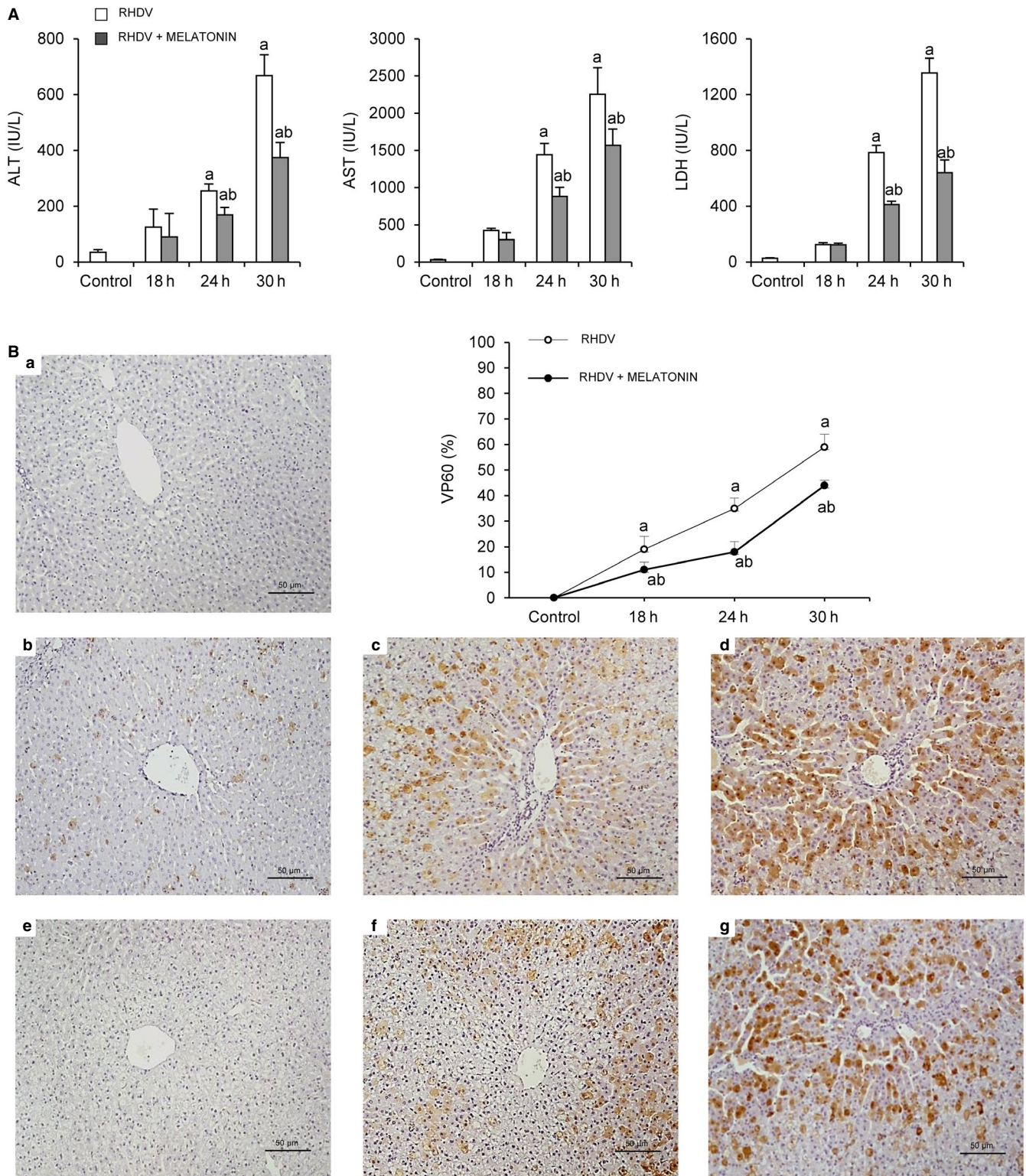


FIGURE 1 Effect of rabbit haemorrhagic disease virus (RHDV) infection and melatonin treatment on liver function and liver expression of the viral capsid protein VP60. A, Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) plasma levels in animals after RHDV infection untreated or melatonin treated were determined. B, Immunohistochemical labelling with VP60 was performed in paraffin-embedded liver tissue sections from (a) control, (b, c, d) 18, 24 and 30 h after infection (hpi) with RHDV, and (e, f, g) 18, 24 and 30 hpi with RHDV + melatonin (Mel), respectively. Original magnification is 200x. Image analysis was performed with ImageJ (v3.91). Values are expressed as means \pm SEM ($n = 6$) and represented as percentage relative to control group. ^a $P < 0.05$, vs Control; ^b $P < 0.05$, vs RHDV at the same time point

(p-IRF3) (Abcam; Cambridge, UK) antibodies at 1:200–1:1,000 dilution with 2.5% non-fat dry milk PBST. Rabbit anti- β -Actin polyclonal antibody at 1:2000 dilution (Sigma-Aldrich, St Louis, MO, USA) was employed as loading control. After TBST washing, membranes were incubated for 1 hour at room temperature with secondary HRP-conjugated antibody at 1:5,000 (Dako, Glostrup, Denmark) and visualized using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden). Specific bands density was measured with ImageJ software (Scion ImageJ Software 1.46a; Bethesda, MD, USA).

2.5 | Immunohistochemistry

Tissue liver samples were recovered, fixed in 10% buffered formalin and paraffin embedded. Sections (4 μ m) were dewaxed and hydrated through graded ethanol, cooked 10 minutes in 25 mmol/L citrate buffer, pH 6.0, immersed in boiling deionized water, letting cool for 20 minutes and finally treated with 3% hydrogen peroxide.³⁸ The slides were incubated with mouse anti-VP60 (Ingenasa; Madrid, Spain), PINK1 (Abcam) or circadian locomotor output cycles protein kaput (CLOCK) antibodies (Thermo Fisher Scientific) overnight at 4°C. Subsequently, EnVision+ system was employed for incubation of tissue sections during 30 minutes. Slides were developed with a 3-3-diaminobenzidine (DAB) solution (Vector Lab, Burlingame, CA, USA), subjected to haematoxylin staining for 10 seconds and mounted. Negative controls were used to evaluate the technique specificity, omitting the primary antibody incubation and incubating with non-immune sera. One of the authors, blinded to the group assignments, was responsible for the evaluation of pathological findings.

2.6 | Transmission electron microscopy (TEM)

Pieces of 1-mm³ were obtained from liver tissues and then introduced overnight into modified Karnovsky fixative [2% glutaraldehyde + 4% buffered formalin (0.1 mol/L phosphate buffer)]. The samples were postfixed for 2 hour at 4°C using a solution of 2% osmium tetroxide, dehydrated with ascending grades of alcohol and, finally, embedded in Epon resin for 72 hour at 60°C. 70 nm width ultrathin sections were generated with an automatic ultra-microtome (Reichert Ultracut E, Vienna, Austria) using a diamond knife and collected on copper grids (200 meshes). Staining was carried out with solutions of uranyl acetate and lead citrate to observe images in a transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at an accelerating voltage of 80 kV.

2.7 | Statistical analysis

Data are expressed as mean values \pm standard error of the mean (SEM) and compared by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test when the analysis indicated the presence of a significant difference. Significance was

accepted when $P < 0.05$. All tests were performed using the statistical package SPSS 22.0 (IBM Corporation, Armonk, NY, USA).

3 | RESULTS

3.1 | Melatonin reduces liver damage and viral capsid protein VP60 labelling in liver of RHDV-infected rabbits

We have previously demonstrated that liver damage in RHDV-infected rabbits increases progressively until 30–36 hpi in parallel to viral replication.^{8,9} In the present study, the activities of ALT, AST and LDH increased significantly from 18 hpi in the liver of RHDV-infected rabbits when compared to the control animals, reaching a maximum at 30 hpi. In comparison with the RHDV groups, melatonin treatment induced a significant decrease of enzyme activities at 18, 24 and 30 hpi (Figure 1A). The presence of the virus in the liver was here confirmed by immunohistochemical analysis of the viral VP60 antigen. Results obtained showed a progressive increase in the extent of labelling in infected rabbits at 24 hpi, mainly found in periportal areas, reaching the greatest expression at 30 hpi (Figure 1B). Melatonin treatment resulted in decreased immunoreactivity with an inhibitory effect of the indole on the replication of the RHDV, causative agent of the severe hepatic injury (Figure 1B).

3.2 | Melatonin regulates altered mitophagy molecular signalling in liver of RHDV-infected rabbits

To evaluate mitophagy involvement in the effects of RHDV infection, we decided to analyse in liver homogenates the PINK1/PARKIN pathway, the mainly described to be involved in FHF progression.^{20,39} Mitophagy activation was confirmed by the significant increased levels of PINK1 and PARKIN, together with other mitophagy receptors, FUNDC1, BNIP3, BNIP3L, VDAC and MFN1 (Figure 2A,B). PINK1 was also determined in liver tissue by immunohistochemistry, detecting first labelling at 24 hpi with marked hepatocytes mainly found in the periportal area, in parallel to the location of the RHDV (Figure 2C). This mitophagy modulation in RHDV-infected rabbits was partially inhibited by melatonin administration, as shown by the reduced expression of mitophagy markers (Figure 2A–C). Accordingly, transmission electron microscopy (TEM) analysis revealed the presence of autophagic vesicles engulfing mitochondria in hepatocytes of RHDV-infected rabbits from 18 hpi, which was also prevented by melatonin treatment (Figure 2D).

3.3 | Melatonin modulates innate immune response in liver of RHDV-infected rabbits

In our FHF rabbit model, we found an overexpression of several innate immune intermediaries, including Toll-like receptor 4 (TLR4),

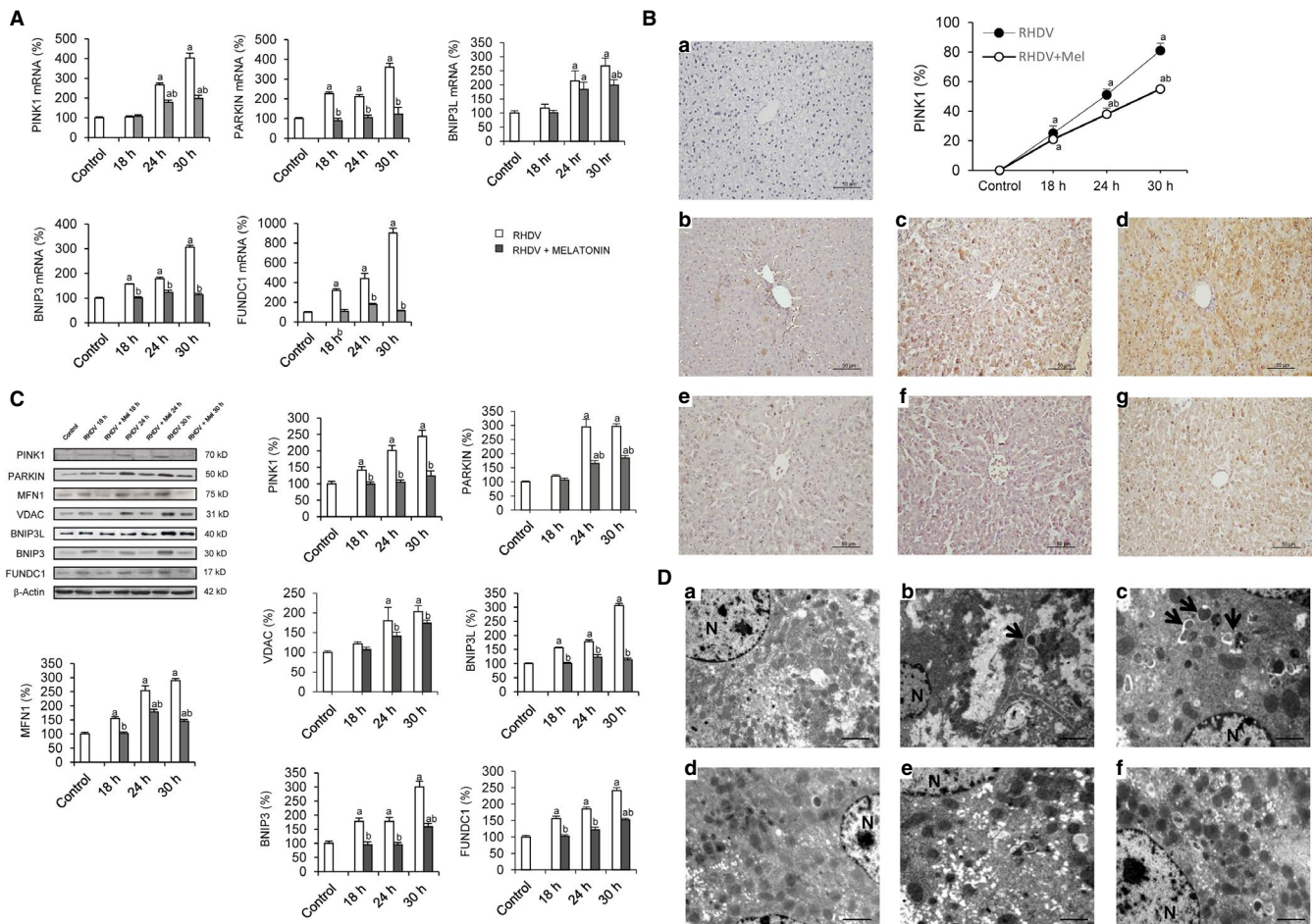


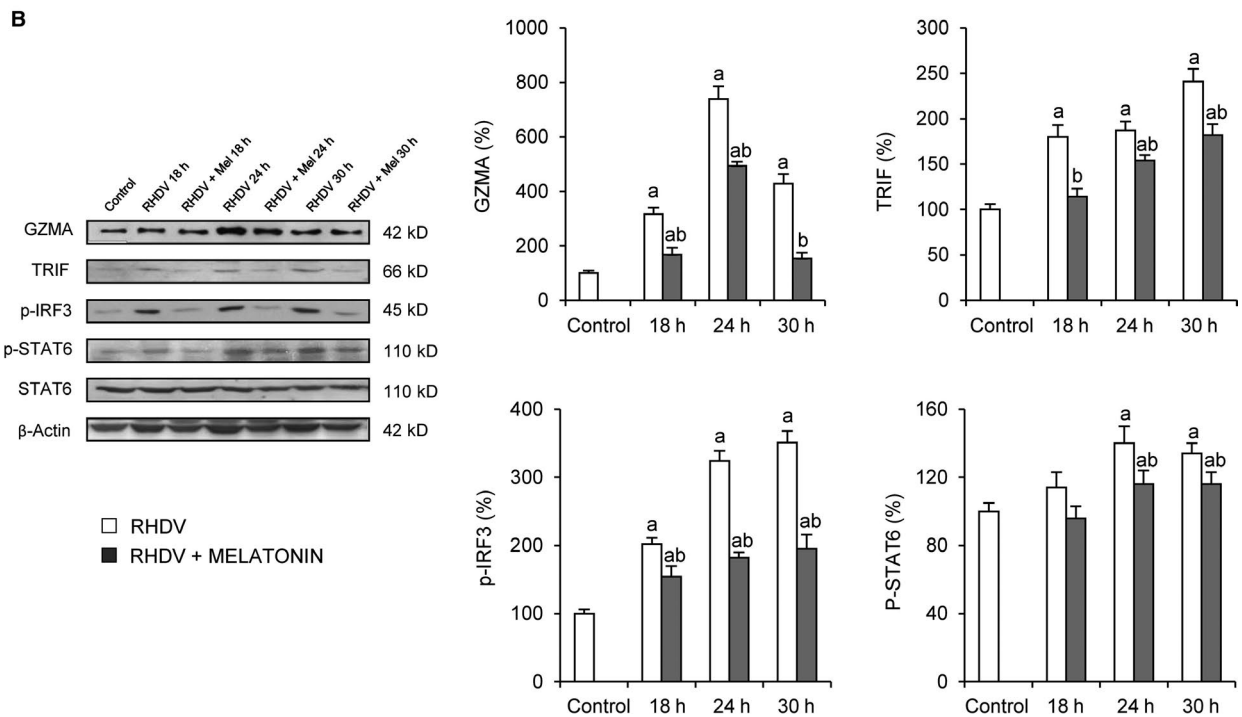
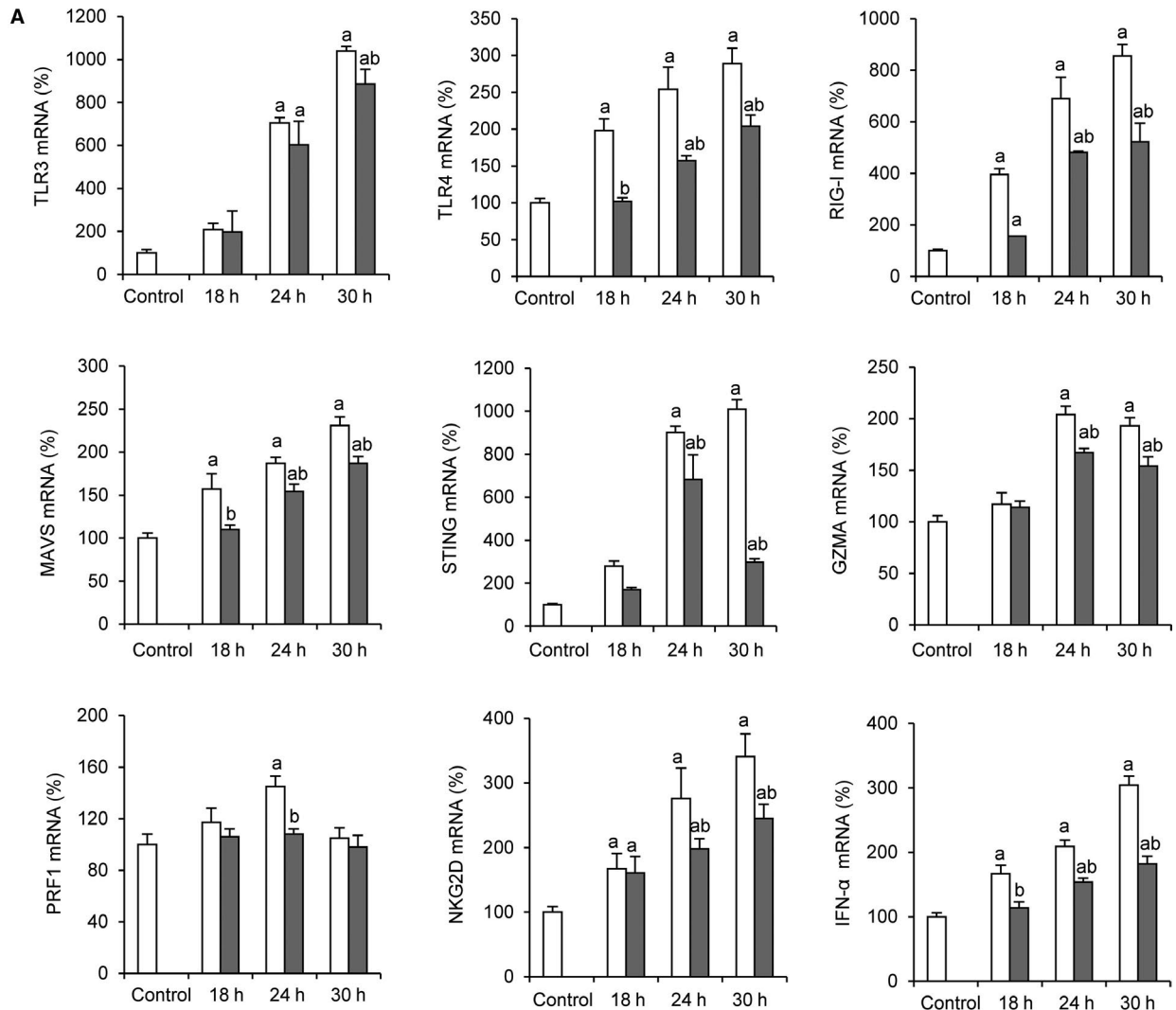
FIGURE 2 Effect of rabbit haemorrhagic disease virus (RHDV) infection and melatonin treatment on mitophagy markers. A, Liver mRNA levels of PINK1, PARKIN, BNIP3L, BNIP3 and FUNDC1 analysed by qRT-PCR. B, Liver protein expression of PINK1, PARKIN, MFN1, VDAC, BNIP3L, BNIP3 and FUNDC1. Representative immunoblots and densitometric quantifications are presented. C, Hepatic PINK1 immunohistochemistry from (a) control, (b, c, d) 18, 24 and 30 h after infection (hpi) with RHDV, and (e, f, g) 18, 24 and 30 hpi with RHDV + melatonin (Mel), respectively. Original magnification is 200x. Image analysis was performed with ImageJ (v3.91). Values are expressed as mean \pm SEM (n = 6) and represented as percentage relative to control group. ^aP < 0.05, vs Control; ^bP < 0.05, vs RHDV at the same time point. D, Representative images from TEM analysis are presented for (a, b, c) 18, 24 and 30 h after infection (hpi) with RHDV, and (d, e, f) 18, 24 and 30 hpi with RHDV + melatonin (Mel), respectively. Arrows indicate mitophagy vesicles. N, nucleus. Scale bar = 2 μ m

retinoic acid-inducible gene 1 (RIG-I), mitochondrial antiviral signalling (MAVS), natural killer group 2D (NKG2D) and interferon α (IFN- α) from 18 hpi; and TLR3, stimulator of interferon genes (STING), GZMA and perforin (PER1) from 24 hpi (Figure 3A). Results were similar to those found in Western blot analysis, where protein levels of GZMA, TRIF, p-IRF3 and p-STAT6, transactivators of the innate immunity, were enhanced in RHDV hepatocytes (Figure 3B). These effects were, in all cases, prevented by melatonin administration to rabbits, hindering such overactivation of the innate immune components (Figure 3A,B).

3.4 | Melatonin prevents NLRP3 inflammasome activation in liver of RHDV-infected rabbits

NLRP3 inflammasome-mediated inflammation is also a process probably involved in FHF highly associated with mitochondria homeostasis through mitophagy modulation.^{17,40} To further evaluate melatonin impact on inflammasome activation, expression of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1 (CASP1) and interleukin 1 β (IL1 β), main components of the NLRP3 inflammasome, was determined. Results showed increased

FIGURE 3 Effect of rabbit haemorrhagic disease virus (RHDV) infection and melatonin treatment on innate immune signalling. A, Liver mRNA levels of TLR3, TLR4, RIG-I, MAVS, STING, GZMA, PRF1, NKG2D and IFN- α determined by qRT-PCR. B, Liver protein expression of GZMA, TRIF, p-IRF3, p-STAT6 and STAT6. Representative immunoblots and densitometric quantifications are expressed as mean \pm SEM (n = 6) and represented as percentage relative to control group. ^aP < 0.05, vs Control; ^bP < 0.05, vs RHDV at the same time point



levels of CASP1, ASC and NLRP3 since 18 hpi, and IL1 β since 24 hpi (Figure 4). Melatonin administration precluded this increase derived from RHDV-induced FHF in rabbit livers, circumventing inflammatory activation (Figure 4).

3.5 | Melatonin restores circadian clock signalling in liver of RHDV-infected rabbits

Alterations in mitophagy and immune and inflammatory responses have been linked to clock machinery disruption.^{22,24} Moreover, recent findings highlight an interesting role of circadian clock on the management of liver injury during FHF.^{35,41} Expression analysis revealed an upregulation of CLOCK and BMAL1 since 18 hpi, and a decrease of REV-ERB α , Period 1/2 (PER1, PER2) and Cryptochrome 1 (CRY1) genes, negative regulators in clock signalling (Figure 5A). Increase in CLOCK expression was also observed by immunohistochemistry (Figure 5B). As in the previous results, melatonin treatment prevented dysregulation of clock genes expression in rabbits with RHDV-induced FHF, partially restoring molecular clock levels (Figure 5A,B).

4 | DISCUSSION

Severity of FHF is related to the complex pathogenesis of this acute injury, where liver transplantation is the only treatment option,

making new pharmacological alternatives an urgent necessity.^{42,43} Viral infection with RHDV has allowed to develop a unique model with histopathological, biochemical and clinical manifestations highly related to those observed in human fulminant viral hepatitis.⁷ Acute liver injury induced by RHDV infection has been previously demonstrated in this animal model by our group, and blood chemistry and immunohistochemical data have shown a damage reduction by melatonin administration.^{5,10} Our findings suggest that melatonin could modulate crucial mechanisms which contribute to severe hepatic damage in a RHDV-induced model of FHF.^{5,10,12-14,34} Here, we explored new molecular pathways accounting for the protective effect of the indole in RHDV-infected rabbits.

Although numerous investigations have studied processes involved in the promotion of viral infection and in the progression of liver injury to FHF, better knowledge of the mechanisms promoting hepatic damage is needed. Within them, mitophagy regulation has been of increasing interest by its role removing damaged mitochondria in injured hepatocytes and preserving mitochondria quality control.^{15,17,20,44} Besides, PINK1/PARKIN, the main pathway in mitophagy induction, seems to play a central role in the response during severe hepatic damage of FHF.^{15,20,39} Our results demonstrated an early augmented expression of PINK1 and PARKIN, mostly marked since 24 hpi, which was accompanied by an increase in the number and content of mitophagy vesicles in liver tissue from RHDV-infected rabbits. Moreover, a greater expression of BNIP3L, BNIP3, FUNDC1 and the mitophagy intermediaries VDAC and MFN1 was also observed during acute damage in rabbit livers. Melatonin

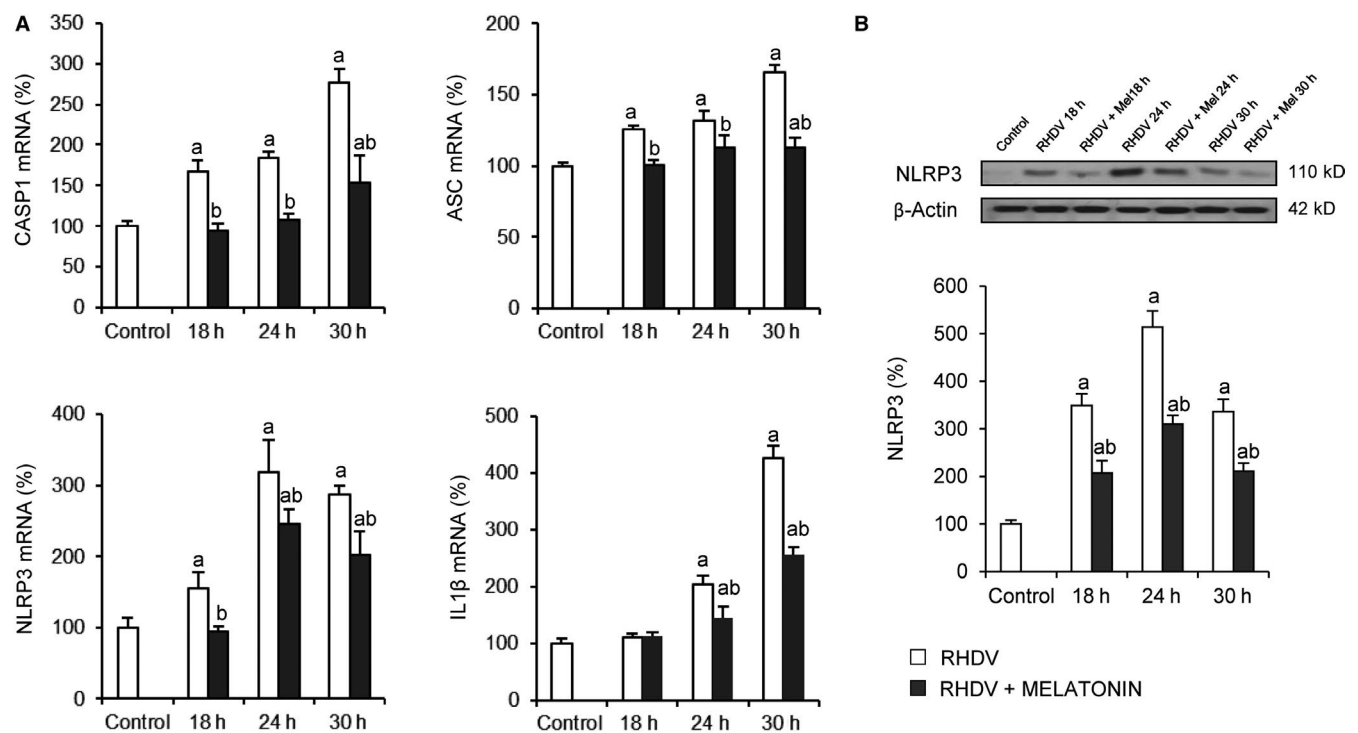


FIGURE 4 Effect of rabbit haemorrhagic disease virus (RHDV) infection and melatonin treatment on NLRP3 inflammasome signalling. A, Liver mRNA levels of CASP1, ASC, NLRP3 and IL1 β determined by qRT-PCR. B, Liver protein expression of NLRP3 by Western blot analysis. Representative immunoblot and densitometric quantification is expressed as mean \pm SEM ($n = 6$) and represented as percentage relative to control group ^a $P < 0.05$, vs Control; ^b $P < 0.05$, vs RHDV at the same time point

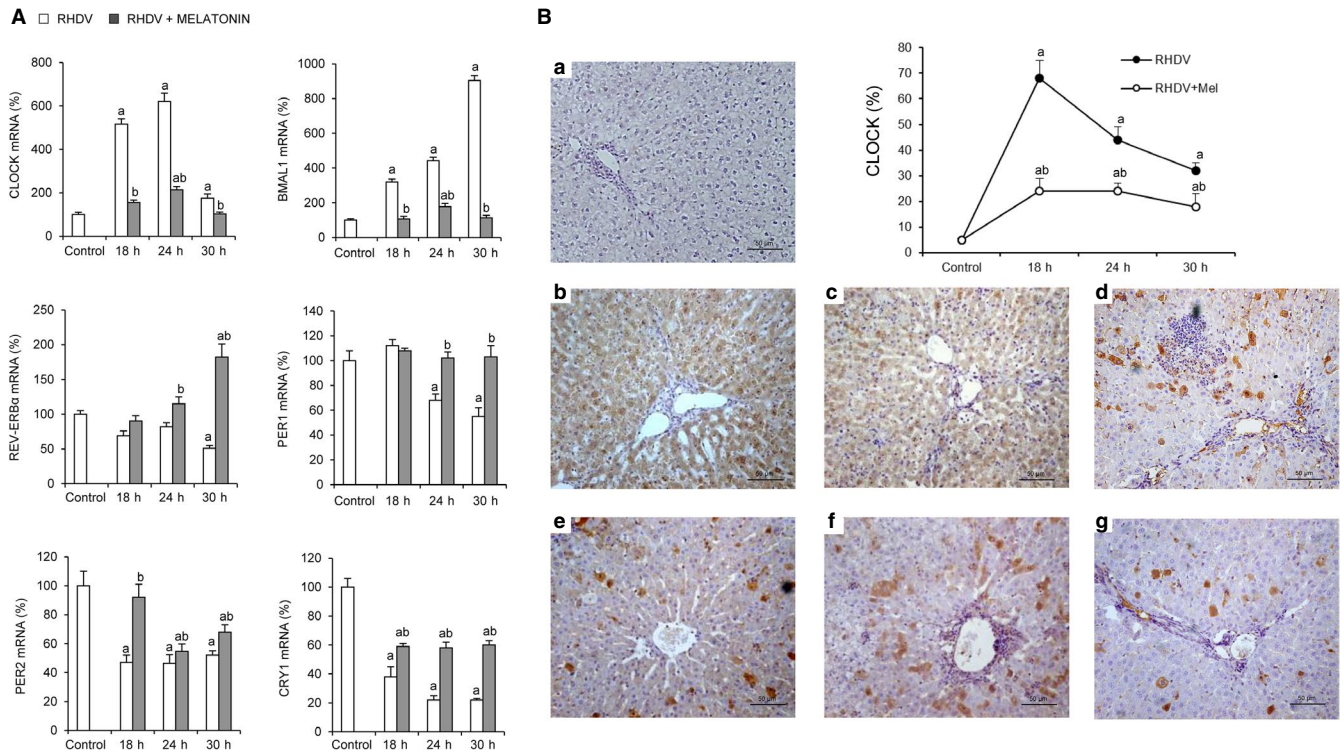


FIGURE 5 Effect of rabbit haemorrhagic disease virus (RHDV) infection and melatonin treatment on the circadian clock signalling. A, Liver mRNA levels of CLOCK, BMAL1, REV-ERB α , PER1, PER2 and CRY1 determined by qRT-PCR. B, Hepatic CLOCK immunohistochemistry from (a) control, (b, c, d) 18, 24 and 30 h after infection (hpi) with RHDV, and (e, f, g) 18, 24 and 30 hpi with RHDV + melatonin (Mel), respectively. Original magnification is 200x. Image analysis was performed with ImageJ (v3.91). Values are expressed as mean \pm SEM (n = 6) and represented as percentage relative to control group. ^aP < 0.05, vs Control; ^bP < 0.05, vs RHDV at the same time point

administration prevented such rise of mitophagy receptors and reduced the number of autolysosomes carrying mitochondria in FHF hepatocytes. Despite most studies highlight a protective effect of mitophagy against liver injury, excessive induction of mitochondria elimination may trigger cell damage by the absence of functional mitochondria.¹⁹ An increment in mitophagy activation has also been reported in an FHF mouse model induced by acetaminophen and, in later studies, the same group found that Parkin knockout mice were less susceptible to acetaminophen-induced injury.^{39,45} Similar to our results, FHF induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS) exhibited mitophagy induction by the increased expression of PINK1 and PARKIN, and the higher content of mitophagy vesicles analysed by TEM. Furthermore, afzelin, the flavonol glycoside, protected liver cells from damage by impairing mitophagy stimulation.¹⁶ These findings reinforce mitophagy implication in severe hepatic lesions derived from viral infection and demonstrate that melatonin could prevent excessive mitophagy induction in the RHDV-induced FHF model.

The role of mitochondria has emerged as a central platform in anti-inflammatory and immune responses.⁴⁶ Previous findings of our group have shown a potential immunomodulatory effect of melatonin in RHDV-infected rabbits by regulating the expression of several cytokines, such as tumour necrosis factor α (TNF- α) and IL6.^{5,34} In addition, a link between innate immunity and mitophagy, comprising the inflammasome component, has been described in

several pathologies, including FHF.^{19,40,47} In the present study, a raised innate immune response was elicited during RHDV-derived hepatic damage, represented by the higher expression of different immune intermediates, such as TLR3/4, RIG-I and MAVS, among others, mainly found from 24 hpi. Prevention of this innate immunity activation was also reached by melatonin administration in RHDV-infected livers. In addition to the well-known role against viral replication, several reports have demonstrated that immune and inflammatory responses also mediate cell damage in FHF induced by treatment with LPS,²⁰ D-GalN/LPS⁴⁸⁻⁵⁰ and TNF- α ⁵¹ and that α -lipoic acid, berberine or crocetin administration to D-GalN/LPS hepatocytes inhibited the inflammatory response⁴⁸⁻⁵⁰ and mitochondria-mediated apoptosis.⁵⁰ Inflammasome activation has been also described as a possible mediator of liver damage in FHF.^{17,40} In our study, the inflammasome intermediaries, NLRP3, ASC, CASP1 and IL1 β , underwent an augmented expression in RHDV livers and melatonin managed to inhibit such FHF-derived effects. Similar results were shown in an FHF model generated by D-GalN/LPS treatment, where exosomes alleviated severe liver damage by hindering inflammasome activation both in vitro and in vivo.⁵² Since a tightly crosstalk has been established between mitophagy-mediated mitochondria homeostasis and the innate immune response and inflammasome,¹⁷ these results strengthen the important and reliable role of the innate immune and inflammasome signalling, along with the previously described mitophagy,

in liver damage progression in RHDV-infected rabbits. Besides, melatonin demonstrated to have a key function by preventing such effects.

Recent studies have evidenced a central role of circadian clock machinery in the modulation of innate immunity and in the maintenance of mitochondrial function.^{1,53} Control of mitochondria dynamics, including mitophagy, has been determined as a dependent process on clock signalling and as a target of BMAL1 protein in mouse livers.^{21,22} In addition, disrupted clock circuitry could lead to excessive immune and inflammatory responses, contributing to severe FHF.^{1,26,41} RHDV-infected rabbits exhibited an augmented expression of the positive clock regulators, CLOCK and BMAL1, highly pronounced since 18 hpi, while levels of REV-ERB α , PER1, PER2 and CRY1 were down-regulated as consequence of RHDV-induced severe hepatic injury. Dysregulation of molecular clock machinery was early prevented by melatonin administration to RHDV rabbits, partially restoring initial expression of clock genes. Circadian rhythms have been suggested to be involved in modulating viral infection through diverse mechanisms including immune and inflammasome responses.^{1,54} Likewise, different studies have shown that loss of preservation of molecular clock signalling contributes to severe liver damage in endotoxin⁴¹ and LPS-induced FHF models.³⁵ Furthermore, REV-ERB α has been associated with mitochondrial biogenesis control²³ and hepatic BMAL1 directly modulated mitophagy and mitochondrial fission in mouse livers.²² Together, these results suggest that alterations on gene expression of the clock machinery may have a key role in the progression of severe hepatic damage derived from RHDV infection.

Although several investigations have established a direct relationship of clock genes with innate immunity and mitochondria homeostasis, REV-ERB α has increasingly acquired interest in this field.^{1,16,55} Protective effects of REV-ERB α have been described in different inflammatory processes, including myocardial infarction⁵⁶ and pulmonary inflammation.⁵⁵ Indeed, a recent research demonstrated that REV-ERB α was responsible for a lower severity of acute hepatic injury in LPS-induced FHF through maintenance of circadian-dependent activity of NLRP3 inflammasome.³⁵ Likewise, decreased REV-ERB α expression was exhibited during D-GalN/LPS hepatic damage in mice livers and afzelin managed to restore REV-ERB α levels, suggesting that this clock gene may participate in the mitophagy regulation exerted by afzelin in FHF.¹⁶ Previous reports of our group have demonstrated a decisive function of melatonin on the modulation of REV-ERB α expression.^{28,30} These findings suggest that melatonin is able to modulate molecular clock signalling, likely preventing mitophagy and innate immune and inflammasome responses in our FHF model induced by RHDV infection. The lack of an in vitro model of FHF of viral origin and the limited number of investigations evaluating melatonin effects on severe hepatic injury makes this study, to the best of our knowledge, be the first one showing such findings.

In summary, even though more studies are needed to deeply understand the evaluated mechanisms, these results reinforce the valuable role of mitophagy and innate immune response, along with

circadian clock signalling, in viral-induced FHF, and highlight the modulatory effects of melatonin on these processes, placing the indole as a potential candidate for therapeutic application in human FHF and reinforcing its potential as an antiviral agent.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

MJT, MA and JGG conceived and designed the study; IC, PFP and BSM performed the research; IC, PFP, BSM and MA were involved in analysis and interpretation of data; and MJT and JGG wrote the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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