Osthole in Bushen Formula May Block HBV Gene Expression through Notch and NF-κB Pathways

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Abstract

Osthole is a natural coumarin in the Bushen formulation. Previous studies reported that it has the potential to downregulate Notch and NFκB, which are important signaling pathways in cells. Therefore, we predicted that ostiole might affect HBV gene expression by down-regulating these two signaling pathways. This study provides experimental protocols to apply osthole to mouse hepatocytes and detect the strength of Notch and NFκB signaling pathways in the cells and HBV gene expression levels. In this work, all possible results were analyzed. Notch signal intensity was measured by assaying NICD, IκB measured NFκB signal intensity, and RT-PCR measured HBV gene expression level. The study of Osthole is meaningful for the research on Chinese medicine. This study will allow a better explanation of the mechanism of Bush's formula and explore the more generalized effects of herbal medicines.

Keywords: Osthole, Bushen formula, HBV gene, Notch, NF-κB

1. Introduction

HBV is a major worldwide health problem and is responsible for nearly one million liver disease deaths each year [1,2]. Chronic hepatitis causes long-term adaptive immunity and is difficult to completely eliminate the virus [3]. In China, the vast majority of patients with liver cancer have a history of chronic HBV infection. CHB has long been treated with traditional Chinese medicine, and previous studies have found that the Bushen formula significantly modifies clinical symptoms and may inhibit HBV DNA replication [4]. *Cnidium monnieri* (L.) Cusson in the Bushen formula is always considered as one of the main herbs. Osthole (OST), the main active ingredient derived from *Cnidium monnieri* (L.) Cusson, is considered to be one of the main active ingredients [5]. However, it is not clear whether it has an effect on HBV genes regulation.

From some other disease studies, Osthole is able to regulate the Notch pathway and NF-κB pathway in some other cells. For example, Osthole can regulate the Notch signaling pathway in microglia cells to inhibits the proliferation [6] and block NF-κB playing an anti-inflammatory role in several diseases [7,8]. As it happens, previous studies have identified Notch signaling as a highly conserved intercellular signaling pathway critical for all aspects of liver function, including development, repair and regeneration, inflammation, and hepatocarcinogenesis [9]. Besides, another important factor in HBV, HNF1α, which can inhibit HBV gene expression and replication by regulating the expression of P65 in the NF-κB pathway [10]. With the mentioned information provided by previous researches, we learned that Osthole has the ability to regulate the Notch and NF-κB pathways and the pathways may inhibit the expression of HBV genes.

2. Hypothesis

We predict that increasing amounts of Osthole blocks both Notch and NFkB pathways resulting in the reduction of chronic hepatitis disease and HBV DNA.

3. Methods

A controlled experimental study was designed in order to test the effect of osthole on the reduction of HBV genes. This method focuses on the measurement of the Notch and NFκB pathways and the expression levels of HBV genes, using continuous osthole stimulation for doses and times gradients under in vitro conditions.

3.1 *Materials*

According to similar researches, hepatocellular carcinoma cell lines HepG2 and HepG2.2.15 can be used, cultured in RPMI medium 1640 (Gibco) supplemented with 10% FBS. All cell lines were treated with 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C with 5% CO2 [11,12].

3.2 Osthole stimulation

The cell will be divided into three main groups, each of which was further divided into three separate groups: (1) negative control: cells treated with PBS/DMSO only, stimulated for one, two and three weeks; (2) positive control: 10 uM entecavir treatment, stimulated continuously for one, two and three weeks;

(3) experimental group: 1nM,10nM,100uM,1uM,10 uM Osthole treatment, continuous stimulation for one hour, two hours, and three hours.

3.3 *Expression levels of HBV*

We use RT-PCR to detect the expression levels of HBV. Using reverse transcriptase to transcribe the mRNA into cDNA and then subject it to PCR.

3.4 *Nfkb activation and treatment*

In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by inhibitory IkB proteins. Pro-inflammatory cytokines, LPS, growth factors and antigen receptors activate IB kinase (IKK), which phosphorylates IkB proteins. Phosphorylation of IkB leads to its degradation, releasing NFκB complexes to translocate to the nucleus, bind to NF-κB DNA response elements, and induce transcription of target genes. Phosphorylated IkB proteins were measured by western blot and the NFκB reporter kit was used to confirm the activity of the NFκB pathway.

3.5 *Notch activation*

Notch receptor binds to the ligand and is enzymatically cleaved to release the transcriptionally active Notch protein fragment (NICD or ICN), which in turn binds to the transcription factor CSL and regulates downstream gene expression. Therefore, we can detect NICD by western blot.

4.Results

Table 1. Possible results

	Assay	Possible result #1	Possible result #2	Possible result #3	Possible result #4	Possible result #5	Possible result #6	Possible result #7	Possible result #8
In vitro model	Osthole blocks NFkB pathway (blocks IkB phosphorylated on or NFkB reporter expression)	+	+	-	-	+	+	-	-
	Osthole blocks Notch pathway (NICD decreases)	+	-	+	-	+	-	+	-
	HBV DNA decrease	+	+	+	+	-	-	-	-
	Supporting Hypothesis ?	**Yes**	**Partially**	**Partially**	**Partially**	**Partially**	**Partially**	**Partially**	**No**

Note: for 'Osthole blocks NFkB pathway', "+" means IkB phosphorylation or NFkB reporter expression statistically significant decrease, "-" means no statistically significant decrease. For Osthole blocks Notch pathway, "+" means NICD statistically significant decreases , "-" means no statistically significant decrease. For HBV DNA decrease, "+" slowly decreases, "-" means no significant decrease.

The following are possible results of Osthole's effect on the Notch and NFκB signaling pathways and on HBV gene expression levels. An overview of all possible results is presented in the Table 1 above.

1) Possible result #1: Applying Osthole to vitro cell models decreases the signal level of Notch and NFκB pathways, and HBV expression level is lowered.

Osthole blocks both the Notch and NFκB signaling pathways and interferes with the regulatory process of HBV genes, and HBV gene expression levels decrease.

2) Possible result #2 Applying Osthole to vitro cell models decreases the signal level of NFκB pathways, but Notch signal level doesn't change significantly, and HBV expression level is lowered.

Osthole only blocks the NFκB signaling pathway, but has no significant effect on Notch pathway. Osthole interferes with the regulatory process of HBV genes, and the HBV gene expression levels decrease.

3) Possible result #3 Applying Osthole to vitro cell models decreases the signal level of Notch pathways, but NFκB signal level doesn't change significantly, and HBV expression level is lowered.

Osthole only blocks the signaling Notch pathway, but has no significant effect on NFκB pathway. Osthole interferes

with the regulatory process of HBV genes, and the HBV gene expression levels decrease.

4) Possible result #4 Applying Osthole to vitro cell models, the Notch and NFκB signal level doesn't change significantly, but HBV expression level is lowered.

Osthole has no significant effect on Notch and NFκB pathways. Osthole doesn't interfere with the regulatory process of HBV genes, but the HBV gene expression levels decrease.

5) Possible result #5 Applying Osthole to vitro cell models decreases the signal level of Notch and NFκB pathways, but HBV expression level isn't significantly lowered.

Osthole blocks both the Notch and NFκB signaling pathways and interferes with the regulatory process of HBV genes, but HBV gene expression levels aren't significantly affected.

6) Possible result #6 Applying Osthole to vitro cell models decreases the signal level of NFκB pathways, but Notch signal level doesn't change significantly, and HBV expression level isn't significantly lowered.

Osthole only blocks the NFκB signaling pathway, but has no significant effect on Notch pathway. Osthole interferes with the regulatory process of HBV genes, but HBV gene expression levels aren't significantly affected.

7) Possible result #7 Applying Osthole to vitro cell models decreases the signal level of Notch pathways, but NFκB signal level doesn't change significantly, and HBV expression level isn't significantly lowered.

Osthole only blocks the signaling Notch pathway, but has no significant effect on NFκB pathway. Osthole interferes with the regulatory process of HBV genes, but HBV gene expression levels aren't significantly affected.

8) Possible result #8 Applying Osthole to vitro cell models, the Notch and NFκB signal level doesn't change significantly, and HBV expression level isn't significantly lowered.

Osthole has no significant effect on Notch and NFκB pathways. Osthole doesn't interfere with the regulatory process of HBV genes, and HBV gene expression levels aren't significantly affected.

5. Discussion

Previous studies have reported that the Bushen formula is able to reduce the expression levels of HBV genes and can be of great help in the treatment of HBV patients [13]. In this study, we select one of the possible active ingredients in the formulation, osthole, and treated the cells with different dose and time gradients to explore the active ingredient of the Bushen formulation that plays a role in HBV downregulation.

It was hypothesized that osthole can block the Notch and NFκB pathway to down-regulate the expression level of the HBV gene, which is a possible potential explanation for Bushen's mechanism for down-regulating the HBV gene.

Possible result #1 appear to be a decrease in HBV expression levels and inhibition of the pathways Nfkb and Notch, which is consistent with our hypothesis. Further study can be done by investigating the ways in which osthole interferes with Notch and NFκB pathways to fully understand the more specific osthole pathway at a molecular level, making it possible to understand which step of the signaling pathway Osthole specifically blocks the signaling molecular transmission. If this result occur, osthole can even be considered in pharmaceuticals for inclusion in the future treatment of HBV.

In possible results #2 and #3, osthole blocks only one of the pathways in Notch and NFκB and downregulates the expression of the HBV gene, partially supporting the hypothesis. Although not completely consistent with the hypothesis, it reveals a pathway through which osthole plays a role in HBV treatment. Follow-up studies can be conducted similar to those in possible result #1 to explore the specific effects of molecular-level. Since previous studies have found experimental support for osthole's downregulating the two pathways in other cells [6,7,8], We can explore the factors that determine whether a drug affects the pathway in different cellular environments. Some small non-coding RNAs are worth focusing on.

Possible result #4 shows that osthole does not block the Notch and NFκB signaling pathways, but still downregulates the HBV gene, partially supporting the hypothesis. This suggests that osthole is an active ingredient in down-regulating gene expression in HBV therapy, which may be achieved by influencing other signaling pathways or biochemical processes. Follow-up research can explore the specific impact of osthole on activities. The initial idea about the experimental design is that the osthole molecules can be traced by labeling the osthole molecules to identify the biomolecules that interact within the cell, and then find the key factors that directly downregulate the HBV gene from these molecules.

Possible result #5 shows that although osthole blocks the Notch and NFκB pathways, it has no significant effect on HBV expression levels, partially supporting the hypothesis. When this result occurs, osthole is not considered an active ingredient in HBV treatment. Since previous studies have demonstrated that the Notch and NFκB pathway can downregulate the HBV gene, when osthole stimulates cells, although the signaling pathway is

blocked, there is a biomolecule that acts as a trans-acting factor acting directly on the HBV gene, resulting in it not being affected by downregulation.

In possible results #6 and #7, osthole blocks one of the pathways in Notch and NFκB, but does not have a significant effect on HBV gene expression levels, partially supporting the hypothesis. This means that osthole affects Notch or NFκB, and signaling pathway blocking affect HBV gene expression. Subsequent research can focus on two aspects, one is the specific mechanism of osthole on its influence pathway at the molecular level; another is that there may be a biomolecule acting directly on the HBV gene without signaling pathway blocking affection.

In possible result #8, osthole neither blocks any of the pathways in Notch and NFκB nor significantly downregulates HBV expression, completely opposing the hypothesis. Excluding experimental errors, It means that our hypothesis is contrary to reality, and osthole is not an active ingredient in the Bushen formula to downregulate the HBV gene. Other molecules in the Bushen formulation should be investigated in the further study, either individually or in combination, to determine the molecule able to downregulate HBV genes. Then using the methods in this paper to explore whether the molecular regulation of the HBV gene is related to Notch and NFκB.

Since the osthole stimulation concentration and time gradient are set in this experiment, the HBV expression levels of cells stimulated by different osthole concentrations and time can be compared with positive or negative controls. On the premise that osthole stimulation does downregulate HBV gene expression levels, we can determine the optimal concentration and duration of osthole downregulation of HBV genes. This is of great significance for the subsequent application of osthole in the clinic.

6.Conclusion

Osthole is an important active ingredient found in Cnidium monnieri, an herb used to treat many symptoms. This study focuses on whether osthole can downregulate the HBV gene by blocking the Notch and NFκB pathways, determining whether osthole plays a role in the treatment of patients with CHB as an active ingredient in Bushen formula that reduce HBV expression.[13] Although experimental validation has not yet been conducted, this paper provides a basis for further HBV treatment and elucidates the mechanism of traditional Chinese medicine Bushen formula. If Osthole does downregulate HBV genes through Notch and NFκB, continuing to study Osthole or its mechanism on pathways may provide new ideas for future new HBV clinical drugs. If it is found that osthole is not an active ingredient in Bushen formulations that downregulate HBV gene expression, then other molecules in the formula can be tested in the same way to find the true active ingredient. Through more research on traditional Chinese medicine, more natural and gentle new treatment methods may be provided for clinical drug treatment.

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