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Comparative Hepatoprotective Effects of Dapagliflozin and Silymarin Against Cyclophosphamide-Induced Liver Injury in Rats: Involvement of Nrf2/HO-1, HNF4α, and HNF6 Pathways

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Abstract

Cyclophosphamide, a common immunosuppressive and chemotherapeutic drug, can cause hepatotoxicity by inducing inflammation and oxidative stress. Dapagliflozin and other sodium-glucose cotransporter-2 inhibitors have anti-inflammatory and antioxidant properties; and, silymarin is a natural chemical derived from the seeds of the milk thistle plant (*Silybum marianum*), which is best known for its antioxidant, anti-inflammatory, and liver protective effects. Objective: By measuring oxidative stress, inflammation, and liver regeneration parameters, special an emphasis on the Nrf2/HO-1 pathway and hepatocyte nuclear factors (HNF4α and HNF6), dapagliflozin's hepatoprotective effects in comparison with silymarin on cyclophosphamide-induced liver injury was evaluated.

Fifty Wistar-Albino rats were randomly allocated into 5 groups (10 animals each): Group I- Negative Control/Distilled water, Group II-(Vehicle Control):Rats orally administrated 2% sodium carboxymethyl cellulose daily for 10 successive days, Group III-(Induction/Cyclophosphamide):Rats intraperitoneally injected with 30mg/kg cyclophosphamide daily for 10 days in a row, Group IV-(Cyclophosphamide+Dapagliflozin):Rats orally received dapagliflozin (3 mg/kg) alongside 30 mg/kg cyclophosphamide intraperitoneally for 10 days, Group V-(Cyclophosphamide+Silymarin):Rats orally received silymarin (200 mg/kg) and 30 mg/kg cyclophosphamide intraperitoneally for 10 consecutive days. At day 11, liver tissue of each rat/group was examined for Nrf2, HO-1, HNF4α, and HNF6 levels.

Cyclophosphamide caused high significant ($p<0.0001$) decrease in mRNA Nrf2, HO-1, HNF4α, and HNF6 levels. In Group IV, dapagliflozin caused the high significant increase ($p<0.0001$) in mRNA of each of the Nrf2 level [(2.182 ± 0.540) *versus* cyclophosphamide (0.244 ± 0.096)], HO-1 [(2.051 ± 0.533) *versus* cyclophosphamide (0.487 ± 0.178)], the mRNA HNF4α [(2135 ± 297.9) *versus* cyclophosphamide (229.5 ± 50.21)], and the mRNA HNF6 level [(3.635 ± 0.610) *versus* cyclophosphamide (0.515 ± 0.255); moreover, in Group V, silymarin caused the high significant ($p<0.0001$) increase each of mRNA of each of Nrf2, HO-1, and HNF4α with very high significant ($p<0.001$) elevate in the mRNA HNF6 level each compared to Group III/cyclophosphamide. Dapagliflozin showing superior upregulation in each of Nrf2, HNF4α, and HNF6 with non-significant differences ($p>0.05$) compared to silymarin.

Dapagliflozin ameliorates cyclophosphamide-induced hepatotoxicity by activating the Nrf2/HO-1 pathway and restoring HNF4α and HNF6 expression, demonstrating comparable or superior efficacy to silymarin. These results underscore dapagliflozin's potential as supplementary treatment to alleviate chemotherapy-induced hepatotoxicity.

Keywords: Cyclophosphamide, Dapagliflozin, Oxidative stress, Nrf2/Ho-1, Silymarin.

Introduction

Cyclophosphamide (Cpd), the oxazaphosphorine derivative of nitrogen mustard, is a prevalently-employed chemotherapy drug for the management of various cancers, including several forms of leukemia, lymphoma, and numerous solid tumors [1, 2]. In addition to its anticancer properties, it also functions as an immunosuppressant and is employed in the management of several non-neoplastic conditions such as systemic lupus erythematosus and rheumatoid arthritis [3, 4].

Though, its clinical application is oftentimes restricted due to a range of adverse effects, including hemorrhagic cystitis, alopecia, and multi-

organ toxicities affecting the heart, testes, and kidneys [5, 6]. Moreover, its therapeutic dose is known to persuade significant hepatotoxicity, which poses an additional challenge to its clinical utility [7, 8].

Upon administration, such drug is metabolized into two key products: phosphoramide mustard and acrolein [9]. The first product exerted its antineoplastic activity by adding an alkyl group to DNA at the N7 position of guanine, thereby inducing tumor cell death. In contrast, the second product (acrolein) is a cytotoxic byproduct that contributes to oxidative stress (OS) *via* lipid peroxidation and the overproduction of reactive oxygen species (ROS) [10, 11]. This hepatotoxic potential substantially-curtail the long-term clinical utility of Cpd and necessitates the exploration of effective hepatoprotective agents to counteract its adverse effects [12].

Sodium–glucose cotransporter 2 inhibitors (SGLT2i) fundamentally-derived for the treatment of type 2 diabetes mellitus (T2DM); and, fulfilled considerable attention for their pleiotropic usefulness beyond glycemic control. Among these, dapagliflozin has demonstrated talented therapeutic qualities. Apart from its ability to reduce blood glucose, it has been demonstrated to minimize OS, inflammation, and apoptosis in various disease models [13]. Furthermore, such drug offers additional clinical benefits, including weight reduction, cardiovascular (CV) protection, and improvement of metabolic parameters [14]. Several preclinical studies have reported that dapagliflozin activates the Nrf2/HO-1 pathway and allays oxidative damage and apoptosis in multiple models of tissue injury, including hepatic and renal insults [15, 16].

Silymarin , a flavonolignan complex derived from the seeds of *Silybum marianum* (milk thistle), a plant belonging to the Asteraceae family, has been extensively used in traditional and complementary medicine due to its well established hepatoprotective properties [17, 18]. Moreover, it is acknowledged for its powerful antioxidant, anti-inflammatory, and membrane stabilizing effects, and has served as a reference compound in numerous experimental studies exploring hepatoprotection against various toxins, including cyclophosphamide [19, 20].

Given their complementary antioxidant and cytoprotective profiles, both dapagliflozin and silymarin were selected for a comparative evaluation against cyclophosphamide-induced liver injury.

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a master transcription factor that maintain cellular redox balance by stimulate the expression of antioxidant defense related genes ([21, 22]. Besides, the Nrf2 activation has been associated with protective sequels in several *in vivo* and *in vitro* models of tissue injury, including nephrotoxicity, thereby suggesting its therapeutic potential in OS-correlated diseases [23]; furthermore, upon activation, the Nrf2 promotes the transcription of several cytoprotective genes, initiating a cellular defense mechanism that enhances adaptation and survival under oxidative or toxic complaints.

Hepatocyte nuclear factors (HNFs) are a group of mutative un-related transcription factors that are in essence expressed in the liver [24]. These factors mount the expression of plethora of hepatic genes related in protein synthesis, glucose metabolism, lipid homeostasis, and detoxification processes [25]. Explicitly, HNF4α has a key part in liver organogenesis during embryonic development and also contributes to the differentiation and function of renal and other epithelial tissues [26–28].

The purpose of this study was to assess and compare the possible hepatoprotective effects of dapagliflozin and silymarin against cyclophosphamide-induced liver injury in rats, with a specific emphasis on the Nrf2/HO-1, HNF4α, and HNF6 signaling pathways.

The experimental protocol was approved by the Local Research Ethics Committee of the College of Pharmacy, University of Baghdad, Iraq. Approval Number: REC062463A, dated March 2, 2024.

Materials and Methods

Animals

Fifty (50) evidently-healthy Wistar-Albino rats aged 5 to 6 weeks old, weighing 150-180g were acquired from the Animal House of the College of Pharmacy/University of Baghdad. The animals were retained in controlled settings, including a 12-hour light/dark cycle; and, a temperature of $24 \pm 2\,^{\circ}C$; and had *ad libitum* access to standard pellet feed and water for the duration of the experiment. Moreover, all experimental rats were kept watch on daily, including weekends and holidays, by trained personnel to assess any signs of distress or morbidity. The endpoints were predefined in accordance with institutional and international welfare guidelines and included any of the following: weight loss of $\geq 20\%$ from baseline or compared to matched controls, severe lethargy, inability to ambulate or reach food/water, persistent recumbency, hunched posture, rough or stained fur, or abnormal/unresponsive behavior. Animals meeting these criteria were euthanized promptly according to the approved Institutional Animal Care and Use Committee (IACUC) protocol to minimize suffering [29, 30].

Materials

The carboxymethylcellulose sodium salt was purchased from HiMedia Laboratories Pvt., India. Cyclophosphamide was obtained from Baxter, Germany, and dapagliflozin was purchased from Sigma-Aldrich, USA. Silymarin was procured from MACKLIN, China. HNF4α levels were measured using a colorimetric method via an ELISA kit obtained from BNTIBODIES ONLINE, Bulgaria.

The GoScript™ Reverse Transcription System (Cat. No. A5000) and GoTaq® qPCR Master Mix (Cat. No. A6001) were purchased from Promega, USA. PCR primers for Nrf2 and HO-1 quantification were supplied by Macrogen, Inc., South Korea. Western blot analysis for HNF6 was performed using a detection kit (Cat. No. E-IRR304A), SDS-PAGE Gel Kit, and antibodies, all obtained from Elabscience, USA

Experimental design

Rats were chosen haphazardly and allotted into 5 groups of 10 rats each (Blinding was applied during outcome assessments to minimize observer bias) as follows:

Group I (Negative Control): Received distilled water (DW) and a normal diet orally.

Group II (Vehicle Control): Received 2% aqueous sodium carboxymethylcellulose (Na^+-CMC) via oral gavage daily for 10 days [31].

Group III (Cyclophosphamide (Cpd)/Induction): Rats injected intraperitoneally (IP) with 30mg/kg Cpd daily for 10 consecutive days [32].

Group IV (Cyclophosphamide+ Dapagliflozin): Rats orally-received 3mg/kg dapagliflozin dissolved in Na^+-CMC alongside 30 mg/kg Cpd IP for 10 days [33, 34]. The dose of dapagliflozin was selected based on previous studies demonstrating antioxidant, anti-inflammatory, and organ protective effects at this concentration in rodent models. The 10-day duration ensured sufficient systemic exposure to modulate relevant signaling pathways [35].

Group V (Cyclophosphamide + Silymarin): Rats orally-received silymarin (200mg/kg) dissolved in Na^+-CMC and 30 mg/kg Cpd IP for 10 consecutive days. The dose of silymarin was selected based on prior hepatoprotective study in rodents by Wang, et al. (2017) who showed that, 200mg/kg provided superior hepatoprotection against triptolide-induced hepatotoxicity via anti-inflammatory, antioxidant, and anti-apoptotic processes [36].

Twenty-four hours after the utmost dose (i.e., at day 11), rats were anaesthetized by diethyl ether, followed by euthanasia by cervical dislocation. Livers of experimental rats/Group were immediately-excised and washed using ice-cold phosphate-buffered saline (PBS; pH 7.4, 4 ºC) to prevent degradation [37].

Although a formal power analysis was not performed, a sample size of 10 rats per group was chosen based on precedent in similar experimental models to ensure sufficient statistical power [38].

Determination of Nrf2/HO-1 by quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from rat liver tissue using Trizol reagent, following the manufacturer's instructions [39]. The RNA concentration and purity were determined spectrophotometrically. Subsequently, 2µg of total RNA was reverse-transcribed into complementary DNA (cDNA), which was then kept at −80 °C for further PCR analysis. Quantitative real-time PCR amplification was performed using a Bio-Rad Real-Time PCR system. Beta-actin was used as the internal housekeeping gene to normalize mRNA expression levels (39). The 2^−ΔΔCT method was used to calculate relative gene expression. Detailed primer sequences and reaction conditions are summarized in Table 1.

Table 1 The primer sequences used in the qPCR assay

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Nrf2	GGAGCAATTCAACGAAGCTC	TAAGTGGCCAAGTCTTGCT
HO-1	CACGCATATACCGCTACCT	TTCATGCGAGCACGATAGAG
Beta actin	CTATCGGCAATGAGCGGTTCC	TGTGTTGGCATAGAGGTCTTTACG

Determination of HNF4α level by ELISA

After euthanasia, a piece of liver tissue of each rat/group was excised and rinsed with ice - cold [(PBS, pH 7.4). Approximately 1g of liver tissue was homogenized in 9mL of PBS using a tissue homogenizer. The resulting homogenate was then centrifuged at 4 °C for 10 minutes. The supernatant was carefully collected and used for the quantification of hepatocyte nuclear factor 4 alpha (HNF4α) levels *via* ELISA, according to the manufacturer's instructions [40].

Determination of HNF 6 by Western blot (W.B) analysis

A section of liver tissue (approximately 0.3g) was homogenized in 1 mL of Thermo Scientific® RIPA lysis and extraction buffer supplemented with 10 µL of sodium orthovanadate (Na_3VO_4) and 10 µL of phenylmethylsulfonyl fluoride (PMSF) to inhibit phosphatase and protease activity, respectively. The homogenate was incubated on ice, followed by centrifugation to obtain the protein rich supernatant.

Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Elabscience, USA), according to the manufacturer's protocol.

For W.B analysis, 10µL of each protein sample combined with sodium dodecyl sulfate (SDS) loading buffer was loaded into the wells of a precast SDS-PAGE gel. After electrophoretic separation, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in TBST buffer for 90 minutes at room temperature with gentle shaking to reduce non-specific binding. It was then incubated overnight at 4 °C with primary antibodies targeting hepatocyte nuclear factor 6 (HNF6) and β-actin (as the internal loading control). On the following day, the membranes were incubated for 60 minutes at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies (dilution 1:10,000). An enhanced chemiluminescence (ECL) detection system was used to visualize protein bands, and signal intensity was captured using the ChemiDoc MP Imaging System. ImageJ was used to software quantify the band intensity [39].

Statistical analysis

The mean ± standard deviation (SD) is used to display all data. A one-way analysis of variance (ANOVA), followed by Tukey's post hoc test were used for statistical comparisons between groups.

(GraphPad Prism software Inc., USA, version 9.5.1) was conducted for analysis. A p-value <0.05 was considered statistically-significant. The levels of significance were represented as follows:

*** p<0.0001 Very high significant.

** p< 0.01 (High significant).

* p<0.05 Significant.

ns = Non-significant (p > 0.05)[41] .

Results and Discussion

1. Effects on the mRNA Nuclear factor erythroid 2-related factor 2 (Nrf2) Level

There were non-statistically significant (p>0.05) (p = 0.2986) difference in the hepatic mRNA Nrf2 levels between rats of Group II (vehicle control 2% Na^+-CMC) (1.741 ± 0.7131) and the negative control/Group I rats (1.557 ± 0.4195), as shown in Figure 1. Furthermore, there was a very high significant (p<0.0001) (p = 0.00004) decrease in the mRNA Nrf2 level in the cyclophosphamide-treated/Group III (0.2447 ± 0.0969) compared to both Group I (1.557 ± 0.4195) and Group II rats (1.741 ± 0.7131). Moreover, Figure 1 also showed that in rats of Group IV/(Dapagliflozin treated), there was a very high significant (p<0.0001) (p = 0.00002) increase in the mRNA Nrf2 level (2.182 ± 0.5404) compared to rats of Group III (0.2447 ± 0.0969). Likewise, in Group V rats treated with silymarin, there was a very high significant (p<0.0001) (p = 0.00003) increase in the mRNA Nrf2 level (1.431 ± 0.2807) compared to Group III (0.2447 ± 0.0969). But there was a high significant (p<0.01) (p = 0.0008) decrease in the mRNA Nrf2 level in rats of Group V (1.431 ± 0.2807) compared to Group IV (2.182 ± 0.5404) Figure1.

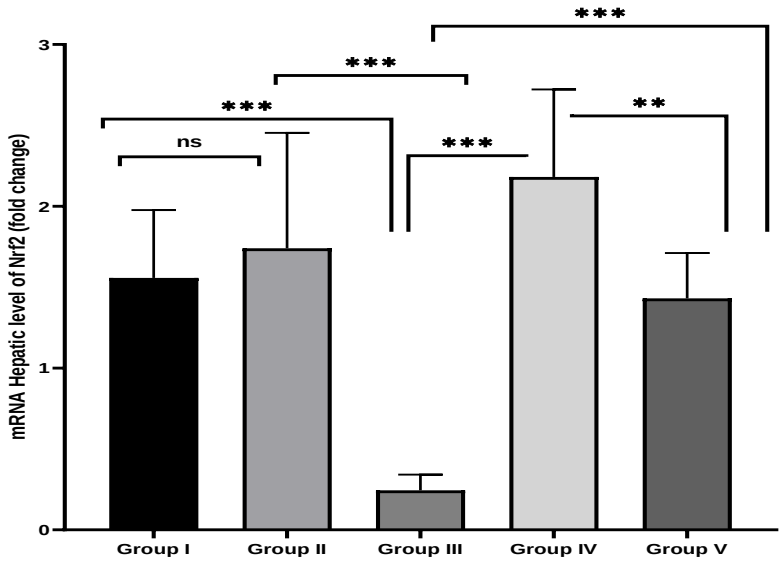

Figure 1. Effect of Dapagliflozin and Silymarin on Nuclear factor erythroid 2-related factor 2 expression in Cpd-induced hepatotoxicity. Data are expressed as Mean±SD. *: The high Significant (P<0.0001), **: Very High Significant (P<0.01), ns: Non-Significant (P>0.05). N=10.**

2- Effects on the mRNA Heme oxygenase (HO) Level

As shown in Figure 2, there were non-significant ($p>0.05$) ($p = 0.4217$) differences in the hepatic mRNA HO-1 levels in Group II (vehicle-control) (1.452 ± 0.5061) in comparison with the negative control Group I rats (1.483 ± 0.146). In contrast, in the Cpd/ Group III rats, there was a very high significant ($p<0.0001$) decrease in the mRNA HO-1 level (0.4878 ±0.1785) compared to each of Group I ($p = 0.00001$) and Group II ($p = 0.00003$), rats respectively, (1.483 ± 0.146) and (1.452 ± 0.5061).

Besides, in rats of Group IV/Dapagliflozin treated, there was a very high significant ($p<0.0001$) ($p = 0.00005$) increase in the mRNA HO-1 level (2.051 ± 0.5333) compared to Group III rats (0.4878 ±0.1785). Similarly, in rats of Group V, Silymarin produced a very high significant ($p<0.0001$) ($p = 0.00006$) increase in the mRNA HO-1 level (1.857 ± 0.6458) compared to the induction/Group III rats (0.4878 ±0.1785) Figure 2. Additionally, such Figure also demonstrate that, there was a non-significant ($p>0.05$) ($p = 0.3591$) difference in the mRNA HO-1 in Group IV hepatic rats (2.051 ± 0.5333) compared to rats of Group V (1.857 ± 0.6458).

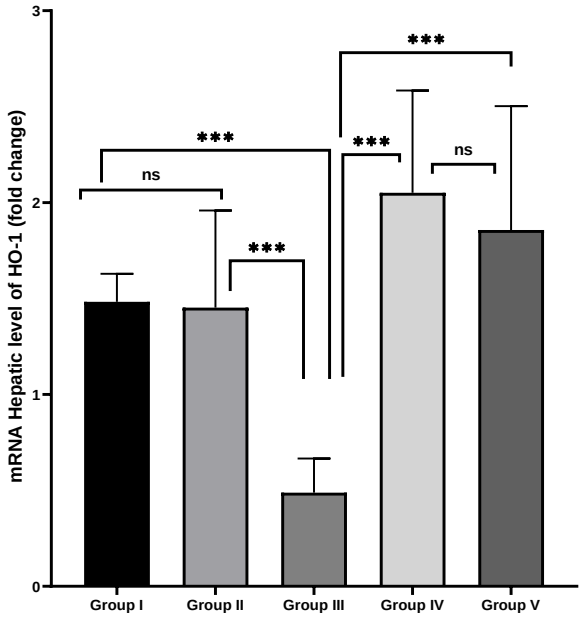

Figure 2. Effect of Dapagliflozin and Silymarin on Heme oxygenase (HO) expression in Cpd-induced hepatotoxicity. Data are expressed as Mean±SD. *: The high Significant (P<;0.0001). **: Very High Significant (P<0.01), ns: Non-Significant (P>0.05). N=10**

3. Effects on Hepatocyte nuclear factor 4alpha (HNF4α)

Figure 3 demonstrate that, there was a high significant ($p<0.01$) ($p = 0.0385$) decrease in the mRNA HNF4α level in rats of Group II (991.3 ± 94.2) compared to Group I rats (1535 ± 380.6). Furthermore, such Figure also showed that, there was a very high significant ($p<0.0001$) ($p = 0.00001$) decrease in the mRNA HNF4α level in rats of Group III (229.5 ± 50.2) compared to Group I rats (1535 ± 380.6); and, there was a high significant ($p<0.01$) in such mRNA level in Group III (229.5 ± 50.2) compared to Group II rats (991.3 ± 94.2). Besides, treatment with dapagliflozin in rats of Group IV, there was a very high significant ($P<0.0001$) ($p = 0.00002$) increase in the mRNA HNF4α level (2135 ± 297.9) compared to Group III rats (229.5 ± 50.2). Similarly, in Group V rats, there was a very high significant ($p<0.0001$) ($p = 0.00006$) increase in the mRNA HNF4α level (1618 ± 593.3) compared to Group III rats (229.5 ± 50.2). Additionally, Figure 3 also revealed that, there was a significant ($p<0.05$) ($p =0.0447$) enhance in the mRNA HNF4α level in Group IV (2135 ± 297.9) compared to rats of Group V (1618 ± 593.3).

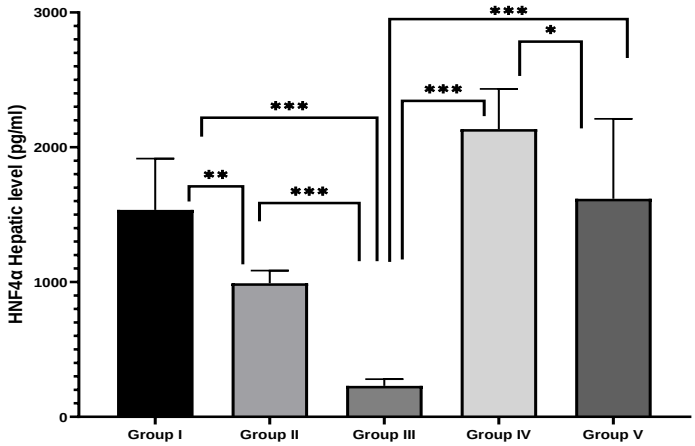

Figure 3. Effect of Dapagliflozin and Silymarin on HNF4*α* Hepatic level in Cpd-induced hepatotoxicity. Data are expressed as Mean±SD. *: The high Significant (P<0.0001). **: Very High Significant (P<0.01), ns: Non-Significant (P>0.05). N=10.**

4. Effects on the Hepatocyte Nuclear Factor 6 (HNF6) Level

Figures 4A showed the image of the W.B. concerning the hepatic expression of HNF6 and β-actin in rats liver homogenates of the various experimental rats' groups. Besides, in Figure 4B showed that, there was a non-significant ($p>0.05$) ($p = 0.2734$). difference in the HNF6 level in rats of Group II compared to Group I. However, there was a high significant ($p<0.001$) ($p = 0.0003$) reduction in the HNF6 level in Group III rats (0.515 ± 0.2558) compared to rats of Group I (2.5 ± 0.5774). Furthermore, such Figures also showed that, in Group IV rats (Dapagliflozin + Cpd), there was a very high significant ($p<0.0001$) ($p = 0.0006$) elevation in the mRNA HNF6 level (3.635 ± 0.6107) compared to rats of Group III (3.635 ± 0.6107). Similarly, in Group V (Silymarin + Cpd), Figure 4B also showed that, there was a high significant ($p<0.001$) ($p = 0.0009$) increase in the HNF6 level (2.308 ± 0.2196) compared to Group III rats (3.635 ± 0.6107). In addition, there were a significant increase ($p<0.05$) ($p = 0.0482$) in the mRNA HNF6 level in rats of Group IV (3.635 ± 0.610) compared to Group V rats (2.308 ± 0.2196), Figure 4B.

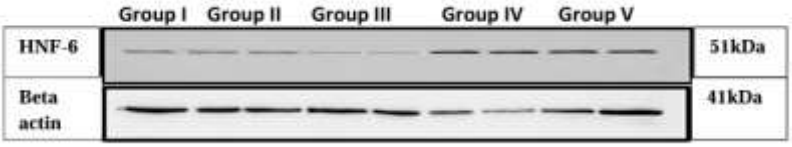

Figure 4A. Western blot images showing hepatic expression of HNF6 and β-actin in different rat liver homogenates groups.

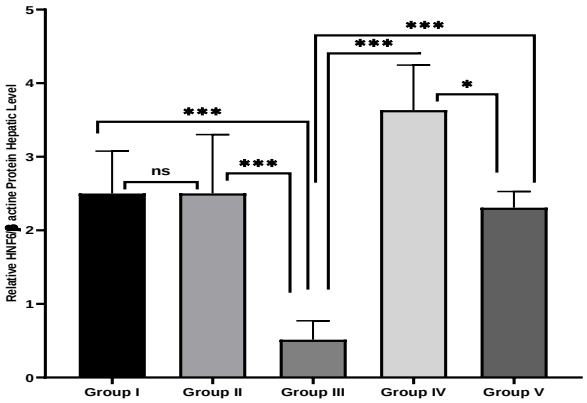

Figure 4B. Level of HNF6 Hepatic level. Data are expressed as Mean±SD. *: The high Significant (P<0.0001). **: Very high Significant (P<0.01), ns: Non-Significant (P>0.05). N=10.**

Discussion

Nuclear factor erythroid 2–related factor 2/ Heme oxygenase-1

The observed suppression of Nrf2 and HO-1 expression in the Cpd-treated rats (Group III) (Figures 1 and 2) underlines the decisive role of OS in the hepatotoxicity induced by such drug. This matches with previous reports that exemplify the involvement of the Nrf2/HO-1 axis as a pivotal component of hepatic antioxidant defense mechanisms [42, 43]. Besides, both dapagliflozin and silymarin significantly-resolved the reduction in these markers, with dapagliflozin exerting a slightly more pronounced effect; and this supremacy, may be attributed to its ability to activate Nrf2 *via* the AMP-activated protein kinase (AMPK) signaling cascade, as supported by earlier studies [44–47]. However, despite these differences, the hepatoprotective profiles of both the dapagliflozin and silymarin appeared broadly comparable in magnitude. Notably, emerging evidence mentioned by researchers suggested the potential synergistic effects when these agents are used concomitantly [46]. The liver protective efficacy of both dapagliflozin and silymarin observed in the current study can be mechanistically explained by their concerted activation of antioxidant defenses, regulation of inflammatory pathways, and modulation of hepatic transcription factors, in line with recent experimental findings of others. Recently, researchers signified that, dapagliflozin upregulates Nrf2 and HO-1 expression, enhancing glutathione activity and superoxide dismutase (SOD) levels, while reducing malondialdehyde (MDA) accumulation and oxidative hepatic injury; and, it suppresses Toll-Like Receptor 4 (TLR4), Tumor Necrosis Factor-Alpha (TNF-α), Interleukin 6 (IL-6), and the PI3K/TGF-β1 pathway, leading to reduction in hepatic inflammation and fibrosis [16]. Additionally, the work of other researchers verified that, dapagliflozin struggled mitochondrial and anti-ferroptotic protection by decreasing H_2O_2 production, stabilizing mitochondrial membrane potential, and activating the AMPK/Sirtuin 1/Peroxisome proliferator-activated receptor gamma co-activator 1-alpha/Forkhead box O1 (AMPK/Sirt1/PGC-1α/FoxO1) signaling axis, whereby magnifying mitochondrial capacity and truncating hepatocyte death [48]. Meanwhile, silymarin activates Nrf2 and prompts antioxidant enzymes; and, its flavonolignans interact with the Keap1-Nrf2 pathway, upregulating antioxidant genes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), Glutamate-Cysteine Ligase (GCL), and HO-1, leading to reduced reactive oxygen species (ROS) and lipid peroxidation; and, it also modulates the protein kinase B/Glycogen synthase kinase 3 beta/Glutathione peroxidase 4 (AKT/GSK3β/GPX4) pathways; where, silibinin stabilizes AKT signaling, inhibits GSK3β, and promotes GPX4 activity, effectively preventing ferroptosis and inflammation in acute liver injury models; besides, silymarin inhibits the triggering activity of NF-κB and reduces proinflammatory cytokines; where, it down-regulates TNF-α, IL-1β, and IL-6, thus mitigating cellular damage and necrosis [49, 50].

Nonetheless, given the context dependent variability in Nrf2 activation where overactivation may paradoxically- promote tumorigenesis or drug resistance in certain settings [51], these findings should be regarded cautiously. Comprehensive mechanistic researches are warranted to delineate the conditions under which Nrf2 activation confers therapeutic benefit versus risk.

Hepatocyte Nuclear Factor 4 Alpha (HNF4α)

Cyclophosphamide (Cpd) administration markedly-downregulated the hepatic mRNA HNF4α level (Figure 3), reflecting impaired hepatocyte differentiation, disrupted metabolic programming, and diminished regenerative potential (Figure 3). Results of this study are consistent with prior investigations linking the HNF4α depletion to compromised hepatic recovery and chronic liver injury [52, 53]. Moreover, in the current study, both dapagliflozin and silymarin restore the mRNA HNF4α level, with dapagliflozin demonstrating a modest advantage over silymarin. This effect may be mediated through the activation of the Sirtuin 1/Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (SIRT1/PGC-1α) pathway, which is known to enhance mitochondrial biogenesis and support hepatocyte function [54]. Besides, mending of the mRNA HNF4α level is critical not only for maintaining hepatocellular identity, but also for orchestrating a transcriptional network involved in lipid metabolism, glucose homeostasis, and xenobiotic clearance. Thus, therapeutic interventions that preserve or restore HNF4α expression may hold substantial promise in mitigating chemotherapy-induced hepatic dysfunction.

Hepatocyte Nuclear Factor 6 (HNF6)

The suppression of the mRNA HNF6 level by Cpd (Group III rats) (Figure 4B) further corroborates its role in maintaining hepatobiliary integrity, particularly through the regulation of bile acid transport and intrahepatic bile duct formation, this aligns with findings from other researchers [55, 56].

The HNF6 regulates genes that involves in bile acid transport, including sodium taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide 1B1 (OATP1B1), and its subduing has been shown to disrupt bile acid homeostasis [55]. Moreover, Cpd-induced

hepatotoxicity has previously been associated with bile acid dysregulation and impaired expression of transporter which further supporting the observed alterations in HNF6 expression [57].

While both agents (dapagliflozin, and silymarin) reversed this downregulation (Figure 4B), the second agent elicited a more robust restoration of the mRNA HNF6 level. This may be due to its well established choleretic activity and modulatory effects on bile acid synthesis and secretion [58]. Unlike silymarin, dapagliflozin may exert minimal direct influence on bile acid metabolism which is supported by multiple studies suggesting its primary mechanisms lie outside the hepatobiliary axis [59–61]. Importantly, the crosstalk between HNF4α and HNF6 in governing bile acid homeostasis and liver regeneration underscores the therapeutic relevance of restoring both transcription factors to achieve comprehensive hepatic protection [62, 63].

Clinical Perspective

The results of this study support the repurposing potential of dapagliflozin beyond its approved indications for the management of type 2 diabetes mellitus (T2DM) and heart failure (HF). In this preclinical model, dapagliflozin demonstrated significant hepatoprotective activity, likely mediated by antioxidant and transcriptional regulatory mechanisms. Given the growing clinical concern regarding chemotherapy associated hepatotoxicity, particularly with agents like cyclophosphamide (Cpd), these results carry translational relevance. Patients undergoing long term chemotherapeutic regimens, especially those with pre-existing hepatic impairment or polypharmacy involving hepatotoxic agents, may benefit from adjunctive therapies that preserve liver function. Incorporating dapagliflozin as a co-treatment could enhance hepatic resilience, reduce treatment interruptions, and improve overall patient outcomes in oncology settings.

However, several clinical questions remain unresolved, including appropriate dosing strategies, timing of administration (pre-treatment versus concurrent use), and the safety profile in non-diabetic populations. Therefore, well designed randomized clinical trials are essential to assess the therapeutic efficacy, pharmacokinetics, and long term of dapagliflozin safety in cancer patients at risk of hepatotoxicity.

Limitations and Future Directions

Several limitations of the current study should be addressed. **First**, the only usage of male rats prevents the evaluations of potential sex-specific variations in drug metabolism, OS responses, and hepatic regeneration. **Second**, the short study duration may not adequately-reflect the chronic or cumulative effects of cyclophosphamide (Cpd) exposure or the long-term efficacy of the interventions. **Third**, although molecular and biochemical analyses were employed to infer mechanistic pathways, the absence of histopathological evaluation limits the ability to corroborate these findings at the tissue level. Furthermore, while prior literature supports the involvement of AMPK and SIRT1 signaling, this study did not directly assess upstream regulators or downstream phosphorylation events.

To build on the current findings, future investigations should incorporate both sexes, adopt extended study durations, perform detailed histological assessments, and employ molecular tools (e.g.immunofluorescence) to directly evaluate signaling pathway activation. Such approaches will strengthen the mechanistic underpinnings and translational applicability of dapagliflozin and silymarin in hepatic protection.

Conclusion

Findings of the present study, revealed the critical involvement of transcription factors Nrf2/HO-1, HNF4α, and HNF6 in mediating oxidative stress responses and liver regeneration during cyclophosphamide-induced hepatotoxicity. Dapagliflozin demonstrated superior efficacy over silymarin in restoring Nrf2/HO-1 and HNF4α levels, suggesting its potent antioxidant and regenerative effects. While both agents improved HNF6 expression, silymarin exerted a stronger effect, possibly due to its choleretic activity. Collectively, these results highlight the multifaceted hepatoprotective roles of dapagliflozin and silymarin via modulation of key transcriptional pathways, with dapagliflozin showing broader metabolic and antioxidative benefits. The dual mechanisms involving antioxidant defense activation, inflammation inhibition, ferroptosis prevention, and support of hepatocyte nuclear factor expression provide a comprehensive mechanistic basis for the hepatoprotection observed in this study.

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Conflicts of Interest

There is no conflict of interest disclosed by the authors.

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Ethics Statements

The research has been approved by the Research Ethics Committee at the College of Pharmacy, Baghdad University. REC062463A in 2-3-2024.

Author Contribution

The following is how the writers attest to their contribution to the paper: Nada N. Al Shawi conceived and designed the project; data collection: Afrah Thiab; analysis and interpretation of results: Nada N. Al-Shawi, Afrah Thiab; the final draft of the paper was approved by all authors after they had assessed the findings.

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التأثيرات الكبدية الوقائية المقارنة للداباغليفلوزين والسيليمارين ضد إصابة الكبد المستحدثة بالسيكلوفوسفاميد في الجرذان: دور مسارات Nrf2/HO-1و HNF4α وHNF6

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الخلاصة

ان السيكلوفوسفاميد هو دواء شائع يُستخدم كمثبط لجهاز المناعة وكمادة كيميائية لعلاج الأورام، إلا أنه قد يؤدي إلى تسمم في الكبد نتيجة تحفيزه للالتهاب والإجهاد التأكسدي..

أظهرت الدراسات أن دواء داباغليفلوزين، وهو أحد مثبطات ناقلات الصوديوم والجلوكوز، يتمتع بخصائص مضادة للالتهاب ومضادة للأكسدة، في حين يُعرف نبات السليمارين بفعاليته في حماية الكبد من الأضرار.

تهدف هذه الدراسة إلى تقييم الفعالية الوقائية لداباغليفلوزين بالمقارنة مع السليمارين في الحد من إصابة الكبد الناتجة عن استخدام السيكلوفوسفاميد، وذلك من خلال قياس مؤشرات الإجهاد التأكسدي والالتهاب وتجديد أنسجة الكبد، مع التركيز على مسارات الإشارات المضادة للأكسدة المعروفة بمسار العامل النووي المُحفّز لإنزيم الهيم أوكسيجيناز، بالإضافة إلى دراسة عوامل النسخ النووية في الخلايا الكبدية، وهي العامل النووي الكبدي ٤ ألفا والعامل النووي الكبدي ٦.

تم تقسيم خمس مجموعات من ذكور الجرذان، بحيث ضمت كل مجموعة عشرة جرذان، إلى المجموعات التالية: مجموعة سليمة (بدون علاج)، مجموعة أعطيت مادة ناقلة، مجموعة تلقت السيكلوفوسفاميد بجرعة ثلاثين مليغرامًا لكل كيلوغرام من وزن الجسم، مجموعة تلقت السيكلوفوسفاميد مع داباغليفلوزين بجرعة ثلاثة مليغرامات لكل كيلوغرام، ومجموعة تلقت السيكلوفوسفاميد مع السليمارين بجرعة مئتي مليغرام لكل كيلوغرام.

وبعد مرور عشرة أيام، تم تحليل أنسجة الكبد لقياس مستويات التعبير الجيني للعامل النووي المضاد للأكسدة وإنزيم الهيم أوكسيجيناز، إلى جانب قياس مستويات العامل النووي الكبدي ٤ ألفا والعامل النووي الكبدي ٦ باستخدام تقنيات تحليل حيوية مختلفة النتائج: أظهر السيكلوفوسفاميد انخفاضًا كبيرًا في مستويات العوامل المذكورة، مما يشير إلى حدوث تلف في أنسجة الكبد. في المقابل، ساهم كل من داباغليفلوزين والسليمارين في استعادة هذه المؤشرات الحيوية، حيث أظهر داباغليفلوزين قدرة أعلى على رفع مستويات العامل المضاد للأكسدة وإنزيم الهيم أوكسيجيناز. كما أدى إلى زيادة ملحوظة في مستويات العامل النووي الكبدي ٤ ألفا والعامل النووي الكبدي ٦، متفوقًا على السليمارين في تحسين مستوى العامل النووي الكبدي ٦.

يُسهم داباغليفلوزين في التخفيف من التسمم الكبدي الناتج عن السيكلوفوسفاميد من خلال تنشيط المسارات المضادة للأكسدة واستعادة التعبير الطبيعي للعوامل النووية الخاصة بالكبد، مما يُظهر فعالية مماثلة أو متفوقة على السليمارين. وتشير هذه النتائج إلى إمكانية الاستفادة من داباغليفلوزين كعلاج مساعد للحد من الأضرار الكبدية المصاحبة للعلاج الكيميائي.

الكلمات المفتاحية : السيكلوفوسفاميد، الداباغليفلوزين، الاجهاد التاكسدي ، مسار العامل النووي المحفز لانزيم الهيم أوكسيجيناز ، السليمارين