DOI: https://doi.org/10.31351/vol34iss2pp108-115

Effects of Different Doses of Quercetin on Apoptotic Markers and Liver Enzymes in Liver Injury Induced by Interferon Beta-1b (Betaferon) in Male Rats in Comparison with Silymarin

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Received 23/10/2023, Accepted 20/2/2024, Published 256/2025

Abstract

The disease-modifying drug for the treatment of multiple sclerosis was interferon beta-1b (Betaferon). It's demonstrated efficacy in relapse multiple sclerosis, But, there are many difficulties in treating this disease, including side effect of Betaferon (hepatotoxicity or liver injury), which present as brief, mild elevations in serum aminotransferase enzymes (alanine aminotransferase, aspartate aminotransferase) levels that appear 3 to 12 months after starting treatment. The antioxidant is quercetin which acts as an anti-oxidant and anti-inflammatory agent, it also has antiviral, anticancer and cardiovascular properties. The study aimed to investigate the apoptotic potential of betaferon on liver cells and to evaluate the hepatoprotective effect of quercetin , by down-regulation of apoptotic biomarkers caspase-3 and caspase-9.The study was employed on 36 healthy male rats for 17 days; and the animals were divided into six groups (6 rats in each group) given daily doses by Intraperitoneal route as follows: Control group; (rats were administered distilled water), Betaferon group or induction group; (rats were administered Betaferon in a dose of 250 µ/kg for 16 days), treatment groups Betaferon + quercetin which divided into three subgroups according to the dose of quercetin (25, 50, and 100mg/kg); (Rats were pre-treated by a dose of 25,50,100mg/kg of quercetin solution for 6 days then continue administering the same dose of quercetin plus Betaferon 250µ/kg on day 7 for 10 days). and Silymarin group; (Rats were pre-treated by a dose of 100mg/kg/day of silymarin suspension I.P for 6 days then continue administering the same dose of silymarin plus Betaferon 250µ/kg daily for 10 days). In the current study, liver function enzymes such as alanine aminotransferase and aspartate aminotransferase also apoptotic markers which include caspase-3 and caspase-9 were measured. Betaferon significantly-increased liver apoptotic markers and also cause elevation in the liver enzymes ($p<0.0001$) compared to the control group. Quercetin significantly-reduce these biomarkers (caspase-3,caspase-9) and also restoring liver enzymes to its normal level. Quercetin showed antioxidant, anti-inflammatory and antiapoptotic effects on the liver tissue of rats, suggesting it has a beneficial effects on minimizing liver injury caused by Betaferon.

Keywords: Drugs induced liver injury, Interferon beta -1b (Betaferon), Liver injury, Multiple sclerosis, Quercetin.

Introduction

Betaferon (IFNβ-1b) is used to treat the signs and symptoms of multiple sclerosis (MS) in order to decrease the frequency of clinical exacerbations [(1)]. The IFNβ-1b refer to immunomodulatory drug class [(2)] that can induce liver injury (DILI) [(3)]; where, patients receiving IFNβ-1b have been documented to experience severe hepatic damage, including cases of hepatic failure, some of which have been attributed to autoimmune hepatitis [(4)]. Although the exact mechanism by which IFNβ-1b causes hepatic injury is unknown , but it is possible that it damages hepatocytes directly or triggers an immune response against the liver [(5)].The enhanced suppressor cell activation, suppression of cytotoxic T-cells, cytokine alterations, and effects on the blood-brain barrier (BBB) may all contribute to IFNβ's immunomodulatory effects. Numerous studies have demonstrated that the majority of MS patients gain from these immunomodulatory effects, but this medication may also cause or make other autoimmune diseases worse, such as thyroid problems, hepatitis and Induction of autoimmune hemolytic anemia (AIHA) during Betaferon treatment [(6)(7)]. Furthermore, hepatotoxicity usually affects women more severely than men, the cause

of these gender-based differences is not well known (in the current study male rats were used, to avoid the physiological variability associated with female estrous cycles and variations in female hormones level which make data more difficult to analyze and the results will become not accurate) [8] but, it has been suggested that hepatotoxicity may be predisposed by having a lower body mass index (BMI) and a higher level of treatment adherence [5]. Also, the concentration of estrogen receptor in the liver differ between males and females and estrogen seems to activate Kupffer cells, which are located in the liver and this caused increase inflammation and necrosis of hepatocytes in females which can affect the results of this study; female rats were avoided in the current study. In addition, the female predominance of drug-induced liver damage may be due to sex variations in drug absorption, metabolism, and excretion, which have been widely-documented in rat models as well as in humans [9].

By scavenging free radicals and preventing the oxidation of various components, Quercetin (QCT) exhibits antioxidant defense and as such, it is a helpful agent for preventing oxidative stress (OS) in many brain cells; and the antioxidant defense of QCT against OS action is the major mechanism of its protective effect [10]. This study aimed to investigates the hepatoprotective and the anti-apoptotic activity of QCT; in addition, this study aimed to investigate the ability of QCT to regulate the hepatic miRNAs expression against IFNβ-1b-induced liver damage in male Albino rats in comparison with silymarin.

Materials and Methods

Chemicals and drugs

The Betaferon drug (IFNβ-1b) was purchased from Bayer Pharmaceutical Company (Germany) prepared as a solution by dissolving it in D.W. (250µ/1ml), and administered by I.P route. The QCT (purity > 98%) was purchased as yellow powder from Pure chemistry (Germany). It was dissolved in 0.1% dimethyl sulfoxide (DMSO) which is used as a solvent based on previous studies [11,12].

Calculation of the drug's dose

The experimental animals were administered different doses of IFN-β depending on the weight of each animal (after conversion to animal dose using animal equivalent dose equation) :

Animal equivalent dose (mg/kg) = Human dose (µg/kg) * Km ratio [13]

K m ratio = Human km / Rat km which mean :Human km = 37, Rat km = 6

The maximum dose of betaferon in adult humans that weight (70 kg) is (250 microgram) [14] and we chose the maximum dose of the drug in order to facilitate the accurance of hepatotoxicity.

250 µg 70kg

x µg 1 kg

which means approximately (3.571 µg/kg) human dose.

Animal equivalent dose = 3.571 × 37/6

= 22.01 µg /kg (maximum dose of Betaferon in rat).

So 22.01 µg /kg is used to calculate the dose for each animal according to their weight.

Study design

Thirty six (36) healthy male rats of approximately (9-13weeks old) with a weight of (160 -185 g) were used in this study. The experimental rats were taken from the animal house of the Iraqi Center for Cancer Research and Medical Inheritance/Mustansiriya University. Animals were housed in (20 x 25 x 35 cm) plastic cages. The animals were kept in clean, dry housing at a temperature of 25 °C with a humidity level of 40–50% and a light/dark cycle of 12 hours before to the start of the study protocol. The Mustansiriya University College of Pharmacy's Ethical Community granted its permission.

Rats were randomly-divided into 6 groups each group containing six animals of (160-185g) weight; and, the study continue for 17 days, and according to the Ethical Committee on Animal Care (file No.7 in 13 November 2022) as follows :

I) Control group (n=6); each rat was **I.P**-injected with distilled water (D.W) **(**0.5ml/day) for 16 days.

II) The interferon beta-1b (IFNβ-1b) (the induction group) (n=6); Rats were **I.P**-injected with a maximum dose of IFNβ-1b (Betaferon 250µ/kg) daily for 16 days.

III) The IFNβ-1b (Betaferon) + Quercetin [QCT (25mg)] treated group (n=6); Rats were pre-treated with a dose of 25mg/kg/day of QCT solution **I.P** for 6 days then continued administering the same dose of QCT plus a maximum dose of IFNβ-1b (Betaferon 250µ/kg) daily for 10 days.

IV) IFNβ-1b (Betaferon) + Quercetin [QCT (50mg)] treated group (n=6); Rats were pre-treated with a dose of 50mg/kg/day of QCT solution **I.P** for 6 days then continued administering the same dose of QCT plus a maximum dose of IFNβ-1b (Betaferon 250µ/kg) daily for 10 days.

V) IFNβ-1b (Betaferon) + Quercetin [QCT(100mg)] treated group (n=6); Rats were pre-treated by a dose of 100mg/kg/day of QCT solution **I.P** for 6 days then continued administering the same dose of QCT plus a maximum dose of IFNβ-1b (Betaferon 250µ/kg) daily for 10 days.

VI) Silymarin (S100) group; Rats were pre-treated by a dose of 100mg/kg/day of silymarin suspension **I.P** for 6 days then continue administering the same dose of silymarin plus a maximum dose of IFNβ-1b (Betaferon 250µ/kg) daily for 10 days. (silymarin is used as a reference drug because it has a well-known hepatoprotective effect as mentioned

from previous studies [15,16]; and, the dose of 100mg/kg of silymarin was the choice from the study of Mahli, A. *et al* (2005) [17].

All rats were anesthetized on the last day of the experiment (day17) by injection of combined ketamine/xylazine which is the preferred injectable anesthetic in rats until the rat loses the ability to move (80 mg/kg ketamine+ 10 mg/kg xylazine) [18], and blood samples were collected from the right ventricle of the heart by using a 5mL syringe and then centrifuged to separate the serum.

Biochemical analysis

At day 17, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities; and, the apoptosis markers (caspase-3 and caspase-9) were measured.Measurements of the liver enzyme aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were done according to manufacturer procedure using sandwich enzyme-linked immunosorbent assay (ELISA) technique [19,20]. The serum caspase-3 and caspase-9 levels were determined using the Sandwich ELISA technique [21,22]; and the procedure employed was according to manufacturers' instructions.

Statistical analysis

Statistical analysis of this data was performed using the statistical Analysis System-version 9.1). Numerical data were described as mean and standard deviation (SD). Analysis of variance (ANOVA) was used for comparison among more than two groups. P-value <0.05 is considered as a significant difference.

Results

Figure (1), showed that there is a significant elevation in serum ALT level ($P \leq 0.0001$) in IFNβ-1b-injected animals (group II) in comparison with that level in the control (group I), QCT (groups III, IV, V) and S100 (group VI) treated animals. While the level of ALT activity was observed to be non-significantly different ($P > 0.05$) between IFNβ + QCT (25,50,100) and S100 groups as shown in Table (1).

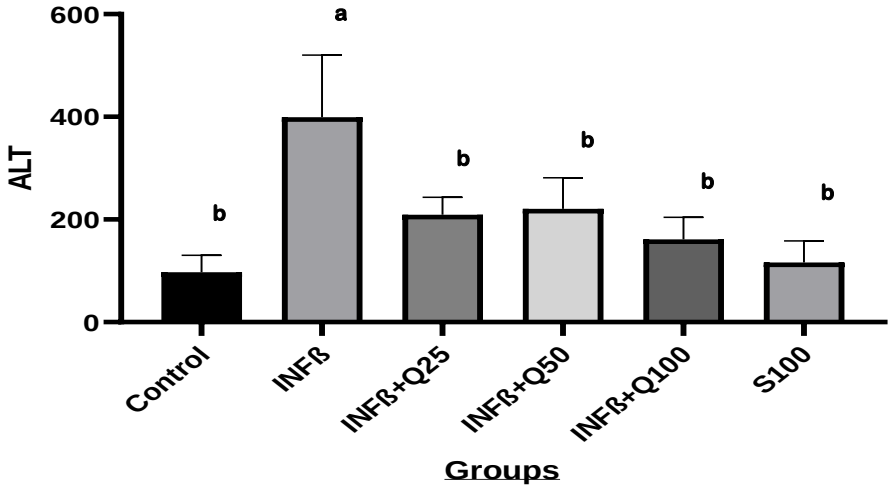

Figure 1.The Effects of Quercetin and silymarin on Serum Alanine Aminotransferase (ALT) Activity Level in IFNβ-1b (Betaferon)-induced Hepatotoxicity.
*** = Significant (P≤0.05)**

Table 1. Effects of Different doses of Quercetin and silymarin on Serum Alanine aminotransferase (ALT) Level in IFNβ-1b (Betaferon)-induced Hepatotoxicity .

Groups (ALT)	(Mean±SD)	p-value
Control	97.00 ±33.40^{b}	0.003**
IFNβ	399.33 ±290.86^{a}	
(Q) 25+ IFNβ	209.33 ±33.80^{b}	
(Q)50+ IFNβ	220.50±60.810^{b}	
(Q)100+ IFNβ	161.16 ±43.16^{b}	
S100	116.16 ±42.13^{b}	

(Means followed by different letters are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different).

Serum aspartate aminotransferase (AST) levels represented as mean±SD in figure (2), there is significant elevation ($p<0.0001$) in serum AST level in rats injected with IFNβ-1b (group II) in comparison with the corresponding serum level of

control (group I), all different QCT groups (groups III, IV, V) and S100-treated group.

Moreover, there is a very high-significant difference in AST serum levels in IFNβ-1b compared to such serum level in control rats' group; also between IFNβ and IFNβ + QCT groups and also between IFNβ and S100 group ($p<0.0001$). While the level of AST activity showed to be non-significantly different in both IFNβ+QCT and S100 groups in comparison with control as shown in Table (2).

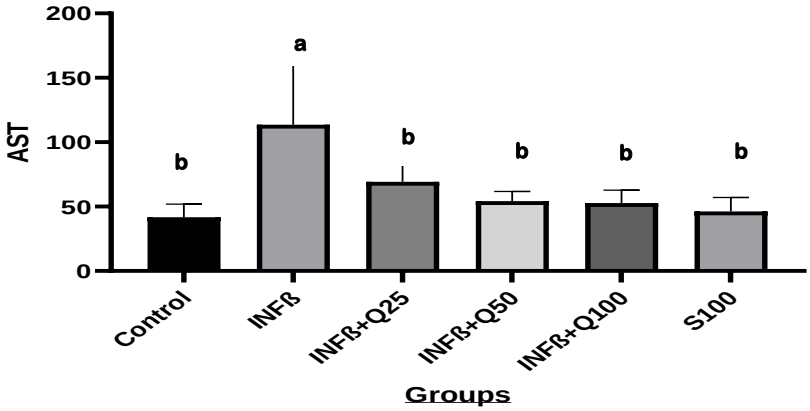

Figure 2. The effect of quercetin and silymarin on serum AST activity in IFNβ-1b (Betaferon)- induced hepatotoxicity.* = Significant (P≤0.05).

Table 2. Effects of Different doses of Quercetin and silymarin on Serum Aspartate aminotransferase (AST) Level in IFNβ-1b (Betaferone)-induced Hepatotoxicity.

Groups (AST)	(Mean±SD)	p-value
Control	41.83 ±10.10[b]	0.001**
IFNβ	113.50±49.70[a]	
Q25+ IFNβ	69.33 ±12.86[b]	
Q50+ IFNβ	54.16 ±7.65[b]	
Q100+ IFNβ	52.66 ±10.09[b]	
S100	46.33 ±10.80[b]	

(Means followed by different letters are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different).

Figure 3. showed that, there is a significant elevation in caspase-3 ($p<0.0001$) in IFNβ-1b (Betaferon) treated animals (group II) in comparison with such serum level to the control (group I), all different QCT groups (groups III, IV, V) and S100 group. There is very highly-significant differences in serum caspase-3 levels between the IFN-β (group II) and control (group I) as well as between IFNβ and IFNβ + all different QCT groups (groups III, IV, V) also between IFNβ and S100 group (group VI) ($P<0.0001$). In addition, there is a significant differences in serum caspase-3 levels among all different QCT groups (groups III, IV, V). While serum levels of this enzyme were observed to be non-significantly different in both IFNβ + QCT 50 and S100 groups as shown in Table (3).

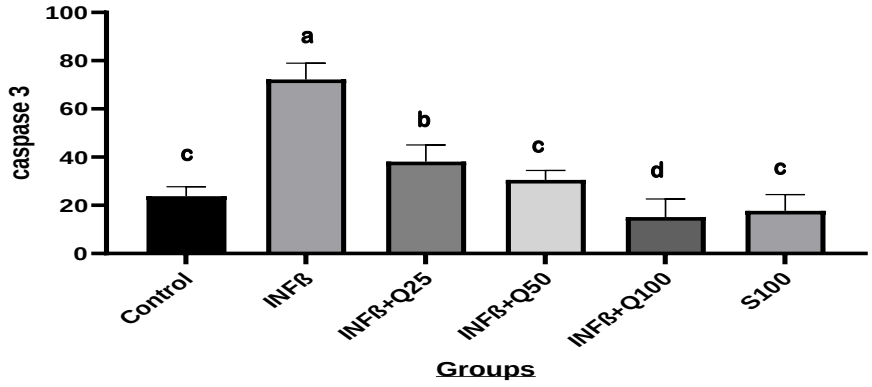

Figure 3. The effect of Quercetin and silymarin on Serum Caspase-3 Level in IFNβ-1b (Betaferon)-induced Hepatotoxicity.* = Significant (P≤0.05)

Table 3. Effects of Different doses of Quercetin and silymarin on Serum Caspase-3 Level in IFNβ-1b (Betaferon)-induced Hepatotoxicity.

Groups (caspase-3)	(Mean±SD)	P-value
Control	23.81 ±3.89[c]	0.001**
IFNβ	72.22 ±6.76[a]	
Q25+ IFNβ	38.14 ±6.86[b]	
Q50+ IFNβ	30.52 ±3.94[c]	
Q100+ IFNβ	15.15 ±7.45[d]	
S100	17.71 ± d6.74[c]	

(Means followed by different letters are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different).

Besides, figure (4) showed that there is a significant elevation in caspase-9 ($p<0.0001$) in IFNβ-1b (Betaferon) treated animals in such serum level compared with such level in the control (group I), all different QCT groups (groups III, IV, V) and S100 group of rats. There is very highly-significant differences in caspase-9 levels between IFNβ-1b and control group as well as between IFNβ and IFNβ + all different QCT groups also between IFNβ-1b and S100 group ($P<0.0001$). In addition There is a significant difference in caspase-9 serum level among all different QCT groups (groups III, IV, V); while, the serum level of this enzyme was observed to be non- significantly different in both IFNβ + QCT 25 and S100 groups as shown in Table (4).

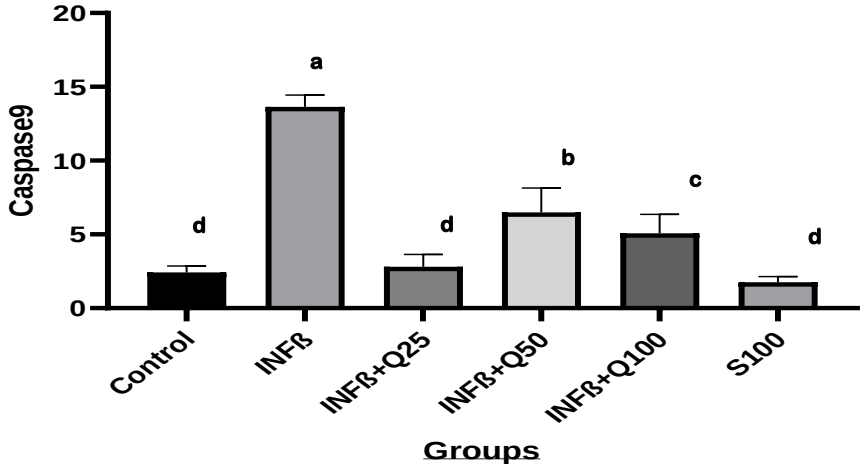

Figure 4. The effect of Quercetin and silymarin on Serum Caspase-9 Level in IFNβ-1b (Betaferon)-induced Hepatotoxicity.
*** = Significant (P≤0.05)**

Table 4. Effects of Different doses of Quercetin and silymarin on Serum Caspase-9 Level in IFNβ-1b (Betaferon)-induced Hepatotoxicity

Groups (caspase-9)	(Mean±SD)	p-value
Control	2.41 ±0.44 d	0.001**
IFNβ	13.64 ±0.80 a	
Q25+ IFNβ	2.80 ±0.84 d	
Q50+ IFNβ	6.48 ±1.65 b	
Q100+ IFNβ	5.08 ±1.27c	
S100	1.75 ± 0.39 d	

(Means followed by different letters are significantly different according to Duncan's multiple range comparisons (DMRTs), Means followed by the same letter are not significantly different).

Discussion

The current study is the first to assess the ability of QCT to protect the liver from damage brought on by IFNβ-1b (Betaferon), which increased OS, apoptosis, and inflammation in the liver tissue of rats, that led to liver damage which is considered one of the possible mechanisms of this drug to cause hepatotoxicity.

Oxidative stress (OS), hepatocyte apoptosis and inflammation, as well as a rise in AST/ALT levels, are indicators of hepatocyte necrosis; moreover, increased apoptosis destroys liver cells, releasing excessive levels of liver enzymes (ALT, AST) into the bloodstream, which can be evaluated as indications of liver injury [23].These effects are all put out as potential justifications for IFNβ-1b - induced hepatotoxicity [24] .The effects of QCT on IFNβ-1b-induced liver damage was examined in this study. Hepatocytes are the main location of aminotransferases enzymes [25]; where, serum AST and ALT levels increased in tissues rich in aminotransferases, notably liver cells when the tissues are damaged or destroyed or when cell membrane permeability changes, allowing leakage into the bloodstream [26].

Similar to other earlier studies [27,28,29], the present study found that administration of IFNβ-1b significantly- elevated the serum activity of ALT and AST in comparison with the negative control group of rats. Furthermore in the pilot study, I.P administration of maximum dose of IFNB-1b to Wistar rats significantly-increased serum ALT and AST levels ; and because of the increased activity of AST in hepatocytes and its release after liver injury, serum AST levels normally-rise quickly after acute hepatocellular in jury, reaching a higher level than ALT at first ; and because of its longer plasma half-life, ALT will become higher than AST within 24 to 48 hours of injury [30].

Interestingly, the mean serum levels of AST and ALT in the group pre-treated with (25mg/kg) of QCT (group III) before injection with IFN-β (group II) were significantly lower than in the IFNβ-1b (group II). By increasing the dose of QCT to (50mg/kg) and (100 mg/kg), the serum AST and ALT decreased to a level non-significantly different from the negative control group. In the same manner, S (100mg/kg) administration normalized the serum AST and ALT. IFN-β promoted apoptosis by increasing both caspase-3 and 9 significantly in comparison with the negative control group,the proposed mechanism is that Betaferon induced oxidative stress which cause increased mRNA expression of caspase-3 in the hepatic tissue that may play an important role in the induction of apoptosis of hepatocytes because caspase-3 is the key element of apoptosis. It has been shown that betaferon elevates intracellular ROS levels resulting in dissipation of mitochondria membrane potential that activates caspase-3 and caspase-9. Thus, the increased caspase-3 gene expression following administration of rats with betaferon may confirm severe pathological changes in the hepatic tissues [31].

Caspase-9 begins apoptosis, which is then carried out by caspase-3 [32]. where, the current study approved this by significant increase in caspase-3 activity among rats treated with maximum dose of IFNβ-1b. Moreover in this study, pre-treatment of three groups of rats with different doses of QCT (25mg/kg, 50mg/kg, and 100mg/kg) significantly decreased the apoptotic markers caspase-3 and 9 compared to those serum levels in the induction group (Betaferon group). Additionally, there was no significant different (P>0.05) shown compared to the negative control group. The S100 group showed comparative results to the QCT (groups III, IV, V) pre-treated groups.

Conclusions

The study revealed that Quercetin has a hepatoprotective effect against IFNβ-1b (betaferone)-induced hepatotoxicity by improving liver injury, significantly normalizing liver enzymes activities of (ALT and AST) and by decreasing apoptotic biomarkers caspase-3 and caspase-9. The highest dose of QCT (100 mg/kg) produced the best results. In addition, this study shows that although the protective effect of QCT is nearly similar to that of silymarin but silymarin give the best results.

Acknowledgment

The authors are extremely-grateful to Mustansiriyah University/ College of Pharmacy/ Pharmacology and Toxicology Department and the animal house of the Iraqi Center for Cancer Research and Medical Inheritance / Mustansiriya University for providing the support and resources necessary to complete this work.

Conflicts of Interest

There is no Conflict of interest regarding the publication of this manuscript.

Funding

There is no financial support from any institution.

Ethics Statements

Animals were housed according to the Ethical Committee on Animal Care (file No. 7 in 13 November 2022) in Mustansiriyah University /collage of Pharmacy/pharmacology and toxicology department.

Author Contribution

Study conception and design: :Noor kamel Obead, Inam Sameh Arif, Huda Jaber Waheed; Data collection: Noor kamel obead; Analysis and interpretation of results: Noor kamel Obead; Draft manuscript preparation : Noor kamel Obead; All authors reviewed the results and approved the final version of the manuscript.

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تاثير الجرعات المختلفة من الكيرسيتين على مساروموت الخلايا المبرمج وانزيمات الكبد في إصابة الكبد الناجمة عن الإنترفيرون بيتا-١ب بالمقارنة مع السليمارين في ذكور الجرذان

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الخلاصة

يعد الإنترفيرون بيتا-١ب (بيتافيرون) علاج لمرض تصلب الاعصاب المتعدد حيث أظهر هذا الدواء فعاليته في العلاج، ولكن هناك العديد من الصعوبات في علاج هذا المرض، بما في ذلك الآثار الجانبية للبيتافيرون (السمية الكبدية أو إصابة الكبد) ، والتي تظهر بشكل ارتفاعات قصيرة وخفيفة في إنزيمات الكبد (alanine aminotransferase, aspartate aminotransferase) حيث يظهر هذا الارتفاع بعد ٣ إلى ١٢ شهرا من بدء العلاج. مضاد الاكسدة هو كيرسيتين والذي يحتوي على عدد من الفوائد الصحية ، بما في ذلك كونه مضادا للأكسدة ، و مضاد للالتهابات وخصائص مضادة للفيروسات والسرطان والقلب والأوعية الدموية. تهدف الدراسة إلى التحقيق في موت الخلايا المبرمج التي يسببها بيتافيرون على خلايا الكبد وتقييم حماية الكيرسيتين للكبد، عن طريق تقليل المؤشرات الحيوية للاستماتة caspase-3,caspase-9 ، تم تطبيق هذه الدراسة على ٣٦ ذكور الجرذان الصحية لمدة ١٧ يوم وتم تقسيم الجرذان الى ٦ مجموعات (6 جرذان في كل مجموعة) اعطيت هذه المجموعات الجرعات يوميا عن طريق الحقن داخل الصفاقI.P على النحو التالي: **مجموعة السيطرة** (تم اعطاؤها الماء المقطر), **مجموعة البيتافيرون أو مجموعة الحث** (تم حقنها بدواء البيتافيرون بجرعة٢٥٠ مايكرو/كغم لمدة ١٦ يوم),**مجموعات العلاج وهي مجموعات بيتافيرون+كيرسيتين** (تم تقسيم هذه المجموعة الى ٣ مجموعات فرعية حسب جرعة الكيرسيتين (٢٥، ٥٠ ، ١٠٠ ملغم/ كغم). عولجت الجرذان هذه مسبقا بجرعات ٢٥ ، ٥٠ ، ١٠٠ ملغم/ كغم من محلول كيرسيتين لمدة ٦ أيام ثم تم الاستمرار في إعطاء نفس جرعات كيرسيتين بالإضافة إلى بيتافيرون٢٥٠ مايكرو/كغم في اليوم السابع لمدة ١٠ أيام).و**مجموعة سيليمارين** (تمت معالجة الجرذان مسبقا بجرعة ١٠٠ ملغم / كغم / يوم من سيليمارين المعلق لمدة ٦ أيام ثم تم الاستمرار في إعطاء نفس جرعة سيليمارين بالإضافة إلى بيتافيرون ٢٥٠ مايكرو / كلغم يوميا لمدة ١٠ أيام). في الدراسة الحالية تم قياس إنزيمات الكبد(alanine aminotransferase, aspartate aminotransferase) ومساروموت الخلايا المبرمج (caspase-3,caspase-9). البيتافيرون سبب زيادة في مستوى انزيمات الكبد ومستويات الانزيمات الخاصة بمسار موت الخلية المبرمج بالمقارنة مع مجموعة السيطرة. ان كيرسيتين قلل بشكل كبير من هذه المؤشرات الحيوية (caspase-3,caspase-9) وأعاد إنزيمات الكبد إلى مستواها الطبيعي. وعليه أظهر كيرسيتين تأثيرات مضادة للأكسدة ومضادة للالتهاب والاستماتة على أنسجة الكبد، مما يشير إلى أن له فائدة في تقليل إصابة الكبد التي يسببها بيتافيرون.

الكلمات المفتاحية:الادوية التي تسبب اصابة الكبد, انترفيرون بيتا-١ب (بيتافيرون).اصابة الكبد.تصلب الاعصاب المتعدد.كيرسيتين.