

# Inhibitory Effect of Bovine Lactoferrin on Human Hepatitis B Virus Replication in HepG2.2.15 Cells

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## Abstract

**In this study, the effect of bovine lactoferrin on HBV replication in HBV-producing HepG2.2.15 cells was analyzed. MTT assay was used to determine the non-toxic concentrations of BLF to the culture cells. The results showed that cytotoxicity increased as the concentration of BLF increased. However, no apparent cytotoxicity was observed with concentration of BLF less than 6.25 mg/ml. The HepG2.2.15 cells were subsequently incubated with BLF of two non-toxic concentrations. mRNA expression level of HBV core protein and surface protein were examined by real-time RT-PCR. The results showed that the mRNA level of both HBV core protein and surface protein were reduced in cells incubated with both non-toxic concentrations of BLF. Our findings suggested that BLF was able to inhibit HBV replication. BLF could be a new candidate of anti-HBV drugs.**

**Keywords:** bovine lactoferrin; HBV; HepG2.2.15.

## 1. Introduction

Hepatitis B virus (HBV) is a DNA virus of the Hepadnaviridae family. The spherical HBV particle consists of an outer lipid envelope and an icosahedral nucleocapsid enclosing an open circular partially double-stranded DNA genome with viral DNA polymerase [1-3]. Globally, hepatitis B is one of the most common infectious diseases. Estimates indicate that at least 2 billion people have been infected with HBV, with over 378 million people being chronic carriers (6% of the world population). There are approximately 4.5 million new HBV infections worldwide each year, and 15–40% of those infected would develop cirrhosis, liver failure, or hepatocellular carcinoma [4]. Currently, there are seven drugs

licensed for treatment of HBV infection in the United States. However, none of them is able to clear the infection while treatment side-effects are continuously emerging [3]. Therefore, it is necessary to identify new compounds to improve current anti-HBV therapy. Bovine Lactoferrin (BLF) is an 80-kDa iron-binding glycoprotein of the transferring family present in cow's milk [5]. It consists of a single polypeptide chain of 689 amino acids. BLF is folded into 2 lobes, each of which is capable of binding one metal ion [5,6]. BLF is thought to have antiviral activity against herpes simplex virus, human cytomegalovirus, herpes virus, hepatitis C virus, hepatitis G virus and human immunodeficiency virus through inhibition of virus-host interaction or direct interaction between BLF and the viral particles [7,8]. Recently, the inhibitory effect of BLF on HBV replication has been reported [9]. However, the data reported from HepG2 cells need to be validated as these cells are not susceptible to HBV infection although they are able to support HBV replication intracellularly [10,11]. In this study, we examined the effect of BLF on HBV in the HBV-producing HepG2.2.15 cell line.

## 2. Materials and Methods

### 2.1 Cell culture

The HepG2.2.15 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1× minimal essential medium nonessential amino acid (Gibco, invitrogen), and 150 µg/ml of Geneticin (Gibco, invitrogen), at 37 °C under 5% CO<sub>2</sub> in air.

### 2.2 MTT assay

To determine the non-toxic concentrations of BLF (Westland, New Zealand) to the culture cells, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay was used [12]. The HepG2.2.15 cells were incubated with two-fold serial

dilutions of BLF (from 50 to 1.5675 mg/ml in culture medium) in 96-well microplates. After 24 h, 10  $\mu$ l of 5mg/ml MTT dissolved in PBS buffer was added to each well. The plates were incubated for 3 h at 37 °C, and then culture medium containing MTT was removed and 100  $\mu$ l of dimethylsulfoxide was added to each well to dissolve formazan. Plates were read at 570 nm against a 660 nm reference wavelength on a micro plate reader (Benchmark Plus). The cell viability was expressed as a percentage of the control. Reagents concentrations were considered non-toxic if the corresponding cell viability was greater than 95%.

### 2.3 Real-time RT-PCR

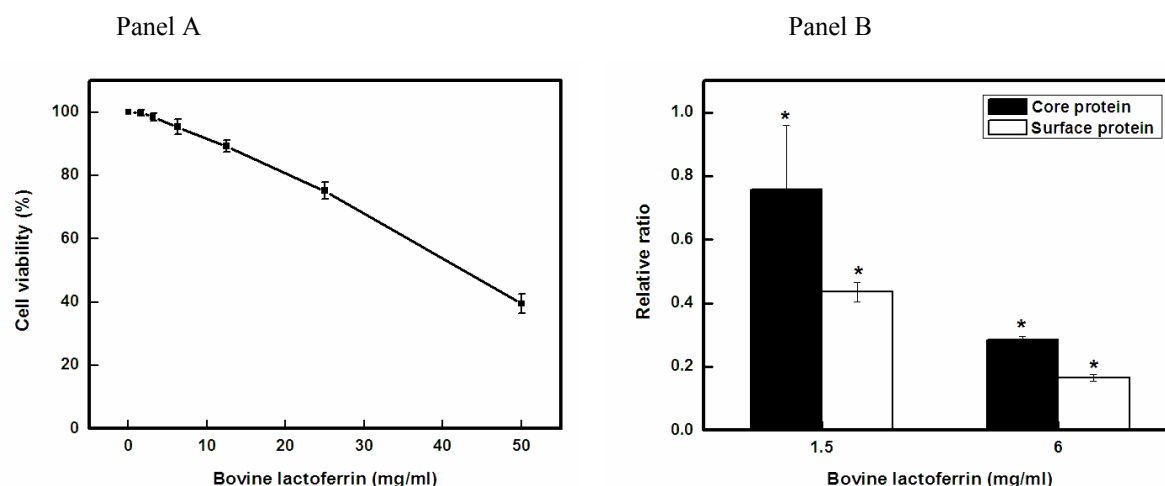
HepG2.2.15 cells were subsequently incubated with two non-toxic concentrations of BLF for 24 h. Total RNA of cells was isolated using RNeasy Mini kit (Qiagen) by following the manufacturer's protocol. The quantity and quality of RNA was determined by examining the absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm. Real-time RT-PCR was performed by utilizing IQ5 multicolor real-time PCR detection system (Bio-Rad) with iScript Onestep RT-PCR kit (Bio-Rad). Reaction mixtures were initially incubated for 10 min at 50 °C and 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The dissociation analysis was routinely carried out by acquiring fluorescent reading for 1 °C increase from 55 to 95 °C. Microsoft Excel formatted data including amplification analysis, experimental report, melting curve analysis and threshold cycle

number were provided automatically by IQ5 optical system software version 2.0 (Bio-Rad). The fold changes were calculated using the following formula:  $Sample \Delta Ct = Ct_{sample} - Ct_{rsD}$ ;  $\Delta\Delta Ct = Sample \Delta Ct - control \Delta Ct$ ; the fold of sample versus control =  $2^{-\Delta\Delta Ct}$ .

### 3. Results and Discussions

The MTT assay showed that cytotoxicity increased proportionally with the increase in the concentration of BLF. However, no apparent cytotoxicity was observed with concentration of BLF less than 6.25 mg/ml (Panel A, **Fig. 1**). We thus subsequently incubated with HepG2.2.15 cells with BLF of two non-toxic concentrations: 1.5 mg/ml and 6 mg/ml. The HBV nucleocapsid is formed by the core protein and surrounded by a lipid bilayer envelope containing HBV surface protein. Core protein and surface protein are indicative of HBV infection. The results of real-time RT-PCR analysis showed the mRNA levels of both HBV core protein and surface protein were reduced in cells incubated with BLF (Panel B, **Fig. 1**). In particular, incubation of Hep- G2.2.15 cells with 6 mg/ml of BLF caused the decrease in mRNA level of the two proteins to about 80% (Panel B, Fig. 1). In addition, the mRNA expression of surface protein was more potent to BLF compared with that of core protein (Panel B, Fig. 1). Our findings therefore suggest that BLF was able to inhibit HBV replication.

In conclusion, BLF could be a new candidate of anti-HBV drugs. However, the detailed mechanism of action needs further investigation.



**Fig. 1 Inhibitory effect of BFL on HBV replication.** Panel A. Cell viability of HepG2.2.15 cells after 24 h incubation of bovine lactoferrin. Panel B. Real-time RT-PCR analyses of genes encoding HBV core protein and surface protein. Asterisks indicate significant difference at  $p < 0.05$ .

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