Nutrition 91–92 (2021) 111466

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Hepatocyte growth factor and fish oil facilitated reversal of D-galactosamine–induced toxicity in primary hepatocyte cultures of albino mice

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ARTICLE INFO

Article History:
Received 7 August 2021
Accepted 23 August 2021

Keywords:
Hepatocytes
HGF
Fish oil
D-galactosamine
Regeneration

ABSTRACT

D-galactosamine (Ga1N), a well-known hepatotoxic agent, induces liver injury resembling human viral hepatitis usually followed by the regeneration processes. Hepatocyte growth factor (HGF) is a cytoprotective factor involved in regeneration of the injured liver. However, the effects of exogenous HGF remain poorly understood because of its rapid clearance by the liver. This study was undertaken to find out whether HGF and fish oil facilitated the reversal of GalN–induced toxicity in primary hepatocyte cultures of albino mice. Primary hepatocytes cultures were established from mice liver tissue. The study involved the effect of GalN on hepatocytes and also determination of the protective role of fish oil on hepatocyte cultures. Cell proliferation tests and liver function tests were done to determine the degree of GalN effect on cultured hepatocytes. Biochemical parameters of cultured cells were also performed to check the recovery effect of fish oil on GalN–induced hepatotoxicity. The combination of Ga1N and HGF triggered cell proliferation in primary hepatocyte cultures specifying activation of regeneration through HGF. However, hepatocyte function tests revealed that although the regeneration process was initiated, its function was slightly altered by Ga1N. Therefore, to control its effect at a functional level, we tested fish oil doses and indicated its influence. This work can be a useful tool for studying hepatotoxic-induced cell regeneration in vitro; moreover, the data indicates that HGF and fish oil has hepatoprotective activity against Ga1N and may aid as a suitable adjuvant in clinical conditions associated with liver damage.

Introduction

Liver tissue has a remarkable regenerative capacity to injuries caused by viral hepatitis and surgical resection. Such proliferation can also be induced under experimental conditions by partial hepatectomy and with hepatotoxic chemical agents like tetrachloromethane, GalN, thioacetamide, acetaminophen, and carbon tetrachloride [1,2]. GalN is frequently used as a model hepatotoxin in animal experiments in vitro as it causes biochemical features closely resembling those seen in human viral hepatitis [2]. Studies have shown that primary hepatocyte culture can serve as an excellent model for studying the mechanisms that regulate cell proliferation and differentiation [3]. The proliferative process in toxic hepatitis has been less widely investigated [4]. In vivo GalN hepatotoxicity has been associated with rapid and extensive depletion of hepatic uridine nucleotide that causes hepatocyte necrosis [5,6]. However, the exact mechanism of Ga1N-induced hepatic necrosis is still unclear. After GalN injury, the liver responds by activation of progenitor cells that proliferate and then differentiate into mature hepatocytes [7,8]. An important role in this regeneration process is played by cytokines and growth factors. Liver injuries trigger some critical elements that make hepatocytes competent to fully respond to these substances [9,10,11] in which liver cells switch from a quiescent state to a proliferative state and re-enter the cell cycle. Many growth factors regulate this process by providing both stimulatory and inhibitory signals for hepatocyte proliferation. The initial regeneration process is implicated to be stimulated by tumor necrosis factor-α and interleukin-6 [12,13]. Hepatocyte growth factor (HGF) and ligands of the epidermal growth factor receptor, of which epidermal growth factor and transforming growth factor–α (TGFα), stimulate further replication. It is not known whether HGF

Abbreviations: Ga1N, D-galactosamine; HGF, Hepatocyte growth factor; TGFα, Transforming growth factor-α; ω-3 PUFA, Omega-3-polyunsaturated fatty acids; TGF-β, Transforming growth factor- β; LDH, Lactate dehydrogenase; ALP, Alkaline phosphatase; GGT, γ glutamyl transferase; PCM, Plain culture medium; FBS, Fetal Bovine serum; GOT, Gglutamate oxaloacetate transaminase; GPT, Glutamate pyruvate transaminase

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https://doi.org/10.1016/j.nut.2021.111466
0899-9007/

and TGFα have identical or complementary functions in hepatocyte replication. However, activation of signaling by either of these two receptors leads to activation of NF-κB, STAT3, phosphoinositide-3-kinase, and eventually Akt, leading to hepatocyte proliferation [14]. The final suppression of cell growth at a set point is actuated by TGFβ and activin [15,16].

Several studies have demonstrated the involvement of HGF to stimulate liver regeneration following partial hepatectomy [14,17,18]. In the liver, HGF is produced by non-parenchymal cells (especially perisinusoidal, Kupffer, and endothelial cells) and is one of the major hepatocyte mitogens that does its activity through activation of hepatocyte Growth Factor receptors that are also called tyrosine-protein kinase Met. Despite the important role of HGF in regeneration, long-term effects of exogenous HGF remain poorly understood. Most of the studies related to hepatic injury and liver regeneration have been focused on in vivo animal models.

Our study focuses on the effects of D-galactosamine and HGF on cultured hepatocyte cells using various parameters of liver metabolism. We also tested the potential protective effect of fish oil against liver injury induced by Ga1N in primary cultures as fish oil supplements contain saturated fats and oxidized lipids that interfere with cell metabolism rendering some biological benefits [19,20]. Also, omega-3−polyunsaturated fatty acids have shown to support liver regeneration and functional revival in living liver donors after resections for liver transplantation [21] and were also found effective for the treatment of patients after hepatectomy [22].

Methods

Animals

Albino mice were housed under controlled temperature (25°C ± 1°C) and light-dark cycle periods (12 h), with free access to a standard laboratory mice pellet (Hindustan Lever, Bangalore, India) and water under strict hygienic condition. All animals received care according to the guidelines for laboratory animals specified by the Committee for the Purpose of Control and Supervision of experiments on Animals. Ethical approval was obtained from the Institutional Animal Ethics Committee (Ref no: GU/Zoo/2013-14/04 dated May 14, 2013) before the design of the experiment.

Hepatocyte isolation and establishment of primary cultures

Hepatocyte primary cultures used for the present study were established as follows. The liver tissues of adult rats were removed under aseptic condition and were transferred to a beaker containing phosphate buffer saline having 0.5% of antibiotic solution (Himedia, A007). The osmolality was adjusted to ~315 mOsm/kg and to pH of 7.4. After the final wash and further incubation for 5 min in an antibiotic solution, the tissues were cut into small fragments of ~2.0 mm^3. To isolate liver cells, an enzymatic dissociation technique using 0.01 U/mL collagenase prepared in phosphate buffer saline was used. The tissue fragments were kept in dissociating media for 5 min and were later triturated 15 times using a 10 mL glass pipette. The dissociated cell suspension thus obtained was filtered through a nylon mesh (100 μm) and centrifuged at 1000 rpm for 3 min. The pelleted cells had top light yellowish cell fraction, which exclusively yielded hepatocytes. This fraction was carefully aspirated separately into centrifuge tubes using a Pasteur pipette and was washed twice in L15 culture medium having 0.2% antibiotics, ~315 mOsm/kg, and a pH of 7.4; cell viability was checked using the trypan blue dye exclusion technique. The cell suspension with more than 95% viable cells was used for inoculation. Cell density count was performed using a hemocytometer, and cells were plated at a density of 2×10^5 cells/mL in each well of collagen-coated multiwell plates containing inoculating culture media consisting of L15 basal medium along with 15% Fetal Bovine Serum (FBS, Sigma, F4135), 20 ng/mL HGF (Sigma, H9661), 0.2% antibiotics with ~315 mOsm/kg osmolarity, and pH of 7.4. All multiwell plates were incubated at 37°C, and the media were replenished at 2-d intervals. Cultured cells were examined daily with an Olympus inverted microscope to observe the condition of the culture. Once the monolayer was formed, the cultures were replaced with a fresh growth medium (L15 along with 10 % FBS, 20 ng/mL HGF, 0.2% antibiotics with ~315 mOsm/kg osmolarity, and pH of 7.4). These cultures were used for further experimentation.

Effect of GalN on cultured hepatocyte

Primary hepatocytes monolayer cultures obtained as described above were used for this experimentation. Control culture plates received plain culture media (PCM) (L15+ HGF 20 ng/mL + FBS 10% and antibiotics) without GalN, whereas experimental sets received PCM with different concentration of GalN (1, 5, 10, 15, 20, 30, 40, 50, 60, and 70 mM). Cultures were viewed and transferred to an incubator at 37°C. After 24 h of incubation, these cultures were assayed for cell proliferation using Water Soluble Tetrazolium salts test (WST test) and liver function enzyme tests like lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), and total bilirubin.

Protective role of fish oil on hepatocyte cultures exposed to GalN

In the second set of experiments, the protective role of two different concentrations of fish oil (10 and 20 μg/mL) on hepatotoxicity was tested individually as well as in combination with GalN (40 mM prepared in PCM) on hepatocyte culture. The experiment was initiated by converting the fish oil tablets (Maxepa, Merck, India) into salt form by the methanolic potassium hydroxide method [23] to dissolve fish oil in culture media. The fish oil salt form was sterilized by ultraviolet exposure for 24 h. The powder form of fish oil was reconstituted in L15 medium at two different concentrations (10 and 20 μg/mL). Control cultured plates received PCM (L15 + HGF 20 ng/mL + FBS 10% with antibiotic), while the experimental culture plates were grouped into six. The first group (hepatocyte primary culture plates n = 3) were exposed to GalN (40 mM) prepared in PCM. The second and third groups of cultures received fish oil of 10 and 20 μg/mL concentration reconstituted in PCM. The fourth and fifth culture groups were exposed to GalN (40 mM) prepared in PCM having two different fish oil concentrations of 10 and 20 μg/mL. Biochemical estimation such as albumin, LDH, total bilirubin, ALP, glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) were assayed to check the recovery effect of fish oil on GalN-induced hepatotoxicity in the primary hepatocytes cultures incubated up to 24 and 48 h.

Cell viability and proliferation test

WST assay is a biochemical test for detecting changes in cell proliferation viz−a-viz cell toxicity. Cell viability and proliferation were assayed using a cell counting kit (Sigma, 96992). The assay is based on the conversation of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to an orange formazan product by the cellular dehydrogenase of live cells. The orange formazan product is soluble in a tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells. The cell counting test was conducted according to the manufacturer's instructions.

Biochemical Assays

The effect of GalN and HGF on cultured hepatocyte function was assessed by measuring the release of LDH into the culture medium and was used as a marker for cytotoxicity. LDH activity was analyzed in the culture media and in cell homogenates using a commercial kit from Crest Biosystems (LDH (P-L) kit). Enzyme activities such as ALP, GOT, GPT, and bilirubin (Total) were assayed by using a kit from Crest Biosystems (ALP (DEA) kit, GOT (IFCC) kigt, GPT (IFCC) kit, BIL (J&G) kit). To evaluate the functional capacity of cultured hepatocytes, the amount of albumin secreted into the culture medium during the incubation period was measured using a commercial kit from Crest Biosystems (ALB kit).

Statistical analysis

All experiments were performed in triplicates using hepatocyte primary cultures. All values were expressed as mean ± standard error. The statistical significance was analyzed by using Student's *t* test. Statistical significance was set at $P < 0.05$.

Results

In our experiment, we assessed the effect of GalN to induce hepatocyte toxicity and the possible role of HGF to trigger cell regeneration by exposing the primary cultures of hepatocytes to different concentration (1, 5, 10, 20, 30, 40, 50, 60, and 70 mM) of GalN along with HGF (20 ng/mL) in their growth medium for 24 h. Increments ($P < 0.05$) in cell density were observed, in comparison to the control after 24 h incubation with 20, 30, 40, 50, 60, and 70 mM GalN concentration in the growth medium (Fig. 1).

Biochemical assays such as LDH, ALP, GGT, and total bilirubin were done to assess the extent of D-galactosamine toxicity on cultured hepatocyte function. Lower concentrations of Ga1N (10, 20, and 30 mM) did not exhibit any significant effect on primary cultures of hepatocytes, but higher concentrations (40, 50, 60, and 70

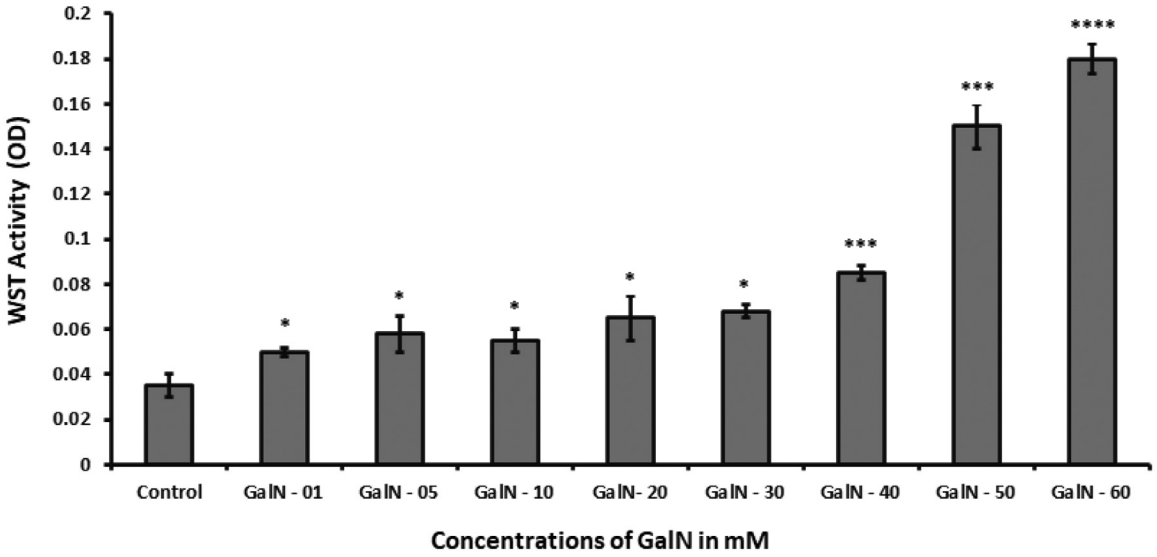

Fig. 1. WST test on hepatocytes cultured in different concentrations of GalN (1, 5, 10, 20, 30, 40, 50, 60, and 70 mM) prepared in plain culture medium. All data represent mean value ± standard error (n = 3). Student's *t* test significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared with its control (C).

mM) showed significant increments ($P < 0.05$) of LDH activity after 24 h of incubation (Fig. 2A) in cells as well as in media compared with the control.

GalN concentrations of 30, 40, 50, 60, and 70 mM in the media showed a significant increase ($P < 0.05$) of ALP activities assayed from media and from cells (Fig. 2B). Significant changes in GGT activities were observed when assayed from media of cultures exposed to GalN, except for 30 mM concentrations (Fig. 2C), as compared with the control.

All concentrations of Ga1N showed significantly less bilirubin when assayed from cells as well as media when compared with the control (Fig. 2D). Figures 3A, B, and C and 4A, B, and C represent the effect of fish oil on biochemical parameters after 24 and 48 h of culture incubations with media composition with GalN, fish oil, and both, in combinations. Overall results indicate recovery of all biochemical parameters of cultured cells (Fig. 3A, B, C and Figure 4A, B, C) under the influence of fish oil.

Discussion

In vitro studies on the liver provide an appropriate alternative method to replace animal models owing to ethical issues;

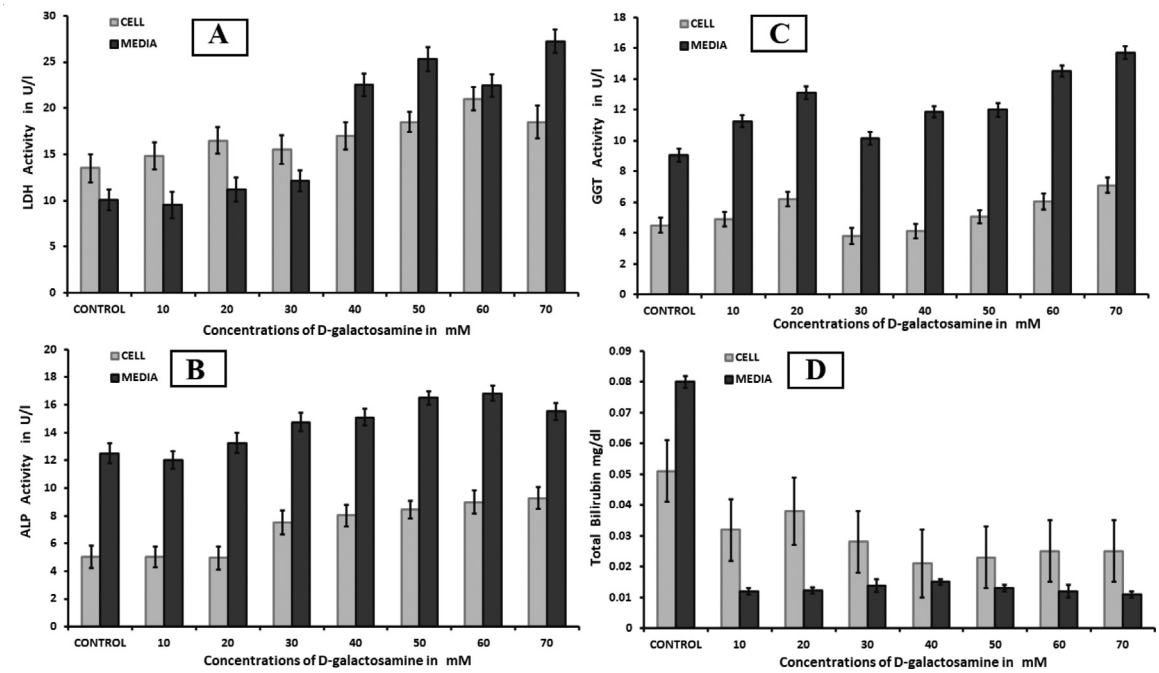

Fig. 2. Effect of GalN of various concentrations (10, 20, 30, 40, 50, 60, and 70 mM reconstituted in plain culture media) on (A) lactate dehydrogenase, (B) alkaline phosphatase, (C) γ-glutamyl transferase, and (D) total bilirubin production after 24 h in the culture media and in cell homogenates. All data represent mean ± standard error (n = 3); C, control.

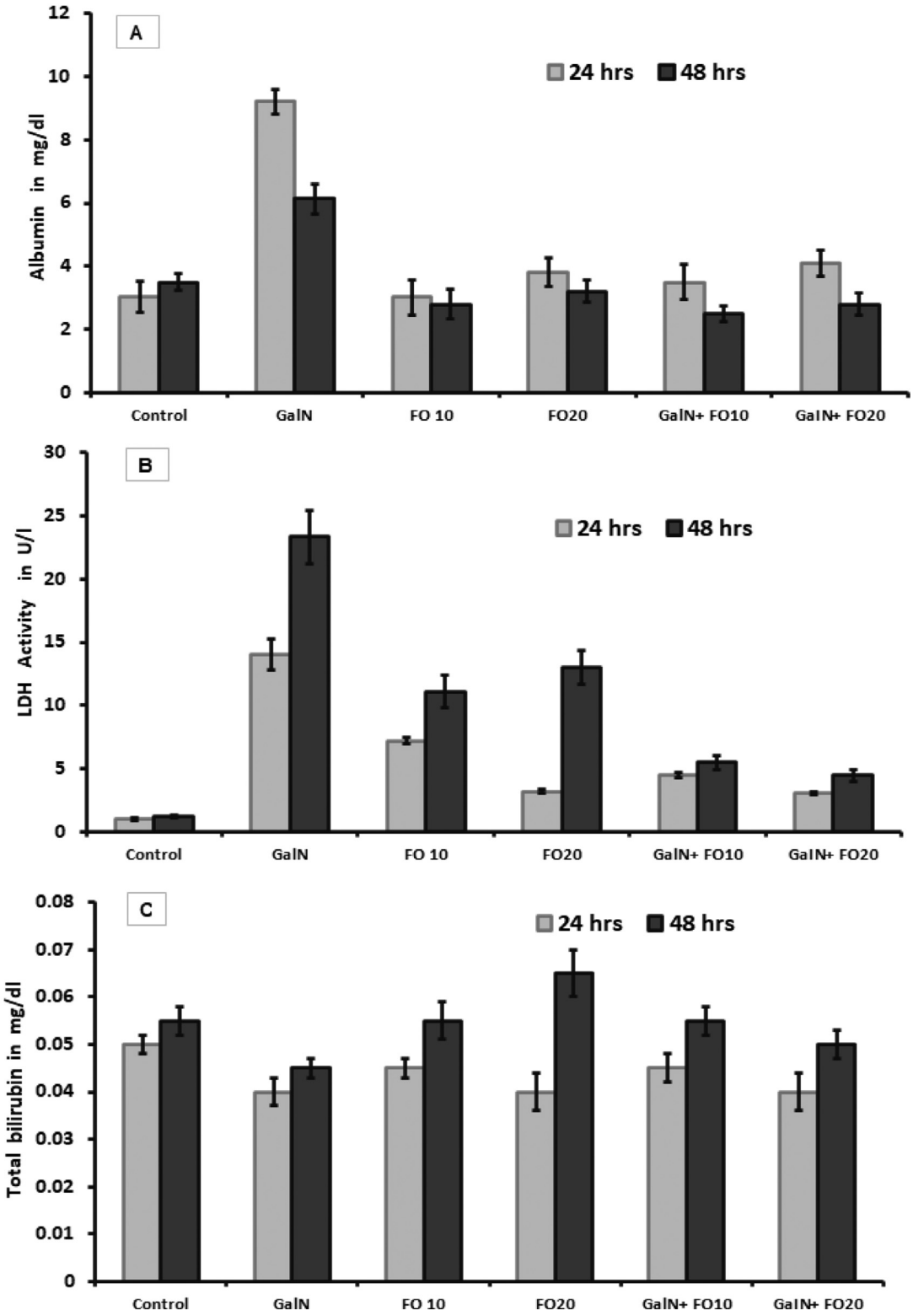

Fig. 3. Effect of fish oil (10 and 20 μg/mL represented as FO10 and FO20) on biochemical parameters of hepatocytes exposed to GalN (40 mM prepared in plain culture media) after 24 and 48 h. (A) Albumin, (B) lactate dehydrogenase, and (C) total bilirubin. Control plates received plain culture media. All data represent mean ± standard error (n = 3). C, control; GalN, D-galactosamine; FO, fish oil; GalN+FO, D-galactosamine plus fish oil.

moreover, cell culture depicts true activity and functions that they display in their natural environment and thus provide a platform for drug toxicity screening, as well as the study of liver regeneration, metabolism, or disease [24,2]. In this study, we could isolate hepatocytes with 95% viability based on an enzymatic dissociation technique using collagenase.

In the present study, we verified the effect of GalN to induce hepatocyte toxicity and the possible role of HGF to trigger cell

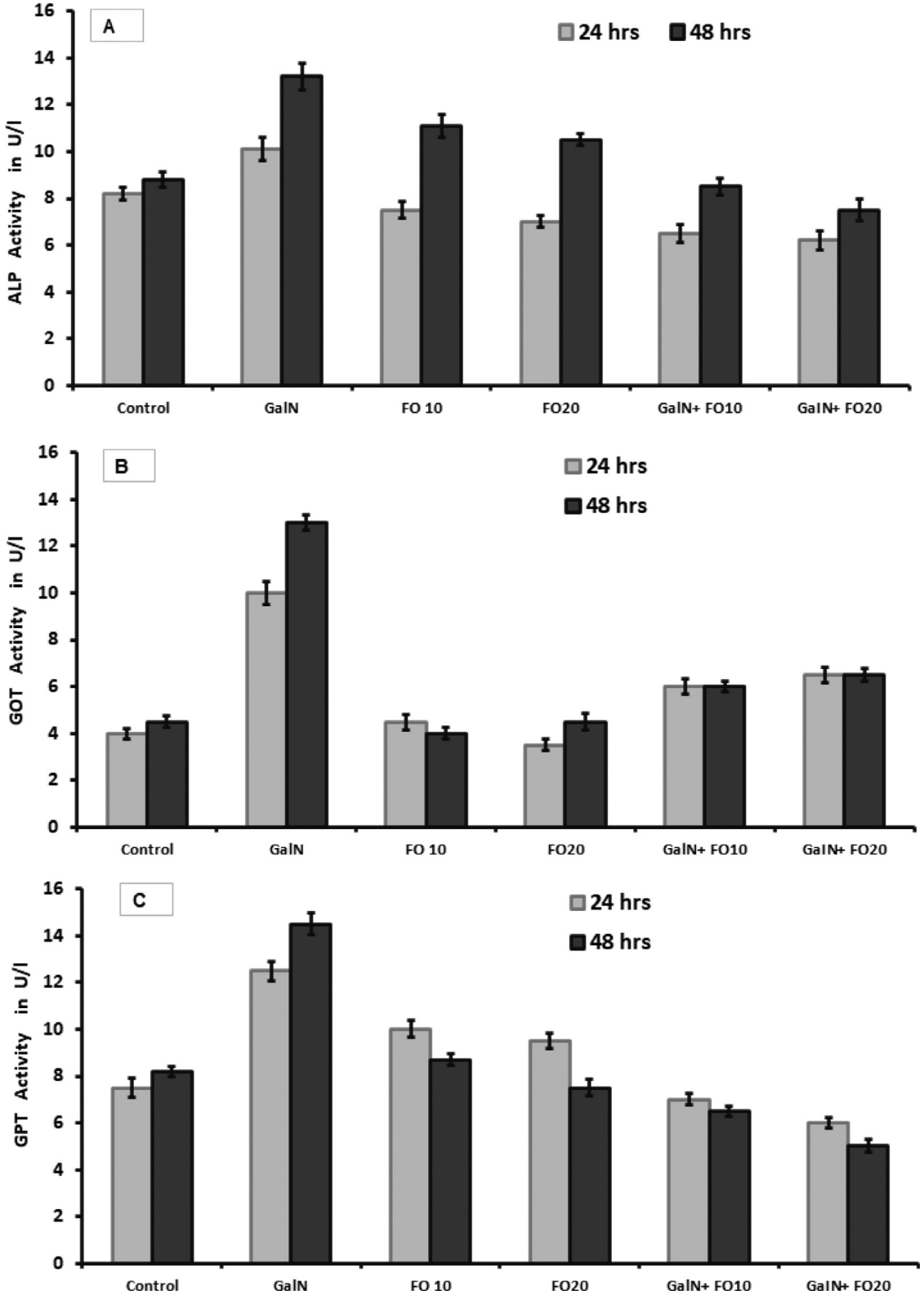

Fig. 4. Effect of fish oil (10 and 20 μg/mL represented as FO10 and FO20) on biochemical parameters of hepatocytes exposed to GalN (40 mM prepared in plain culture media) and incubated for 24 and 48 h. (A) Alkaline phosphatase, (B) glutamate oxaloacetate transaminase, and (C) glutamate pyruvate transaminase. Control plates received plain culture media. All data represent mean ± standard error (n = 3). C, control; G, GalN; FO, fish oil; GalN+FO, GalN plus fish oil.

regeneration in vitro. The combination of Ga1N and HGF triggered cell proliferation within 24 h when compared with the control group. Cleavage of tetrazolium salt (WST-8) by cellular dehydrogenase proceeds only in viable cells, thus the increase in activity induced by Ga1N in this study indicates expansion in the number of viable cells, which specifies that HGF promotes activation of regeneration process leading to the proliferation of hepatocytes. However, hepatocyte biochemical tests revealed that although the regeneration process was initiated by HGF, its function was slightly affected by the toxic effect of GalN. An increase in the activity of LDH, ALP, and GGT in this study indicates hepatocyte injury, which is in accordance with the previous reports of in vivo studies [2,25]. In viral hepatitis, a deficit of total bilirubin metabolism occurs; in the present study, total bilirubin secretion by hepatocyte was reduced, indicating Ga1N-induced hepatocyte injury at the functional level. To control the Ga1N effect at a functional level, we tested the influence of fish oil. Few reports have shown that lipid emulsion has a protective effect against liver toxicity [1,25]. While comparing the effect of Ga1N and HGF alone with the effects of Ga1N along with HGF and fish oil for 24 and 48 h, we found that fish oil improves the function of hepatocytes. However, the protective effect of fish oil was dose-dependent, and a lower dose of 10 and 20 μg/mL participates in improving the hepatocyte function.

Albumin synthesis is a widely used marker for testing hepatocyte functional capacity. Hepatocyte culture exposed to Ga1N shows impaired albumin production [2]. However, in this study, we observed that Ga1N in combination with HGF induces increased albumin production in primary culture. This can be attributed to the regeneration process triggered by HGF as a recovery effect to Ga1N toxicity, which leads to a significant increase in cell proliferation as a result, the production of albumin also increases. However, the addition of fish oil to GalN in combination with HGF normalized the albumin production to that of the control.

GalN induces a marked change in enzyme activity during hepatic injury [26]. A similar finding was observed in this study wherein the activities of LDH, ALP, GOT, and GPT increased slightly. The rise in activity may be interpreted as a result of cellular leakage and loss of functional integrity of cell membrane. The activity induced by GalN tended to decline with fish oil. Nevertheless, a significant reduction was reached at a concentration of 10 and 20 μg/mL after 24 and 48 h of incubation. Determination of bilirubin serves as an index for the assessment of hepatic function. Changes in the level of total and direct bilirubin indicate disturbance of hepatocellular function. The increase level of direct bilirubin in this study is in agreement with the report of the previous studies that GalN-induced hepatitis is characterized by increased levels of bilirubin in serum [27].

The present work documents that HGF significantly diminishes the extent of hepatocyte injury induced by GalN and triggered the regeneration process. Although the proliferation process was initiated by HGF, its function was slightly altered. However, the addition of fish oil considerably improved the hepatocyte function following the regeneration process. Furthermore, this work has successfully established a model for studying hepatotoxic-induced cell regeneration in vitro and has shown the protective role of HGF and fish oil to injury caused by GalN.

Author contributions

The authors' contributions were as follows: R.R. and S.N.D. jointly designed the experimentation and hypothesis of this work. A.S. conducted experimentation and data acquisition, compiled the initial statistical analysis, and wrote the first draft of the manuscript. R.R. and S.N.D. further refined the manuscript with required statistical analysis and data representation and interpretations of the result. All authors read and approved the final manuscript.

Declaration of Competing interest

Authors declare no conflict of interest.

Acknowledgments

This work has been financially supported by the Department of Biotechnology, Government of India, New Delhi (DBT/PR11635/AAQ/03/448/2008 dated September 9, 2009).

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