

The Effect of Extracted Bacterial *Salmonella enteritidis* Lipopolysaccharide on Inducible Nitric Oxide Synthase in Human Liver Hepatocellular Carcinoma Cell Lines in Induction and Inhibition Conditions

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Hepatocellular carcinoma is the most ordinary type of liver cancer and most commonly appears in a patient with chronic viral hepatitis and with cirrhosis. Lipopolysaccharide motivates the hepatocyte cells and increases the production inducible cytokine with the high production of nitric oxide and reactive oxygen species. In this study, the rate change iNOS searched by adding lipopolysaccharide and the effect of inhibiting this inflammatory enzyme by L-NAME and combined effect of this in HepG2 cancer cells was assessed. *Salmonella enteritidis* lipopolysaccharide is extracted by methanol-chloroform method and silver stained SDS-PAGE electrophoresis bands. Four treatment groups of Human liver hepatocellular carcinoma cell lines stimulated with 100ng/ml lipopolysaccharide, 500µM L-NAME as inhibitor and were incubated for 12 and 24 hours. Variables including both increase and decrease inflammatory factor, iNOS, was assayed. The obtained results showed that initial activity of iNOS in not stimulated in HepG2 cell, 0.902U/ml after stimulation with lipopolysaccharide for 12 and 24hours (0.491U/ml) and (0.433U/ml) decreased and the effect of this inhibitor was also studied. The data showed that reducing iNOS in Human liver hepatocellular carcinoma cell lines correlated with the cell density and duration of incubation with LPS. Inhibition of enzyme associated with inflammation, with inhibitor substances; L-NAME, observed as well as lipopolysaccharide. We can potentially design drugs to treat a variety disease and cancer to use.

Key words: Inducible Nitric Oxide Synthase, HepG2 Cell, *Salmonella enteritidis*- Lipopolysaccharide- SDS-PAGE.

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The most ordinary type of liver cancer is hepatocellular carcinoma. Most cases of Hepatocellular carcinoma are secondary to either a viral hepatitis infection or cirrhosis^{1,2} and Ninety percent of Hepatocellular carcinoma develops in the context of chronic liver disease and mainly in patients with cirrhosis. The hepatitis virus is the

commonest cause of Hepatocellular carcinoma in the world and every year kill more than one million people³.

Human liver hepatocellular carcinoma cell lines (HepG2) are culturable, repeatedly proliferable, immortalized and available. So, they can be easily saved, cultured, proliferated, and tested and they are the good targets for gene therapy in hemophilic patients. They are used in cellular and genetic tests in hepatic diseases, human immunodeficiency virus, liver and breast cancer and many genetic diseases and metabolic studies^{4,5}.

One of the stimulating factors of growth and proliferation and causing malign diseases in cell is lipopolysaccharide (LPS) which is glycolipid part of outer membrane of negative gram bacteria which produce cytokine and vasodilator such as nitric oxide⁶. Lipopolysaccharide be capable of induce macrophages and microglia to produce various types of inflammatory molecules⁷. The production of high amount of nitric oxide happens after the induction of inducible Nitric Oxide Synthase (iNOS) related to cytokine^{8,9}. The produced nitric oxide by iNOS increase gene expression of prostaglandin E2, cyclooxygenase 2 and cell proliferation through activating p38 route and mitogen-activated protein kinases and C-Jun N-terminal kinase 1/2¹⁰.

Nitric Oxide Synthase is most regulated enzymes in biology. There are three known isoforms, two are fundamental (c Nitric Oxide Synthase) and inducible Nitric Oxide Synthase is the third one. Cloning of Nitric Oxide Synthase enzymes indicates that, c Nitric Oxide Synthase include both brain constitutive (Nitric Oxide Synthase 1) and endothelial constitutive (Nitric Oxide Synthase 3), the third is the inducible (Nitric Oxide Synthase 2) gene¹¹.

In contrast to the critical calcium-dependent regulation of constitutive Nitric Oxide Synthase enzymes (n Nitric Oxide Synthase and e Nitric Oxide Synthase), iNOS has been described as calcium-insensitive, likely due to its tight non-covalent interaction with calmodulin (CaM) and Ca²⁺. While evidence for 'baseline' iNOS expression has been elusive, IRF1 and NF- κ B-dependent activation of the inducible Nitric Oxide Synthase promoter supports inflammation mediated stimulation¹². Nitric Oxide plays a

significant role in host immune defense, vascular regulation, neurotransmission, and other systems under normal conditions, aberrant Nitric Oxide expression is thought to cause severe inflammatory disease. Overproduction of inducible Nitric Oxide synthase (iNOS) is especially related to various human diseases such as inflammatory and neuronal disorders because of the upregulation of Nitric Oxide¹³.

Pathologic generation of nitric oxide through increased inducible Nitric Oxide Synthase production may decrease tubal ciliary beats and smooth muscle contractions and thus affect on embryo transport, which may result in ectopic pregnancy¹⁴. The inhibition of production of nitric oxide is done by L-NAME (the special inhibitor of inducible Nitric Oxide Synthase) which causes the decrease of density of produced nitric oxide through this enzyme and avoids the formation of malign diseases^{15,16}. The effect of lipopolysaccharide upon increase and decrease of activity of inflammatory factor of iNOS and also the effects of specific inhibitor of inducible Nitric Oxide Synthase in Human liver hepatocellular carcinoma cell lines were not studied entirely.

In this study, we tried to stimulate inducible Nitric Oxide Synthase by using salmonella enteritidis lipopolysaccharide that is extracted and by using inducible Nitric Oxide Synthase inhibitor inspecting the results in Human liver hepatocellular carcinoma cell lines in inducing condition with lipopolysaccharide in these cells.

MATERIALS AND METHODS

Materials

Medium culture DMEM+L-Glutamine (Gibco, code 12800-116), Quantakine iNOS ELISA kit (R&D system company, Michigan, America), N^o-Nitro-L-arginine methyl ester hydrochloride (L-NAME) substance (Sigma-Aldrich company, code N5751-1G) *Salmonella enteritidis* bacteria of mutant rough pour H.g.m (Iran Pastour institute), and HepG2 liver cells (Iran Pastour institute) that in this study we used them.

Methods

By the methanol-chloroform method we extracted Lipopolysaccharide of *Salmonella enteritidis* which is a cheap, safe and simple

method. Then for purity and accuracy checking of the extract we used Sodium dodesyl sulfate poly acryl-amide gel electrophoresis (SDS-PAGE) and silver staining.

Methanol-chloroform extraction method

For extraction, separation and purification of lipopolysaccharide there are different methods which may use extracting agent such as ether¹⁷, hot-phenol¹⁸, butanol¹⁹, EDTA²⁰ and proteinase K²¹. In this study, we used methanol-chloroform method for its rapid and low cost and also phenol was not used due to its low safety and poisoning. Standard bacterium of *Salmonella enteritidis* was supplied from Iranian reference health laboratory.

SDS-PAGE and Electrophoresis methods

In electrophoresis method, we produced acryl amide gel 12% the following materials were used: acryl 30%, TBE (10X), AMS, TEMED (N, N, N', N'-tetra methyl ethylenediamine), distilled water and in SDS-PAGE method, the separating gel 12% was used. The gel was stained by silver staining methods (Fig. 1) based on Sambrook 2001 protocol²². The exactness of their formed bands were investigated with standard lipopolysaccharide of *salmonella enteritidis* Sigma L-6011-100MG.

Cell culture and laboratory experiments the medium culture is used which is supplemented with amino acid mixtures including 4mmol L-Glutamine and 10% fetal bovine serum, 1000 unit/ml penicillin and 100 µg/ml streptomycin with humid atmosphere 95%, 5% CO₂ in 37°C²³ for the growth of Human liver hepatocellular carcinoma cell lines, and then the cells are incubated.

After culture in T75 model flask and approaching to the density of 85%, they are cultured in the plate and 3hours after the stimulation of cells with lipopolysaccharide, L-

NAME is added. In two times for 12 hours and 24 hours, the treated cell groups are incubated. The amount of one million cells is lysated and its activity of inducible Nitric Oxide Synthase is measured by the intended microplate kits and by the ELISA reader the results are read.

Four treatment groups are categorized as follow

1. Human liver hepatocellular carcinoma cell lines (HepG2)
2. lipopolysaccharide + Human liver hepatocellular carcinoma cell lines (HepG2)
3. Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) + Human liver hepatocellular carcinoma cell lines (HepG2)
4. Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) + lipopolysaccharide + Human liver hepatocellular carcinoma cell lines (HepG2)

Statistical analysis

In this study, we used two-way ANOVA for comparing the means and analyzed of results. The differences between the means were determined with P<0.05 level in different experimentations. Data are expressed as means ± standard errors (SEM) and all of the calculations were assessed by the software SPSS.

RESULTS

Findings of the study demonstrated that activity of inducible Nitric Oxide Synthase in the culture media, inducible Nitric Oxide Synthase in unstimulated Human liver hepatocellular carcinoma cell lines was 0.902U/ml in orders. This amount after the stimulation of the Human liver hepatocellular carcinoma cell lines with lipopolysaccharide for 12 and 24hours decreases as 0.491U/ml. Therefore, in the other experiments,

Table 1. The results of four treatment groups of cancerous cells of HepG2 with lipopolysaccharide and inhibitors during 12 and 24 hours

Treatment groups	12 hours iNOS(U/ml)	24 hours iNOS(U/ml)
1- Human liver hepatocellular carcinoma cell lines	0.902	0.932
2- Lipopolysaccharide + HepG2 liver cells	0.491	0.433
3- L-NAME + Human liver hepatocellular carcinoma cell lines	0.893	0.673
4- L-NAME + Lipopolysaccharide + Human liver hepatocellular carcinoma cell lines	0.714	0.650

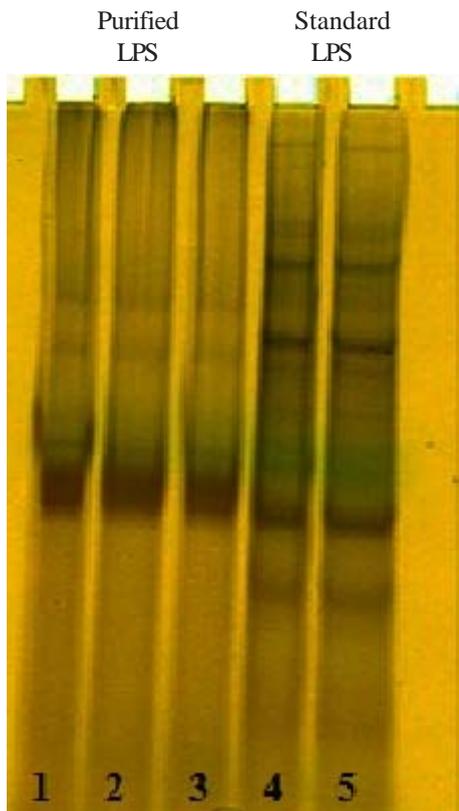


Fig. 1. Bands one, two and three are related to the extracted *salmonella enteritidis* lipopolysaccharide mutant rough pour H.g.m, bands four and five are related to standard *salmonella enteritidis* lipopolysaccharide Sigma L-6011-100MG for support of authenticity of extracted bands

lipopolysaccharide is used as an inhibitor of inducible Nitric Oxide Synthase producing. The inhibitor effect of L-NAME was investigated and the results were shown (Table 1). Treatment with L-NAME as specific inhibitors of inducible Nitric Oxide Synthase enzyme significantly decreases the activity of inducible Nitric Oxide Synthase in stimulated Human liver hepatocellular carcinoma cell lines with LPS, while the same substance does not change the activity of produced inducible Nitric Oxide Synthase in unstimulated cells (Fig. 2).

DISCUSSION

Activity of inducible Nitric Oxide Synthase in the culture media inducible Nitric Oxide Synthase in unstimulated Human liver hepatocellular carcinoma cell lines in this study was 0.902U/ml. This amount after the stimulation of the Human liver hepatocellular carcinoma cell lines with lipopolysaccharide for 12 and 24hours decreases as 0.491U/ml. Treatment with L-NAME as specific inhibitor of inducible Nitric Oxide Synthase enzymes significantly decreases the activity of inducible Nitric Oxide Synthase in stimulated Human liver hepatocellular carcinoma cell lines with lipopolysaccharide. Of course Bultink and et al in 2006 suggested that TNF- α or lipopolysaccharide did not induce any detectable NOx⁻ in circulation of iNOS^{-/-} mice with an iNOS^{+/+} hematopoietic cell population²⁴ and this is agreement with the results of this study. The stimulation of the intended cells and inducing of

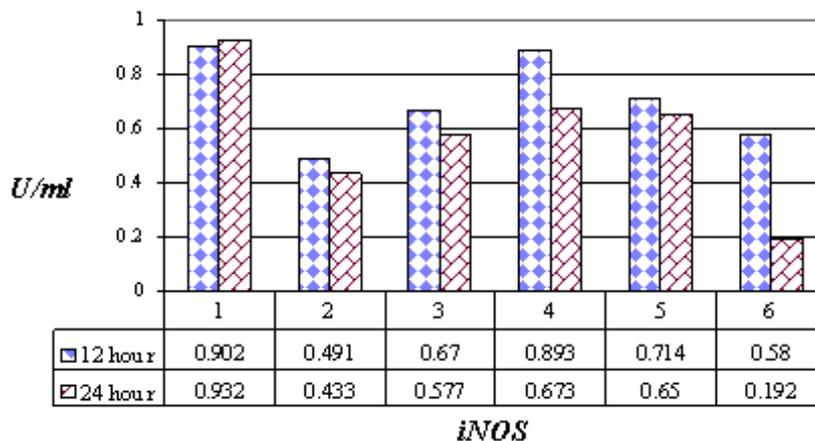


Fig. 2. The activity of iNOS in cancerous cells of HepG2 after 12 and 24 hours of incubation after and before motivation with lipopolysaccharide

inflammatory enzyme was done in this study with 100 ng/ml of the extracted Lipopolysaccharide out of salmonella bacterium which this amount was suggested in a standard way by Nemeth and his colleagues in 2003²⁵. In 2006, Kaptanovic and his colleagues expressed that lipopolysaccharide causes induce of inducible Nitric Oxide Synthase with the movement of NF- κ B into core²⁶. In 2008, Ziwen Liu and et al in their experiments investigated that lipopolysaccharide in RAW264.7 cells, stimulates the gene expression of Cyclooxygenase-2 and inducible Nitric Oxide Synthase with releasing mitogen activated protein kinase and NF- κ B, and some of inhibitors which stop the signal route of mitogen activated protein kinase and the activity of NF- κ B, it also inhibits the gene expression of Cyclooxygenase-2 and inducible Nitric Oxide Synthase²⁷ and it is opposite of result of this study. In 2005, Scott *et al.*, found out that lipopolysaccharide is the starter of an intra cell signaling cascade⁶.

Regarding the measured activities of inflammatory enzyme of inducible Nitric Oxide Synthase in cancerous cells of Human liver hepatocellular carcinoma cell lines in 12 and 24 hours, we found out that the activity of this enzyme in cancerous cells is very high and it is in line with the results of studies done by Dipopolo and Aiwen in 2000 and 2003^{28,29}. In 1999 Nanbo and et al found out that Lipopolysaccharide binding protein is synthesized in hepatocytes and is known to be an acute phase protein. Cytokine-induced production of Lipopolysaccharide binding protein was reported to increase 10-fold in hepatocytes isolated from LPS-treated rats, compared with those from normal rats. Human liver hepatocellular carcinoma cell lines were shown to express CD14. Prestimulation of Human liver hepatocellular carcinoma cell lines with LPS/LBP were augmented cytokine-induced production and gene expression of LBP and CD14^{30,27}.

The activity of inducible Nitric Oxide Synthase in Human liver hepatocellular carcinoma cell lines cancerous cells during 12 hours of incubation with lipopolysaccharide decreased from 0.902 to 0.491 U/ml. regarding this fact that L-NAME is inhibitor and reducing factors of inflammatory enzyme of inducible Nitric Oxide Synthase but lipopolysaccharide of *Salmonella enteritidis* highly reduces the activity of

inflammatory enzyme of inducible Nitric Oxide Synthase. Human liver hepatocellular carcinoma cell lines groups which receive L-NAME for inhibition of inducible Nitric Oxide Synthase; it is observed that by adding lipopolysaccharide to inducible Nitric Oxide Synthase and incubation, not only lipopolysaccharide does not induce inducible Nitric Oxide Synthase but of course helps the inhibitors and reduce inducible Nitric Oxide Synthase in higher activity. L-NAME properly decreased the activity of inducible Nitric Oxide Synthase after 12 and 24 hours before and after the stimulation of Human liver hepatocellular carcinoma cell lines with lipopolysaccharide. In groups which received lipopolysaccharide, the decrease activity was more noticeable.

L-NAME can properly be used in the treatment of many diseases and cancers because of their positive effects on both inflammatory enzymes. Also lipopolysaccharide is a decreasing factor of this enzyme. Regarding this fact, group four, lipopolysaccharide helps more to inhibitor in the stimulation of enzyme, *Salmonella enteritidis* Lipopolysaccharide that it has a special sugar linkage in its Antigen O, can be used in reducing the activity of inflammatory enzyme of inducible Nitric Oxide Synthase as the helper of inhibitors. Also Inhibition of enzyme associated with inflammation, with inhibitor substances; L-NAME, observed as well as Lipopolysaccharide. Because of, Cyclooxygenase-2 and inducible Nitric Oxide Synthase induction by Lipopolysaccharide, are possible and have the synergy effect on one another thus the suggested mechanism of this decrease is that A lipid of lipopolysaccharide consists of exceptional structure including matchless fat acids which are accompanied with hydroxyl groups and they can cause the restraint of Cyclooxygenase-2 with competition by arashidonate and then this changes are effective on inducible Nitric Oxide Synthase. We can potentially design drugs to treat a cancer and diversity disease to use.

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