

## Research Paper

# The Effect of Remdesivir as An Anti-COVID-19 Drug on the Secretion of Inflammatory Markers by Chicken Liver Cells: An In Vitro Study



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**Citation** Akbari Jonoush Z, Mahdavi R, Ghafourian M, Khoshnam SE, Nezhad Dehbashi F, Farzaneh M. The Effect of Remdesivir as An Anti-COVID-19 Drug on the Secretion of Inflammatory Markers by Chicken Liver Cells: An In Vitro Study. *Journal of Inflammatory Diseases*. 2023; 26(4):173-182. <http://dx.doi.org/10.32598/JID.26.4.2>

**doi** <http://dx.doi.org/10.32598/JID.26.4.2>



### Article info:

Received: 01 Jul 2022  
Accepted: 17 Aug 2022  
Publish: 01 Jan 2023

### Keywords:

Hepatocytes, COVID-19, Remdesivir, Inflammatory factors

## ABSTRACT

**Background:** For severe cases of acute respiratory syndrome type 2 (SARS-CoV-2) infection (COVID-19), Remdesivir (RDV) is introduced as an anti-viral drug with side effects. Hepatotoxicity from prolonged exposure to RDV is associated with increased inflammatory factors. In this study, we evaluated the effect of RDV on the secretion of inflammatory markers by chicken liver cells.

**Methods:** In this in vitro study, 20 stage X embryonated chicken eggs were incubated for 10 days at 37.5°C and 60–65% humidity Hamburger–Hamilton stages (stage HH35). Liver cells were grown in a medium containing DMEM/F12+10% fetal bovine serum (FBS). After three days, the culture media was supplemented with four doses of RDV (1.00, 2.00, 3.00, and 4.00 µM). After 24 and 48hs, the viability of the hepatocytes, gene expression of Sox17, CXC motif chemokine receptor 4 (CXCR4), Interleukin-1 (IL-1), Interleukin-6 (IL-6), and TNF-α, and hepatocellular functions (albumin and urea secretion) were assessed.

**Findings:** Each hepatocyte had a prominent nucleus and a nucleolus with a hexagonal shape. The pink tint of the periodic acid Schiff (PAS) positive cells in the PAS staining verified the hepatocytes' glycogen content. Up to 50% of the cells lose viability after 48 hours in the presence of 3 and 4 µM RDV (P<0.001). In the presence of 3 µM RDV, the production and secretion of both albumin (P<0.001) and urea (P<0.05) decreased. Besides, the expression of IL-1, IL-6, and TNF-α significantly increased after treatment with 3 µM RDV (P<0.001).

**Conclusion:** We concluded that RDV therapy altered the expression and function of hepatocyte inflammatory factors.

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## 1. Introduction

The world faced a serious problem in December 2019 with the confirmation of the first cases of severe pneumonia and other respiratory tract diseases in Wuhan, Hubei Province, central China [1]. The International Committee on the Classification of Viruses (ICTV) designated this pneumonia as acute respiratory syndrome type 2 (SARS-CoV-2) [2, 3]. Breathlessness, pneumonia, a dry cough, and fever are COVID-19 symptoms. Most patients improve, but a small percentage develop acute respiratory distress syndrome (ARDS), organ failure, septic shock, and death [4-6]. The COVID-19 virus causes significant liver damage, and the metalloproteinase ACE2 is essential for this liver damage and serves as a functional receptor for the SARS-CoV-2 virus [7]. This enzyme is present on the surface of the respiratory tract epithelium (in alveolar type II cells of the lung), upper esophageal epithelial cells, enterocytes of the ileum and colon, the heart, testicles, pancreatic smooth muscle cells, and endothelium [8, 9]. Cholangiocytes and hepatocytes can express ACE2 significantly [10]. SARS-CoV-2 causes direct cytotoxicity in liver cells due to persistent viral replication [11, 12], and it may cause lysis, necrosis, and apoptosis. Virus-induced cytotoxic T cells can affect sickness severity [13]. Immunological inflammatory responses are another pathogenic route affecting the liver and resulting in liver dysfunction in COVID-19. High levels of inflammatory markers such as C-reactive protein (CRP), ferritin, lactate dehydrogenase (LDH), D-dimers, interleukin-6 (IL-6), and IL-2 have previously validated these phenomena, indicating a clear correlation between the existence of the cytokine storm syndrome and illness severity [14]. COVID-19-positive patients may undergo pharmacological polytherapy treatment, complicating clinical care. In this context, information on the possible hepatotoxicity of the pharmacological substances used is vital to the diagnostic process [15]. Research on the impact of antiviral drugs on this virus has revealed harmful liver side effects of anti-COVID-19 drug therapy; therefore, after drug administration to COVID-19 patients, it is vital to monitor liver function and hepatotoxicity [16, 17]. Remdesivir (RDV) (GS-5734, RDV) is an RNA polymerase inhibitor that inhibits viral RNA production [18]. After RDV was converted into the pharmacologically active analog adenosine triphosphate by the host cells, it competed with adenosine triphosphate for integration by the RdRp complex into the nascent RNA strand, resulting in the cessation of viral RNA production [19]. This medicine was designed and tested to suppress the Ebola virus, and its efficiency

was also proven against the COVID-19 virus, which got food and drug administration (FDA) approval in October 2020 [20, 21]. Although RDV decreases inflammation and shortens hospitalization, previous studies reveal that it induces liver harm (drug-induced liver injury) [22, 23]. Currently, we have insufficient data about the potential hepatotoxicity of RDV. To study the effects of RDV on liver cells in vitro, hepatocytes are a suitable option [24]. Due to the limited availability of human hepatocytes in vitro, animal models can be used [25].

The main advantages of the chick as a model system for experimental study have been, and remain, the ease with which cells and tissues can be labeled, transplanted, and cultured; biodistribution; biocompatibility; low cost; and its similarity to mammalian systems [26]. Liver cells from chicken embryos reproducing in the lab can be used to study RDV medicines [27].

We are considering RDV's effectiveness in COVID-19 patients and COVID-19's impact on liver function, in vitro RDV function in hepatocytes' behavior, and inflammatory markers. This study investigated the effects of RDV on chicken embryo-derived hepatocytes for the first time.

## 2. Materials and Methods

### Ethics statement

Fertilized chicken fertilized eggs (*Gallus gallus domesticus*, White Leghorn) were provided from a commercial breeding farm in Ahvaz, Iran. This company raises breeder birds following industry standards to achieve optimal bird performance. The Animal Research Committee approved all animal experiments in this study at Ahvaz Jundishapur University of Medical Sciences.

### Cell culture media

The conventional medium contained DMEM/F12 (Invitrogen) as a basal medium, 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen).

### Preparation and isolation of the liver tissue from 10-day-old chicken embryos

For this purpose, 20 embryonated chicken eggs (stage X) were incubated for 10 days Hamburger–Hamilton stages (stage HH35) at 37.5°C with a relative humidity of 60-65%. After 10 days, chicken embryos were removed from the eggs, and the liver tissues were isolated

using sterile forceps and put into a sterile tube containing phosphate-buffered saline (PBS). Then, the Trypsin-EDTA enzyme (0.25% Trypsin and 2.25 mM EDTA) was used to dissociate the liver tissue. After 10 min, the liver cells were pipetted, and the enzyme was inactivated with a 10% FBS medium. The dissociated liver cells were centrifuged (1500 rpm) for 5 minutes and suspended in a fresh medium, according to the method described by Lee et al. [28].

### Derivation and culture of hepatocytes

Freshly isolated liver cells were seeded at a density of  $1 \times 10^4$  cells/mL in DMEM/F12+10% FBS medium and incubated at 37°C and 5% CO<sub>2</sub>. The medium was replaced every day. For the subculture, every 5–6 days, colonies were dissociated with trypsin and reseeded onto new plates coated with gelatin (0.1%).

### Periodic acid schiff staining

For periodic acid Schiff (PAS) staining, the hepatocytes were fixed with 4% paraformaldehyde for 20 min and washed with PBS. The periodic acid solution was added for 10 minutes and washed with PBS. Then, Schiff's solution (Sigma-Aldrich) was added for 10 minutes and washed, and the cells were observed under an inverted microscope.

### Measurement of cell viability

We prepared three 25-cm<sup>2</sup> culture flasks containing hepatocytes (cells from the primary culture) to evaluate cell viability. On day 3, the basic medium was supplemented with four concentrations (1.00, 2.00, 3.00, and 4.00 μM) of RDV [29]. The cells were incubated with RDV for 24 and 48 hours, and cell viability was assessed using the trypan blue exclusion test [30].

### Gene expression analysis

qRT-PCR was used to determine hepatocyte gene expression based on the previously described method [31]. Three 25-cm<sup>2</sup> culture flasks were prepared, and hepatocytes were incubated with the best concentration of RDV. Hepatocytes without RDV were used as a control. After 48 h of incubation, the samples were harvested for the gene expression of Sox17, CXC motif chemokine receptor 4 (CXCR4), Interleukin-1 (IL-1), Interleukin-6 (IL-6), and TNF-α. The sequences of the primers are presented in Table 1. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the transcript levels of the target genes.

### Hepatocellular function assays

The culture medium was analyzed for albumin content using the enzyme-linked immunosorbent assay kit and for urea content with the QuantiChrom Urea Assay kit.

**Table 1.** Primer sequences for chicken-related genes

Gene Name	Sequence
GAPDH-Chik-F	GTCTGGAGAAACCAGCCAAGTA
GAPDH-Chik-R	AACCTGGTCCTCTGTGTATCCTA
sox17-Chik-F	TTTATTCCGGATGCTGAGGACTG
sox17-Chik-R	ATCGCTATCTCCGAAGGAT
CXCR4-Chik-F	GAGTGAAGTAGAAGGACGGTATCTC
CXCR4-Chik-R	GGCAAGACAAGTCTACCAAGA
IL6-Chik-F	GCCTTTCAGACCTACCTGGAAT
IL6-Chik-R	CATCGGGATTATACCATCTGC
IL1-Chik-F	TGCAGAAGGTAAGGATGGGA
IL1-Chik-R	CACTGTGGTGTGCTCAGAAT

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

### Statistical and data analysis

All experiments were performed in at least three biological replicates, and quantitative results were reported as Mean±SD. In a normal distribution of quantitative data, a t-test was used to compare two groups; an analysis of variance (ANOVA) was used for more comparisons of two groups. In the case of a non-normal distribution, their non-parametric equivalents were used.  $P < 0.001$  (\*\*\*) and  $< 0.05$  (\*\*) between groups were considered significant. SPSS software, version 21 was used for data analysis.

## 3. Results

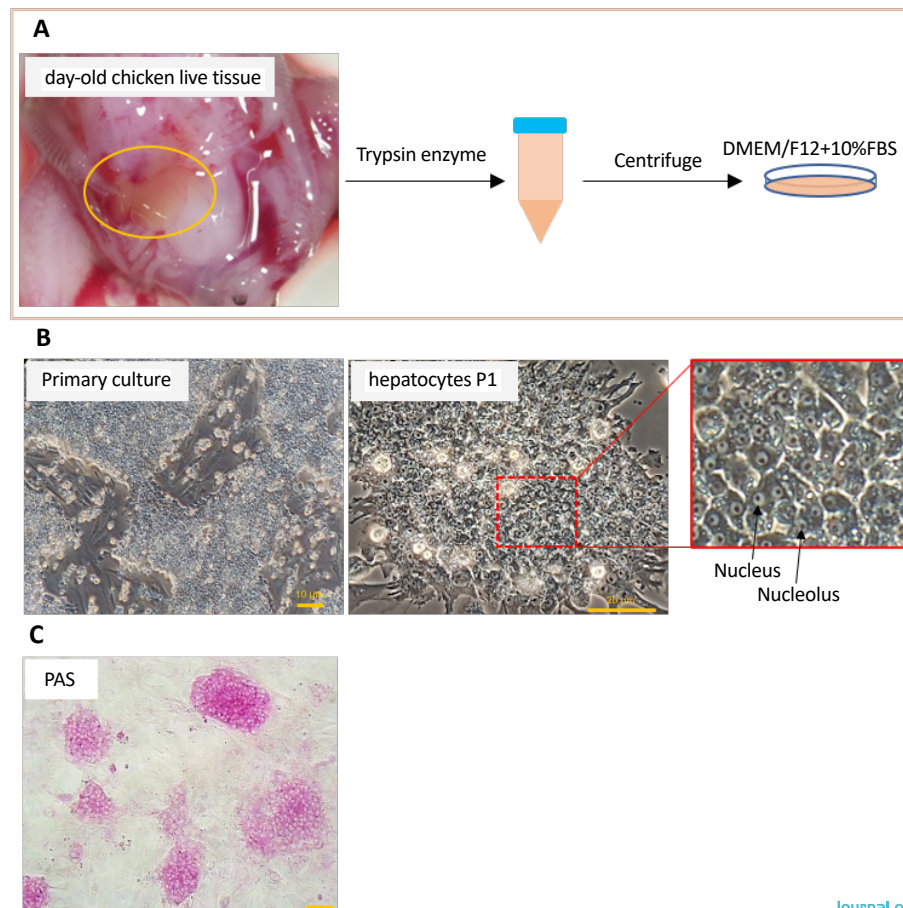
### Culture of hepatocytes from a 10-day-old chicken embryo

First, we isolated the liver tissue from developmental stage HH36 of white leghorn strain eggs according to a previously described protocol [32]. Liver cells were

enzymatically digested with the trypsin enzyme and cultured in plates-or flasks coated with gelatin (0.1%) containing DMEM/F12+10% FBS. Under this condition, chicken hepatocytes' small and thin colonies appeared after 2 days. Each hepatocyte with a hexagonal structure had a large, transparent, and a dark nucleolus. After 3 days, the cells were passaged with the Trypsin enzyme and maintained their structure and proliferation ability after 3 enzymatic passages. In the glycogen staining, the PAS-positive cells had a light pink color that confirmed the presence of glycogen in hepatocytes (Figure 1).

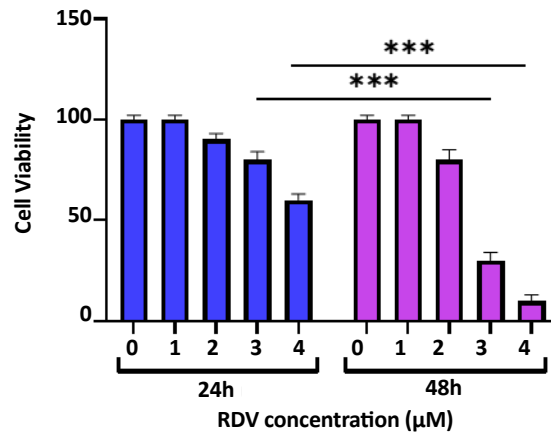
### Viability of hepatocytes following treatment with RDV

On the fourth day of culture, 50% to 60% of the plate contained hepatocytes suitable for evaluating the drug effects. We assessed the cytotoxicity of 4 concentrations (1, 2, 3, and 4  $\mu\text{M}$ ) of RDV on hepatocytes for 24 and 48 hrs. Following staining with trypan blue in the presence of 3 and 4  $\mu\text{M}$  RDV, up to 50% of the cells lost their viability after 48



**Figure 1.** Culture of hepatocytes from a 10-day-old chicken embryo

A) The liver tissue isolated from developmental stage HH36 of White Leghorn strain eggs; B) The image of structure hepatocyte; C) The PAS-positive cells showed a light pink color that proved the presence of glycogen in hepatocytes (passage 1).



**Figure 2.** Viability of hepatocytes following treatment with RDV (the impact of different concentration of RDV on viability of hepatocytes after 24 and 48 h)

hours ( $P < 0.001$ ) (Figure 2). Therefore, we performed further experiments in the presence of 3 µM RDV (after 48h).

#### The expression of hepatocyte-specific genes following treatment with RDV

We measured the expression of two hepatocyte-specific genes, including SOX17 and CXCR4, by RT-PCR analysis with chicken-specific primers. The data revealed that the expression of both SOX17 and CXCR4 significantly decreased after treatment with RDV ( $P < 0.001$ ) in the presence of 3 µM RDV after 48h (Figure 3).

#### The expression of inflammatory markers following treatment with RDV

We detected the expression of inflammatory markers, including IL-1, IL-6, and TNF- $\alpha$ , by RT-PCR analysis with chicken-specific primers. Our data showed that the expres-

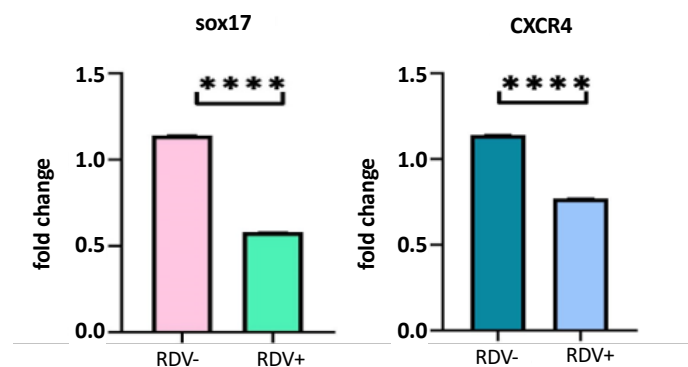
sion of inflammatory markers was significantly higher in the RDV+ group compared to the RDV- group ( $P < 0.001$ ) in the presence of 3 µM RDV after 48 h (Figure 4).

#### Functional assay of hepatocyte functions following treatment with RDV

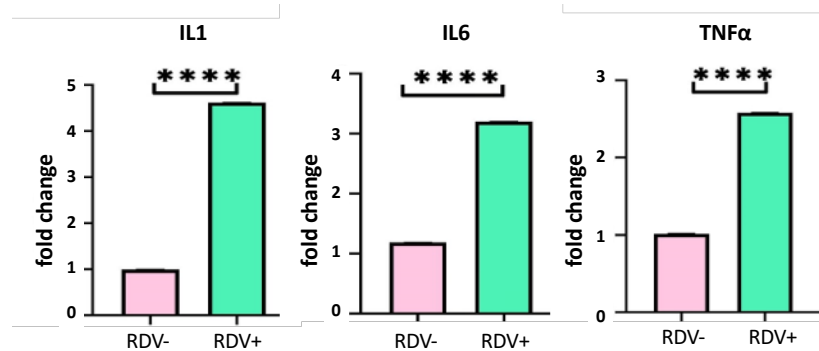
Next, we measured the production and secretion of albumin and urea by hepatocytes. We found that the production and secretion of both albumins ( $P < 0.001$ ) and urea ( $P < 0.05$ ) significantly reduced in the RDV+ group compared to the RDV- group ( $P < 0.05$ ) in the presence of 3 µM RDV after 48 h (Figure 5).

## 4. Discussion

This study explored the effects of RDV on the expression of inflammatory factors in liver cells. COVID-19 individuals may experience liver damage. Due to the



**Figure 3.** The expression of hepatocytes-specific genes following treatment with RDV (the impact of RDV on the expressions of two hepatocyte-specific genes, such as SOX17 and CXCR4, by RT-PCR analysis with chicken-specific primers)



**Figure 4.** The expression of inflammatory markers following treatment with RDV (the impact of RDV on the expressions of inflammatory markers including IL-1, IL6 and TNF- $\alpha$  by RT-PCR analysis with chicken-specific primers)

very few studies on the effects of RDV on liver cells and inflammatory factors, we decided to use the chicken embryo model to determine whether RDV has an adverse effect in the cases mentioned. Accessible, cost-effective, and fast-growing chicken embryos are used in many experiments [33, 34]. After treatment with RDV, we found that the expression of inflammatory markers, including IL-1, IL-6, and TNF- $\alpha$  increased. The expression of two hepatocyte-specific genes, SOX17 and CXCR4, decreased and reduced albumin and urea synthesis by hepatocytes. Our study implies RDV can affect hepatocytes.

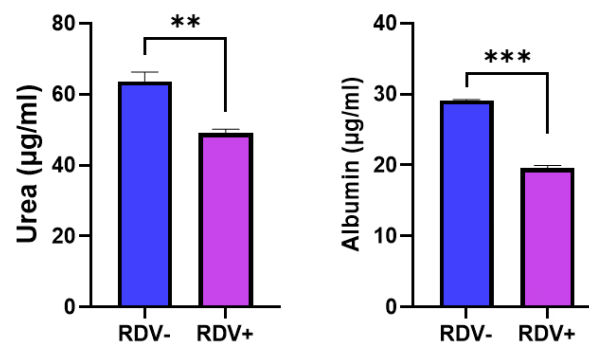
Adverse events were recorded by 66% of patients in the RDV group and 64% of patients in the control group in a trial Wang et al. conducted in Wuhan, China. One of the most frequent side effects in the RDV group was hypoalbuminemia [35].

In contrast to our findings, El-Haroun et al. showed that adult male albino rats treated with RDV had elevated urea and creatinine serum levels [36]. The negative ef-

fect of RDV on the kidneys may result in a decrease in glomerular filtration rate (GFR) and a reduction in creatinine clearance [37]. This discrepancy with our data may be due to differences in the animal model and cell type studied. These researchers proved that RDV caused acute kidney injury, an accumulation of collagen fibers in the interstitium and between glomerular capillaries in the renal cortex, and renal fibrosis as a result of the development of numerous profibrotic factors such as TGF- and TGF-/Smad3 signal pathways, according to another finding in this study [36].

The other study that contrasts with our findings, the YN Li study in China conducted on male C57BL/6 mice, showed that the levels of an inflammatory factor (IL-18, IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ) in high-fat diet (HFD)-induced non-alcoholic fatty liver disease (NAFLD) mice significantly reduced after treatment with RDV [38].

The effects of RDV on mouse preimplantation embryos cultivated in vitro were examined by Yusuke Marikawa et al. At E4.5, the gene expression profiles of im-



**Figure 5.** Functional assay of hepatocyte functions following treatment with RDV

portant cell lineage markers were evaluated to assess the molecular health of blastocysts that had been exposed to RDV. They discovered that RDV dramatically reduced Nanog expression at 2 M and that RDV exposure also down-regulated the expressions of the primitive endoderm (PrE) markers Gata4, Pdgfra, and Sox17, with a 75-80% drop at 4 M [39].

Currently, we have insufficient data about the potential hepatotoxicity of RDV. RDV's hepatotoxicity is difficult to attribute, given its compassionate usage in COVID-19 patients and the disease's liver malfunction, and more studies are needed on the effect of RDV on liver cells and its effect on inflammatory factors [40-42].

## 5. Conclusion

We concluded that following treatment with 3  $\mu$ M RDV, inflammatory factor expression in hepatocytes increased while albumin and urea levels decreased. In this study, our goal was to raise awareness among healthcare professionals about RDV-induced liver injury and its management in COVID-19 patients. An early diagnosis of liver damage can be made in COVID-19 patients with careful and close monitoring of inflammatory factors, and the risk of DILI may be decreased. Before using COVID-19, more investigation is advised to understand better the nature of the risk of hepatotoxicity and drug-drug interactions with RDV.

## Ethical Considerations

### Compliance with ethical guidelines

The Animal Research Committee approved all animal experiments in this study at [Ahvaz Jundishapur University of Medical Sciences](#) (Ethical No. IR.AJUMS.REC.1400.685).

### Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

### Authors' contributions

Caring out the experiment: Zahra Akbari Jonoush and Roya Mahdavi; Original idea, writing–review and editing of the manuscript: Mehri Ghafourian and Maryam Farzaneh; Data collection and the final approving: Seyed Esmacil Khoshnam and Fereshteh Nezhad Dehbashi.

## Conflict of interest

The authors declared no conflict of interest.

## Acknowledgments

We wish to thank all our colleagues at the Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, [Ahvaz Jundishapur University of Medical Sciences](#), Ahvaz, Iran.

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