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ORIGINAL ARTICLE

Reciprocal regulation of glycogen synthase kinase-3 and β-catenin affects cell proliferation of fructose treated rat hepatocytes

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Abstract

High consumption of fructose might lead to obesity, diabetes, and metabolic syndrome in the long term. Recent studies demonstrated the induction of insulin resistance in the liver tissues by down-regulation of insulin signaling pathway elements. Glycogen synthase kinase-3 (GSK-3), one of the insulin signaling elements, suppresses the β-catenin function that is required for cell proliferation and integrity. This study is designed to demonstrate the effects of fructose on the proliferation of rat hepatocytes and its effects on GSK-3 and β-catenin expression. Accordingly, rat hepatocytes were treated with different concentrations of fructose, and cell proliferation was followed with an xCELLigence real-time cell analysis system. Besides, gene and protein expression levels of GSK-3β and β-catenin were evaluated in fructose-treated cells with qRT-PCR and Western blot, respectively. The results demonstrated proliferative effects of fructose at low doses (0-25-50 mM), but cytotoxic properties are pronounced at higher doses (100-150 mM). The IC_{50} value was calculated as 140±7 mM fructose for Clone-9 cells. Molecular effects of fructose over GSK-3β and β-catenin appeared at gene and protein levels at 100- and 150-mM concentrations at which GSK-3β were suppressed. Conversely, high-dose fructose leads to β-catenin induction as a compensatory mechanism to counteract the antiproliferative effects of fructose at these doses. In conclusion, high-dose fructose-induced cytotoxicity activates a compensatory molecular mechanism involving β-catenin induction which might protect the cells in the long-term fructose exposure.

Keywords: Fructose, cytotoxicity, GSK-3β, β-catenin, gene expression

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Introduction

There has been a significant increase in high fructose consumption due to changes in dietary habits around the world. Several studies demonstrated strong evidence for the induction of metabolic disorders such as obesity, diabetes, and metabolic syndrome with a high-fructose-containing diet [1-3]. However, the direct link between excessive fructose intake and the onset of these pathologies is not well-known yet. Most of the metabolic effects of fructose stem from its rapid utilization in the liver tissues since its metabolism bypasses the main regulatory steps of glycolysis. The sudden increase in hepatic intermediates of glycolysis leads to a shift towards the oxidation reactions [4] which are associated with a state of imbalance between reactive oxygen species production and antioxidant capacity. Oxidative stress contributes to the pathophysiological processes of metabolic diseases mainly affecting the liver tissues and also activates proteins involved in proliferation control [5]. It has been shown that low levels of free radicals might increase the antioxidant enzyme activities and promote cell proliferation but at high levels, they activate several redox-sensitive signal transduction pathways to induce apoptosis and necrosis [6]. Fructose has also been shown to mediate cell proliferation in a dose-dependent manner by regulating many signal transduction elements such as insulin signaling and wnt/β-catenin pathways [7,8].

Recently, we have shown the downregulation of the main insulin signaling elements in various tissues with a high-fructose-containing diet [9,10]. Especially, IRS1/PI3K/AKT downstream signaling was repressed with high-fructose. One of the targets of AKT includes glycogen synthase kinase-3 [GSK-3β] which regulates insulin-dependent glycogen synthesis. GSK-3β was also reported to provide a regulatory function over β-catenin protein [11] since it phosphorylates β-catenin for its ubiquitination and degradation through proteasomes [12,13]. β-catenin is the key effector to trigger transcription of specific genes functioning in the promotion of cell proliferation, migration, and invasion [14].

Herein, we hypothesized that fructose could modulate rat hepatocyte cell [Clone-9] proliferation through a mechanism including GSK-3β and β-catenin function. It is, therefore, of interest to investigate whether fructose regulates cell proliferation via insulin signaling intermediate GSK-3β and β-catenin function which might provide new strategies in regulating hepatoma cell growth. To examine the connection between these signaling elements and proliferation reaction, we evaluated the cell growth inhibitory potential of fructose in a dose-dependent manner by a real-time cell analysis system and determined the gene and protein expression levels of GSK-3β and β-catenin.

Materials and Methods

Cell culture

Clone 9 cells, healthy rat liver cells showing normal physiological features were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as recommended using F12K medium (Kaighn's Modification of Ham's F-12 Medium, ATCC® 30-2004™, ATTC, Wesel, Germany) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml). The growth of the cells was continued in a 37ºC incubator (Sanyo MCO 17AIC, USA), providing 95% humidity and 5% CO_2 until they reached 90% confluency. Then, the cells that were removed with trypsin/EDTA solution were subcultured to the new growth media.

Determination of cell proliferation with real-time cell analysis

The effects of fructose on Clone-9 cell viability and the rate of proliferation were analyzed using the real-time cell analysis system (xCELLigence RTCA S16, ACEA Biosciences, USA). Accordingly, Clone-9 cells ($1x10^{+4}$ cells/100 µl F-12K medium) were seeded into the wells of gold-plated 16-well E-plates of the xCELLigence system and treated with different amounts (25-250 mM) of fructose, prepared in the growth medium, at the cell growth was in log phase. The real-time growth dynamics of the cells were measured instantaneously for 72-hours.

Determination of protein expressions of GSK-3β and β-Catenin by Western blot

After determining the IC_{50} value of fructose, Clone-9 cells were treated with different amounts of fructose (0-25-50-100 and 150 mM) for 48-hours in the T25 cell culture plate. After that, cells were scraped from the surface with a cell scraper (Sarstedt, USA) and collected in 100 µL of homogenization medium containing 50 mM Tris, 150 mM sodium chloride, 1 mM EDTA, 1% (w/w) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride, pH:7.4. Cell suspensions were homogenized with Tissue Ruptor™ homogenizer (Qiagen, USA) on ice for one minute and then ultrasonicated (Sonopuls, Bandelin, Germany) for 30 seconds to ensure complete cell disruption. Homogenates were then centrifuged at 1,200g for 10 min at +4ºC and the supernatants were removed for protein determination with the Lowry method [15]. Fifty µg of total proteins were separated with 12% polyacrylamide gel by electrophoresis and transferred onto the polyvinylidene fluoride membranes using a semi-dry electroblotting apparatus (TransBlot Turbo, BioRad, Munich, Germany) after which the membranes were blocked with 5% bovine serum albumin for 1 h. Then, the blots were incubated with primary antibodies of GSK-3α/β (anti-GSK3 α/β rabbit IgG 1:1000, Cell Signaling Technology, Danvers, MA, USA), β-Catenin (anti-β-Catenin rabbit IgG, 1:1000, Abcam, Paris, France) overnight at +4ºC. As an internal control, GAPDH proteins were also labeled with GAPDH (anti-GAPDH Rabbit IgG, 1:5000, Abcam, Paris, France) antibody for the data normalization. After the washing step, horseradish peroxidase (HRP) conjugated secondary antibodies (Goat Anti-rabbit IgG–HRP conjugate; 1:5000, Santa Cruz, Dallas, TX, USA) was applied for 1 h, and then the blots were treated with Clarity™ Western ECL (Bio-Rad Laboratories, Hercules, CA, USA) substrate solution for 5 min. Images of the blots were gained using the ChemiDoc™ MP Chemiluminescence detection system (Bio-Rad Laboratories, Hercules, CA, USA) equipped with a CCD camera. The relative expression of proteins with respect to GAPDH was calculated using the Image J software [16].

Determination of gene expressions with quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from fructose treated Clone-9 cells using miRVANA miRNA isolation kit (Thermo Scientific, Waltham, MA, USA) as described according to the manufacturer's total RNA isolation protocol. After isolation, the amount and the quality of the total RNAs were determined by spectrophotometry at 260/280nm and Qubit 4.0 System (Invitrogen, Carlsbad, CA, USA). Then, 1 µg of total RNA was reverse transcribed to cDNA using a commercial first-strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Expression levels of *gsk-3β* and *β-catenin* were determined with a real-time quantitative polymerase chain reaction (qRT-PCR, LightCycler480 II, Roche, Basel, Switzerland). Accordingly, 1 µl cDNA, 5 µl 2X SYBR Green Master Mix (Roche, Basel, Switzerland) and 2 µl primer pairs of each (Table 1) at 0.5 µM concentrations in a final volume of 10 µl were mixed. qRT-PCR was performed as follows: initial denaturation at 95ºC for 10 min, denaturation at 95ºC for 10 s, annealing at 58ºC for 15 s, and extension at 72ºC for 15 s with 40 repeated thermal cycles measuring the green fluorescence at the end of each extension step. All reactions were performed in triplicates, and the specificity of PCR products was confirmed using melt analysis. The relative expression of genes with respect to internal control glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was calculated with the efficiency corrected advance relative quantification tool provided by the LightCycler-II 480 SW 1.5.1 software (Roche, Basel, Switzerland).

Statistical analysis

Gene and protein expressions of *gsk3β* and *β-catenin* in Clone-9 cells were normalized to corresponding housekeeping gene and protein expression of GAPDH. Data are expressed as the fold-change value over the untreated cells and represented as mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way ANOVA followed by an

appropriate posthoc test (Tukey). Comparisons giving *P*-values less than 0.05 were accepted as statistically significant. All analyses were done using SPSS 21.0 software (IBM Corporation, Armonk, NY, USA).

Results

Time and dose-dependent effects of fructose on Clone-9 cell proliferation

In this study, real-time cell monitoring was performed for Clone-9 cells which were treated with various amounts of fructose to demonstrate time and dose-dependent effects. The xCELLigence® RTCA instrument (Roche, USA) was utilized for this purpose which uses non-invasive electrical impedance monitoring to determine cell proliferation in a real-time and label-free manner. Gold-coated plate wells of the instrument measures the adherent cell amount which is then converted to a cell viability index with its software. Real-time monitoring of cell index provides a dynamic view of cell health which is very valuable especially as the time of incubation is very important. Hence, Clone-9 cells were seeded into the wells of gold-plated 16-well E-plates of the xCELLigence system and treated with different amounts of fructose (25-250 mM) at the log phase and real-time growth dynamics were measured instantaneously over 72-hours.

According to the results, low-dose fructose (25 and 50 mM) administration did not have a significant effect on the growth of Clone-9 cells (Figure 1). Although there was a slight decrease with 50 mM application in the number of cells in a very short time after the application, the cells approached the growth dynamics of the control cells within two hours.

In addition, the cytotoxic effect reaches a much more pronounced level in moderate fructose applications (100 and 125 mM) (Figure 2). It was determined that cell viability was halved within two hours of fructose application, but the number of viable cells approached the control samples 24-hours after the fructose application.

The effects of high-dose fructose (150, 200 and 250 mM) on Clone-9 cell proliferation were much more pronounced and dramatic (Figure 3). Accordingly, 150 mM fructose decreased cell

Table 1. Primer sequences of *gsk3β*, *β-catenin* and internal standard *gapdh* used for the mRNA expression determination with qRT-PCR

Gene	**Forward Primer Sequence (5'→3')**	**Reverse Primer Sequence (5'→3')**
β-catenin	TGTGGTTAAACTCCTGCACC	AAGCAGCTGAACTAGTCGTG
gsk3β	TATGGTCTGCAGGCTGTGTG	CCGAAAGACCTTCGTCCAA
gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT

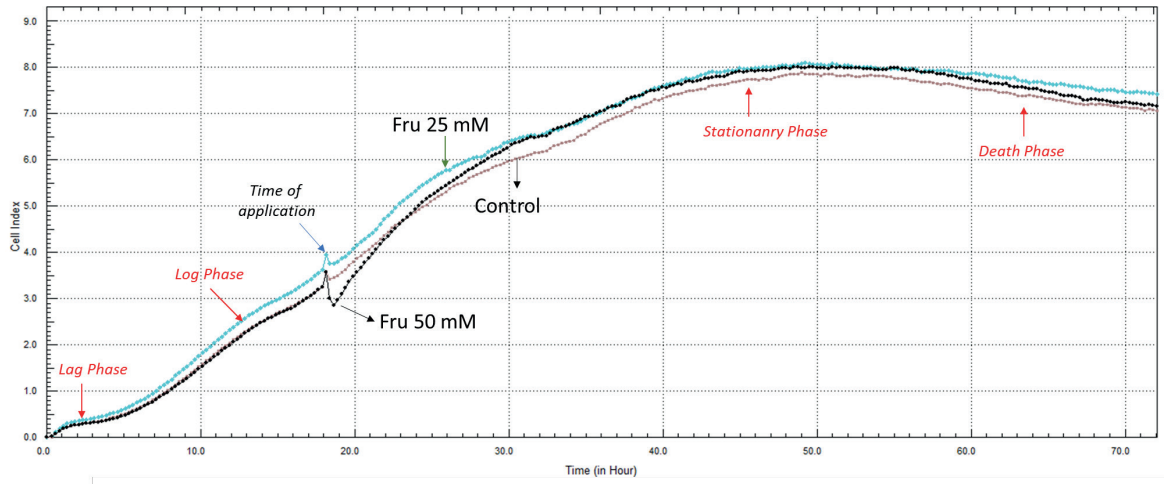

Figure 1. Growth plots of Clone-9 cells treated with low dose (25- and 50- mM) fructose. Lines are shown as the mean of two biological replicates

viability by 80% within two hours. However, unlike the medium-dose applications, cell viability could not reach the behavior of control cells in the later stages. Application of 200- and 250-mM fructose literally ended cell viability, since no living cells were left at these doses, cell recovery did not occur in the later stages.

As can be seen from the real-time cell analysis, the cytotoxic effects of fructose on Clone-9 cells were most evident within two hours after fructose administration. In this context, the IC50 value for the first two hours was calculated as 140±7 mM fructose.

Regulation of GSK-3β and β-Catenin expression with different doses of fructose

In this study, Clone-9 cells were treated with different doses of fructose (0, 25, 50, 100 and 150 mM), and qPCR and Western blot analysis were conducted to reveal GSK-3β and β-catenin expressions. Figure 4 demonstrates the changes in GSK-3β levels and Figure 5 demonstrates the alterations of β-catenin at both gene and protein expression levels, respectively.

According to the results, low levels of fructose (25 mM) did not change the gene and protein expression levels of GSK-3β which is also in parallel with the insignificant proliferative effects at this dose. Fructose exerted slight

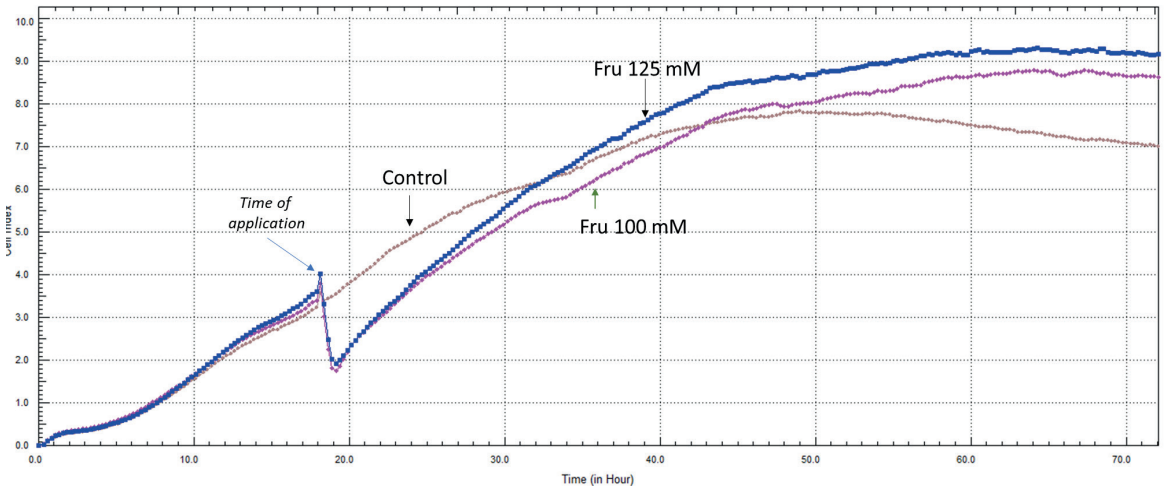

Figure 2. Growth plots of Clone-9 cells treated with medium dose (100- and 125- mM) fructose. Lines are shown as the mean of two biological replicates

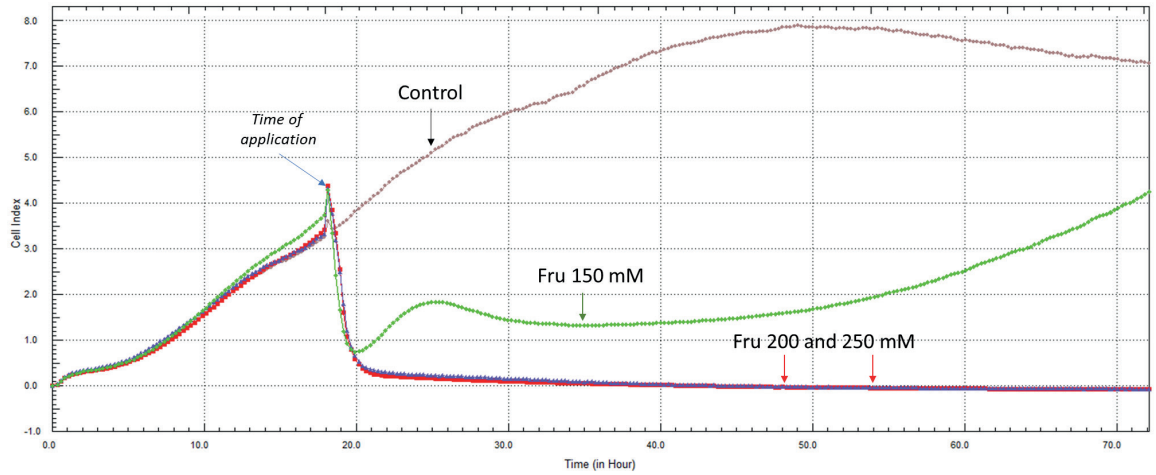

Figure 3. Growth plots of Clone-9 cells treated with high dose (150- , 200- and 250- mM) fructose. Lines are shown as the mean of two biological replicates

cytotoxic effects which also suppressed GSK-3β at both gene and protein levels by 20% at 50 mM concentration. In line with increased cytotoxicity, 100- and 150-mM concentrations further reduced GSK-3β expressions of Clone-9 cells (Figure 4B and 4C).

Considering β-catenin levels, completely inverse associations were observed as compared with GSK-3β in such a way that an increase in fructose concentration augmented the β-catenin levels at both gene and protein levels. Even though low levels of fructose did not modify β-catenin expression significantly, high-fructose (100- and 150- mM) induced β-catenin levels in a dose-dependent manner (Figure 5B and 5C).

Discussion

Fructose is an important ingredient of soft drinks and fast foods, and its increased consumption might contribute to the high prevalence of metabolic disorders worldwide. The metabolic effects of fructose have been mainly well-characterized by insulin resistance, hypertriglyceridemia, and fatty liver [17]. However, it is not well known if there are dose-dependent cytotoxic effects of fructose over hepatic cells and the differential expression of β-catenin and GSK-3β proteins could have a role in proliferation control. Therefore, this study assessed the potential effects of fructose on the rate of hepatocyte proliferation in conjunction with the expression of the antiproliferative β-catenin protein that is thought to be regulated by GSK-3β.

Glycogen synthase kinase 3 is a constitutively active protein kinase [serine/threonine] mainly controlled by IRS1/PI3K/AKT signaling, and phosphorylation by AKT inhibits its activity [18]. In addition to initially reported substrate glycogen synthase, it also phosphorylates several

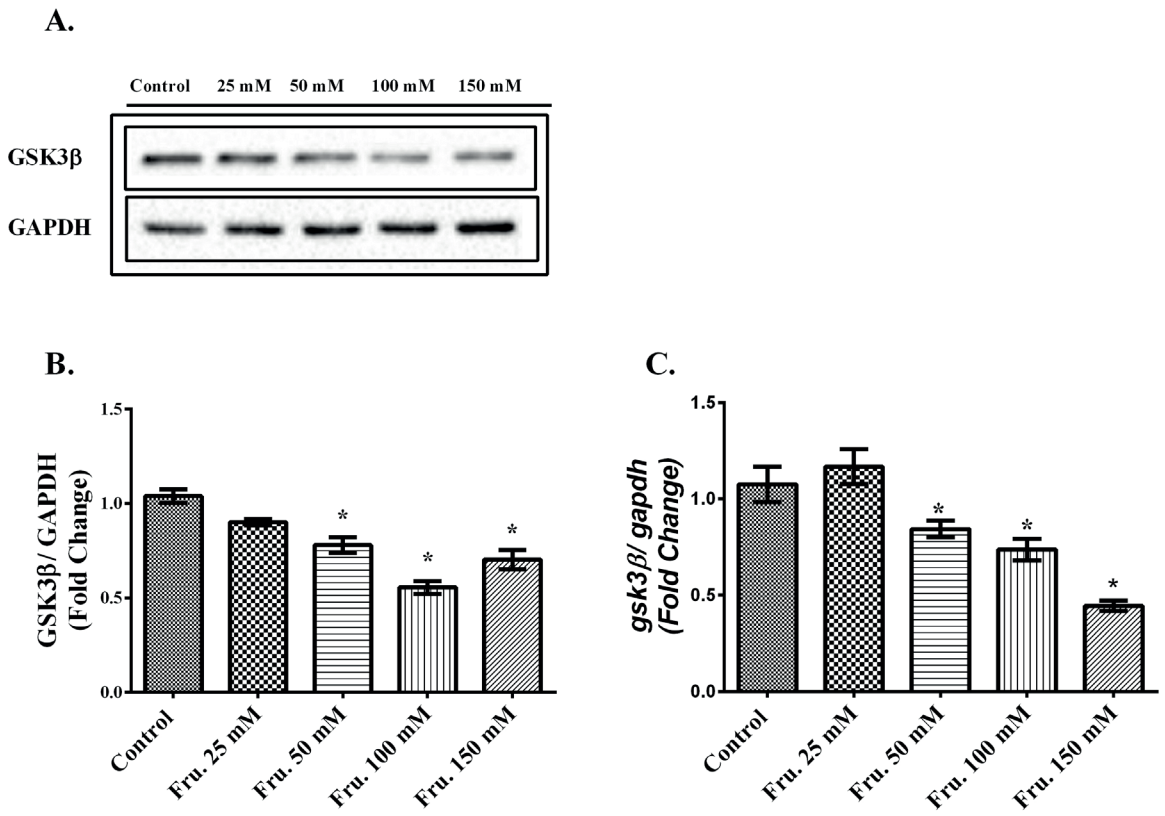

Figure 4. Representative Western blot bands of GSK-3β proteins in fructose-treated Clone-9 cells (A) and its relative protein (B) and mRNA expression levels (C). Data were normalized with corresponding GAPDH and *gapdh* expression and given as fold-change over non-treated Clone-9 cells. Values are given as mean ± SEM and the number of biological replicates was greater or equal to three (n ≥ 3). *$P < 0.05$, significant from the control group. Fru: Fructose

upstream and downstream components of the IRS1/PI3K/AKT signaling network to provide feedback control over its function [19]. Besides, more than a hundred other GSK-3 substrates have also been identified, the phosphorylation of which may result in the suppression of their activity. In mammals, GSK-3 has two closely related isoforms GSK-3α and GSK-3β which are encoded by two distinct genes but catalyze similar substrates. Their activity is found to be modulated by numerous extrinsic factors such as food components, food metabolic products, lipids, and drugs [20], and also it has prominent roles in the NF-κB and the WNT/β-catenin pathways which are often aberrantly regulated in cancer, tumor progression and uncontrolled cell proliferation [21]. Thus, the modulation of GSK-3 activity via natural compounds is still a promising target to various therapeutic approaches for proliferation control.

One of the well-defined targets for GSK-3β is the β-catenin protein having roles in cell proliferation and transition from epithelial to mesenchymal form that is critical for cancer development and metastasis. Activated GSK-3β phosphorylates β-catenin leading to its ubiquitination and degradation which suppresses the transcription of crucial genes in cells [22]. The β-catenin regulates cell adhesion, proliferation, signal transduction, metabolism, and other biological processes and its dysregulation could be associated with some metabolic diseases such as obesity, diabetes, nonalcoholic fatty liver disease, and metabolic syndrome [23]. Together with GSK-3β, β-catenin could play pivotal roles in transmitting various extracellular and intracellular regulatory signals which are critical to cell growth, survival, regeneration, or cell death.

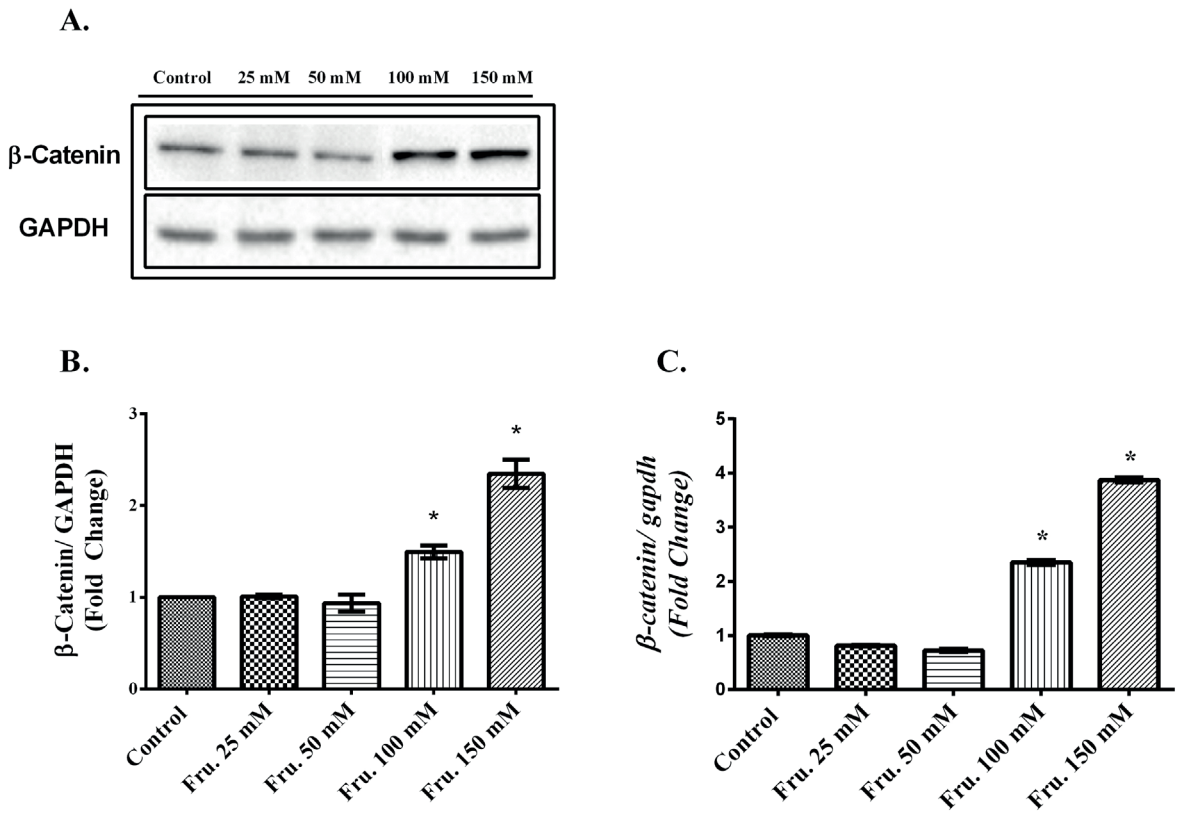

Figure 5. Representative Western blot bands of β-catenin proteins in fructose-treated Clone-9 cells (A) and its relative protein (B) and mRNA expression levels (C). Data were normalized with corresponding GAPDH and *gapdh* expression and given as fold-change over non-treated Clone-9 cells. Values are given as mean ± SEM and the number of biological replicates was greater or equal to three (n ≥ 3). *P < 0.05, significant from the control group. Fru: Fructose

Previously, downregulation in hepatic gene and protein expressions of insulin receptor downstream molecules has been demonstrated in the liver of high-fructose-fed rats [24]. In rat models of fructose-induced metabolic syndrome, we recently showed that hepatic insulin signaling appears to be suppressed due to reduced expression of IRS-1/2 and AKT [25] that would eventually modulate GSK-3 in the long run. Herein, this study demonstrated the proliferative role of low-dose fructose [25 mM] on hepatic cells, but high-doses over 100 mM concentration remarkably induced the cytotoxicity probably due to impaired insulin signaling in association with reduced GSK-3β levels which would eventually induce a compensatory proliferative response by activated β-catenin levels. Thus, this compensatory response of β-catenin might maintain hepatocellular integrity by triggering regeneration rather than promoting apoptosis at high-fructose levels.

Conclusion

In conclusion, the results presented in this study show that hepatic Clone-9 cells respond differently to fructose applications. While low doses and low treatment time augments the cell proliferation, high concentrations above 100 mM significantly reduced cell viability within two hours of incubation. Thus, effective, and non-toxic concentrations of fructose and the duration of treatment need to be carefully titrated for at least in vitro studies. Various molecular and genetic approaches should also be employed to dissect out the molecular mechanisms responsible for fructose-induced cytotoxicity. Especially the functional relationships between GSK-3β and β-catenin should be revealed to better understand the pharmacological activation of the canonical β-catenin pathway which provides proliferation control. Our data offer strong evidence of an interaction between GSK-3β and β-catenin, and we propose that the GSK-3β/β-catenin axis is essential to maintain cell survival in hepatocytes under high-fructose treatments.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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