ORIGINAL ARTICLE

Enhancement of Liver Toxicity on *Diabetes Mellitus* by a Universal Chemical Pollutant (Acrylonitrile) in Rats

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Abstract

The present study was designed to investigate potential liver damage due to acrylonitrile in Streptozotocin induced diabetes in rats. Twenty-four rats were divided into 4 treatment groups. Nondiabetic control rat receiving distilled water, non-diabetic rat receiving acrylonitrile aqueous solution (10 mg/kg/day), diabetic control rat receiving distilled water and diabetic rat receiving acrylonitrile aqueous solution. All groups received the treatment for 4 weeks. The animals were assessed for hepatoxicity markers in serum, oxidative stress markers, CYP2E1 activity and cyanide formation in tissues. Acrylonitrile significantly elevated serum aminotransferase, alanine aminotransferase, total bilirubin levels, triglycerides and total cholesterol in diabetic groups as compared to normal control group. Antioxidant markers like glutathione showed significant decline while a significant increase in malondialdehyde, superoxide dismutase and catalase in diabetic rats treated with acrylonitrile. CYP2E1 activity was observed in acrylonitrile – exposed nondiabetic and diabetic groups as compared to control. Cyanide formation was raised in both the nondiabetic and diabetic groups as compared to control group. Acrylonitriles can produce acute hepatic injury, induction of *diabetes mellitus* type II, and accomplish the CYP2E1 enzyme which sequentially leads to generation of oxidative stress and its metabolic product–cyanide that may potentiate the oxidative stress posing more deleterious effect.

Keywords

Acrylonitrile; Streptozotocin; Diabetes Type 2; Cytochrome P450 2E1; Cyanide; Liver toxicity

Introduction

Acrylonitrile (ACN) is extensively utilized in the manufacturing of several industrial products like nitrile rubbers, plastics, synthetic fibers and fumigants of insect killers for grain[1]. In addition, it also finds use as an intermediate in the synthesis of antioxidants, surface coatings, adhesives, and dyes[1]. Acrylonitrile is also used in the manufacturing of dialysis tubing possessing high permeability[2], and cigarette smoke[3]. Metabolism of ACN in human is through two main metabolic pathways (i) Glutathione conjugation and (ii) oxidation by Cytochrome P450. The conjugation of ACN with glutathione leads mainly to produce

2-cyanoethyl mercapropuric acid (N-acetyl-S-2-cyano ethyl cysteine[4,5]. Cytochrome P-450 oxidation yield 2-cyanoethyleneoxideandcyanide (CN) that undergoes conjugation with reduced glutathione to yield multiple metabolites including cyanide and thiocyanate[6].

It was observed that ACN induced acute toxicity in rats is characterized by damage to the tissues of vital organs which includes liver, lung, brain and kidney[7-12], stomach[13], and lysis of adrenal cortex[14].

One of the hallmarks of diabetes is the requirement of multiple medications with long-term administration which may consequently lead to unavoidable complications[6,15]. A recent study highlighted that in drug induced diabetes in rats as well as normal rats, the level of enzymes responsible for hepatic drug metabolism was reduced. The decreased enzymes included glutathione reductase, glutathione peroxidase, p-nitrophenol glucuronosyl transferase, arylsulphotransferase I and II[16].

In contrast, Sindhu *et al.*[17] studied the expression of major isozymes of CYP450 in Streptozotocin (STZ) induced diabetes with simultaneous treatment with insulin. They reported upregulation of microsomal activity of CYP1B, CYP1A, heme oxygenase (HO)-2 proteins and CYP1A2-dependent 7-ethoxyresorufin O-demethylase (EROD) in the diabetic rats. The expression of CYP1A, CYP1B1 and HO-2 had been improved by insulin and there was a noticeable induction in CYP2B1 and 2E1 proteins in diabetic animals. Treatment with insulin caused total and partial restorations of CYP2E1 and CYP2B1 proteins, respectively. Similarly, insulin could in part improve CYP2C11 protein while diabetic state decreased it by > 99%. The decisive disclosure of this study is that overall changes in the expression of different isozymes of CYP450 exist in diabetic rats which can be evidently ameliorated by insulin therapy[18,19]. This study was performed to assess the potential liver damaging effect of ACN and enzyme induction *per se* in *diabetes mellitus* (DM) leading to supplementary deleterious effect in the presence of DM.

Materials and Methods

Animals

Male Sprague Dawley rats weighing 150–200 g were obtained from the animal facility of King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. The animals were provided with standard pellet diet and water *ad libitum*. They were kept at typical conditions (room temperature of 25 ± 2°C, 45–55% relative humidity and 12 h dark/light cycle). Procedures involving animals and their care were conducted in conformity with the institutional guidelines of King Abdulaziz University, Jeddah, Saudi Arabia.

Induction of Diabetes

Type 2 diabetes was induced by injecting freshly prepared STZ, dissolved in cold citrate buffer (0.1M), pH 4.5 at dose (35 mg/kg) as a single intraperitoneal injection to overnight-fasted rats. The rats were tested for successful induction of diabetes after 2, 5 and 8 days of STZ injection by determining fasting blood glucose levels. Rats with blood glucose levels ranging between 250–350 mg/dl were selected[18]. Fasting blood samples were collected from the tail vein of the overnight (12–15 h) fasting rats. The blood glucose level was analyzed using One Touch Ultra (LifeScan/Johnson & Johnson, Milpitas, CA, USA).

Animal Treatments

Twenty-four rats were divided into four groups (six animals per group). Groups 1 and 2 were normal healthy animals while groups 3 and 4 were diabetic rats. Briefly, group 1 served as negative control receiving only distilled water for 4 weeks. Group 2 was administered aqueous solution of ACN orally (10 mg/kg/d) for 4 weeks. This dose represents one tenth of oral LD50 of ACN in rats[19]. Group 3 served as a diabetic group receiving only distilled water for 4 weeks. Group 4 served as diabetic group exposed to ACN under the same conditions as group 2 (10 mg/kg/d for 4 weeks).

Preparation of Tissue and Serum Samples

Blood samples were collected from the retro-orbital plexus followed by centrifugation at 3000g for 15 min for serum separation[19]. The serum was stored at -80°C until the assessment of hepatotoxicity markers. Rats were sacrificed and liver tissues were dissected and homogenized at 1:5 (w/v) with the phosphate-buffered saline (PBS) pH 7.4.

Evaluation of Hepatotoxicity Markers

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides

and total bilirubin were assessed colorimetrically using commercially available kits (Spectrum Diagnostics, Cairo, Egypt). Liver index was calculated as liver weight/ body weight X 100.

Assessment of Oxidative Stress Markers

Hepatic tissues homogenate was used for determination of lipid peroxides by assessing the level of thiobarbituric acid reactive substances (TBARS) expressed as nmol malondialdehyde (MDA)/gram tissue. Reduced glutathione (GSH) level and superoxide dismutase (SOD) and catalase (CAT) activities were determined in liver tissues using commercially available kits (Biodiagnostics, Cairo, Egypt)[18]. The protein content in the hepatic homogenates was determined according to the method of Lowry *et al.*[19,20] using bovine serum albumin as a standard.

Cytochrome P450 2E1 (CYP2E1) Activity

Colorimetric assay was used to measure CYP2E1 activity through hydroxylation of p-nitrophenol according to Chang *et al.*[21]. Briefly the assay monitors the formation of "p-nitrocatechol" from "p-nitrophenol" due to the enzymatic action of CYP2E1. Then, the product "p-nitrocatechol" was acidified by trichloro acetic acid followed by neutralization with 2N NaOH and then assessed colorimetrically at wavelength 535 nm.

Cyanide Determination

CN^- concentration in the hepatic homogenates (10% w/v in PBS) was determined electrochemically as described by Abreu and Ahmed[22]. Briefly, 2 mL of the homogenate was added to 2 mL 4 N H_2SO_4 contained in the outer chamber of a Conway microdiffusion cell (Thomas Scientific, Swedesboro, NJ USA). The inner chamber contained 2 mL 0.1 N NaOH. Silicone grease was used for sealing the cells with a glass cover and rotating at 0.8×g for 2h. In the inner chamber of the Conway microdiffusion cell Silver sulfide electrode model 9416BN and double junction reference electrode model 9202 (Orion Research Inc., Cambridge, MA, USA) were placed to which 50 µL KAg $(CN)_2$ indicator solution was added. The indicator solution was prepared by adding 1.25M Na_2HPO_4, 0.55M NaOH, and 0.46 mM KAg $(CN)_2$ to make up final volume of 100 mL. Millivolts were determined using a Hanna pH meter (model 8417). Serially diluted standards of NaCN were used to construct the standard curve. The CN^- content of samples was determined from this standard curve.

Statistical Analysis

The results were expressed as mean ± standard error of measurement and statistically analyzed using one-way analysis of variance (ANOVA) followed by student Tukey–Kramer post hoc test. P values ≤ 0.05 were considered significant. The percentage of change was calculated as follows:

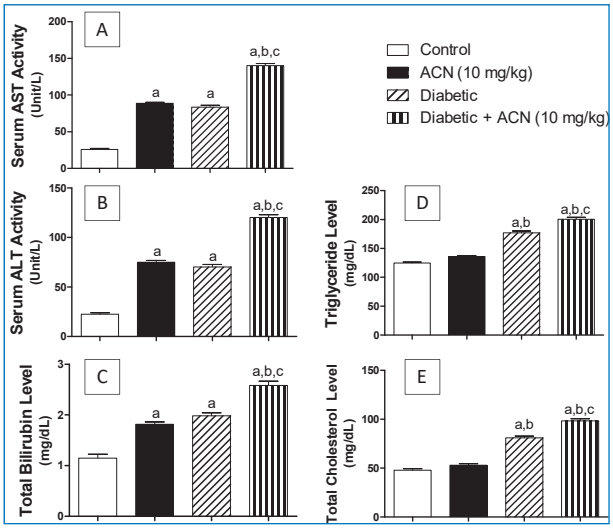
Percentage Change = (treated control)/(control) ×100.

Results

Hepatotoxicity Markers

As shown in Figure 1A, normal rats exposed to ACN have shown more than a 3-fold increase in serum AST level, compared to the control group. The same effect was obtained in diabetic rats, where AST level was elevated by a comparable extent. However, when diabetic rats were exposed to ACN, the combined effect was greatly higher, resulting in about a 5-fold increase in serum AST level, compared to the control group. Interestingly, exposure of diabetic group to ACN significantly elevated AST by about 57% and 67%, when compared to ACN-exposed nondiabetic and diabetic groups, respectively. It is worth noting that analogous patterns of effects were observed for ALT and total bilirubin levels. There were significant and comparable increases in their serum levels in ACN-exposed and diabetic rats, compared to the control group. Additionally, exposing diabetic animals to ACN markedly increased serum levels of ALT and total bilirubin, compared to all other groups, as shown in Figure 1B, and 1C, respectively.

Regarding serum triglycerides level, ACN-exposed nondiabetic exposed animals failed to show any significant change when compared to the control group, as shown in Figure 1D. However, diabetic rats have shown a significant rise of serum triglycerides by about 42%, compared to the control. This effect was further magnified when diabetic animals were exposed to ACN, where serum triglycerides level was significantly elevated by about 60% compared to the control and 13.2% compared to the diabetes-alone group. A similar pattern of observations was obtained



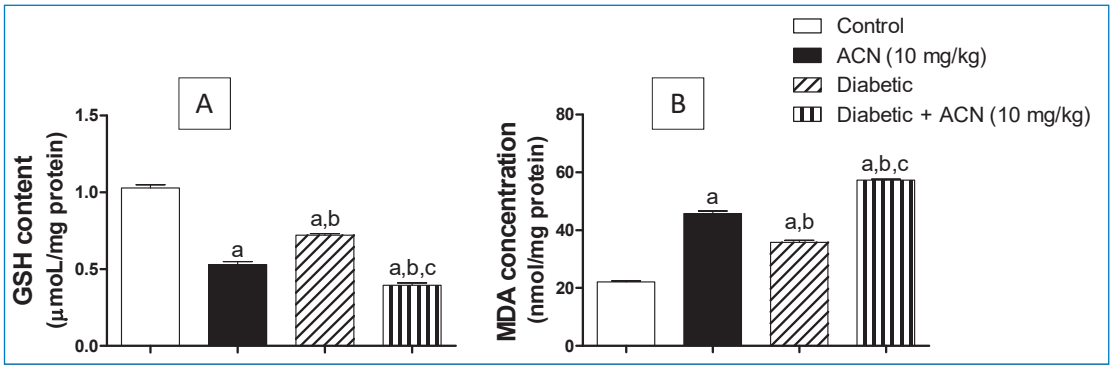
Each value represents mean ±Standard deviation
n = 6
Statistical analysis was carried out using ANOVA followed by student Tukey–Kramer post hoc test.
aSignificantly different from the control at p < 0.05
bSignificantly different from acrylonitrile-exposed nondiabetic group at p < 0.05
cSignificantly different from the diabetic group at p < 0.05

Figure 1. Effects of acrylonitrile and diabetes on serum hepatotoxicity markers: aspartate aminotransferase (Panel A), ALT (Panel B), total bilirubin (Panel C), triglycerides (Panel D) and total cholesterol (Panel E) in normal and STZ-induced diabetic rats.

with total cholesterol level. As detected in Figure 1E, no change was observed in ACN-exposed nondiabetic group, with a significant elevation in diabetic animals by 69%, compared to the control group. Importantly, diabetic animals exposed to ACN have shown more than a 2-fold increase of serum level of total cholesterol.

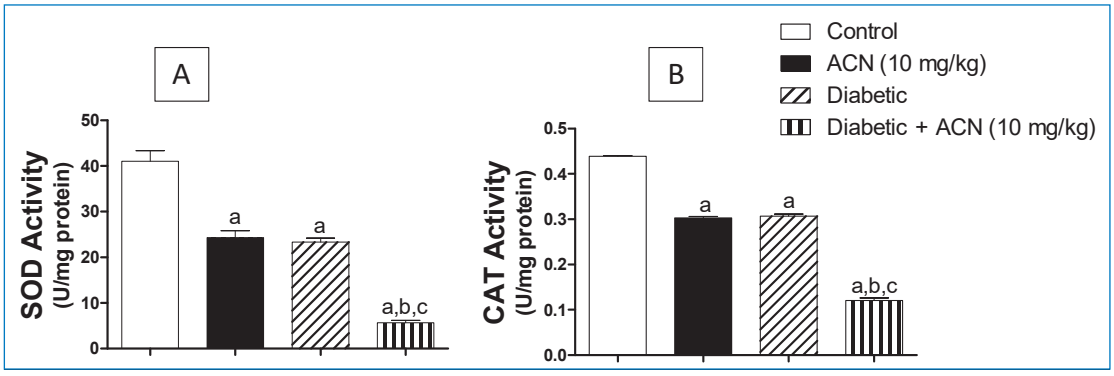
Oxidative Stress Markers

Oxidative status was evaluated by measuring the hepatic content of GSH as an antioxidant marker and MDA concentration as a lipid peroxidation marker, in addition to the enzymatic activities of two antioxidant enzymes: SOD and CAT, in hepatic tissue homogenates. As shown in Figure 2A, ACN caused a significant depletion of GSH by about 50% compared to the control group. Diabetes itself induced oxidative imbalance as manifested by a significant reduction of GSH content by about 27% compared to the control. However, when diabetes was combined with ACN exposure, the net effect was markedly significant from all tested groups, as GSH was reduced by about 60% compared to the control. These observations were further reinforced by measuring MDA as lipid peroxidation marker, as demonstrated in Figure 2B. Animals exposed to ACN have significantly increased MDA concentration to almost two times the control value. In diabetic rats, MDA concentration was elevated by about 60% when compared to the control group. However, exposure of diabetic rats to ACN resulted in an amplified effect on MDA concentration that was significantly higher than



Each value represents mean ±Standard deviation
n = 6
Statistical analysis was carried out using ANOVA followed by student Tukey–Kramer post hoc test.
aSignificantly different from the control at p < 0.05
bSignificantly different from acrylonitrile-exposed nondiabetic group at p < 0.05
cSignificantly different from the diabetic group at p < 0.05

Figure 2. Effects of acrylonitrile and diabetes on oxidative stress markers: reduced glutathione (Panel A) and lipid peroxidation malondialdehyde (Panel B) in hepatic tissue homogenate of normal and STZ-induced diabetic rats.



Each value represents mean ±Standard deviation
n = 6
Statistical analysis was carried out using ANOVA followed by student Tukey–Kramer post hoc test.
aSignificantly different from the control at p < 0.05
bSignificantly different from acrylonitrile-exposed nondiabetic group at p < 0.05
cSignificantly different from the diabetic group at p < 0.05

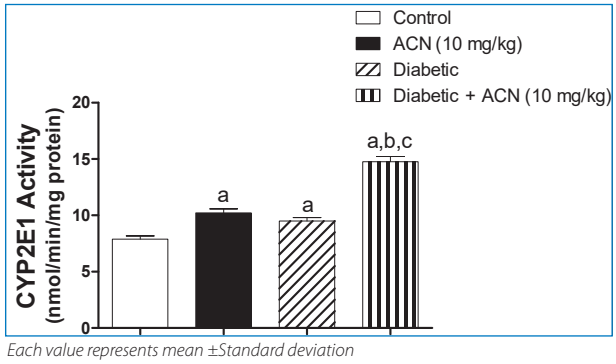
Figure 3. Effects of acrylonitrile and diabetes on antioxidant enzyme activities: superoxide dismutase "SOD" (Panel A) and catalase "CAT" (Panel B) in hepatic tissue homogenate of normal and STZ-induced diabetic rats.

all other groups (control, ACN-exposed nondiabetic and diabetic animals).

Concerning SOD enzymatic activity, exposure of normal animals to ACN significantly decreased SOD activity by about 41% compared to the control, as shown in Figure 3A. A similar effect was detected in the diabetic group, where SOD activity was dropped by an analogous degree. However, the net effect of diabetes and ACN exposure was much more significant than all other groups; being able to reduce SOD activity to about 14% of the control value. Parallel to these findings, CAT activity was decreased in a comparable pattern, as shown in Figure 3B. Acrylonitrile-exposed nondiabetic and diabetic groups have demonstrated about a 30% reduction compared to the control group, while in diabetic animals exposed to ACN, a significant decrease of CAT activity by about 72% of the control value was observed.

Cytochrome P4502E1 (CYP2E1) Activity

As shown in Figure 4, CYP2E1 activity was significantly increased by 29% and 20% in ACN-exposed non-diabetic and diabetic groups, respectively, compared to the control. No significant difference was detected between these two groups; ACN-exposed non-diabetic and diabetics. It is worth mentioning that exposure to ACN in diabetic rats markedly activated CYP2E1 by about 82% compared to the control group. Interestingly, this effect was also significant in comparison to the other groups (ACN-exposed non-diabetic and diabetic groups).

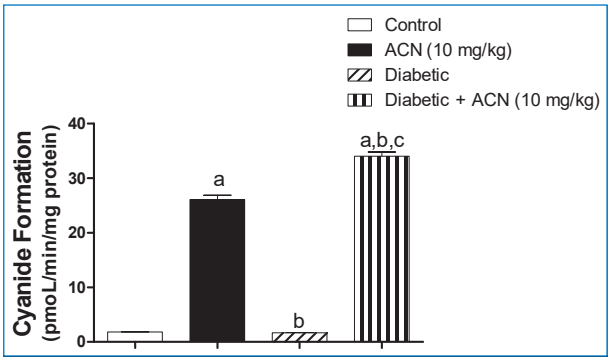


Each value represents mean ±Standard deviation
n = 6
Statistical analysis was carried out using ANOVA followed by student Tukey–Kramer post hoc test.
aSignificantly different from the control at p < 0.05
bSignificantly different from acrylonitrile-exposed nondiabetic group at p < 0.05
cSignificantly different from the diabetic group at p < 0.05

Figure 4. Effects of acrylonitrile and diabetes on CYP2E1 activity in hepatic tissue homogenate of normal and STZ-induced diabetic rats.

Cyanide Formation

Cyanide concentration in the hepatic homogenates was assayed electrochemically and results were illustrated in Figure 5. In the absence of ACN, cyanide formation was negligible in both the control and diabetic groups. As a result of ACN exposure in non-diabetic rats, its metabolic product – cyanide – was produced in a significant amount (> 20-fold), compared to the control. Moreover, exposure to ACN in diabetic



Each value represents mean ±Standard deviation
n = 6
Statistical analysis was carried out using ANOVA followed by student Tukey–Kramer post hoc test.
aSignificantly different from the control at p < 0.05
bSignificantly different from acrylonitrile-exposed nondiabetic group at p < 0.05
cSignificantly different from the diabetic group at p < 0.05

Figure 5. Effects of acrylonitrile and diabetes on cyanide production in hepatic tissue homogenate of normal and STZ-induced diabetic rats.

rats obviously caused more than a 30-fold increase in cyanide production, compared to the control group, an effect that was also significant when compared to ACN-exposed non-diabetic rats.

Discussion

Several worldwide research studies have revealed that environmental pollutants potentially act as a contributing determinant for DM. Chronic exposure to the pesticides and herbicides often creates disturbance in glucose metabolism and is also capable of inducing insulin resistance[23,24]. Moreover, organic pollutants are lipophilic in nature and the likelihood of obesity potentiates the ability of the body to store these toxic chemicals which in turn are proficient in inducing insulin resistance as well as DM type II (DM2) in a dose and perhaps time dependent manner[25,26].

The results of this study decisively illustrate that ACN alone quite significantly produces acute injury to hepatocytes of rats as revealed by an increased level of not only liver enzymes but also the serum levels of total cholesterol and triglycerides. It is noteworthy that these parameters were also augmented in a similar manner in drug induced diabetic rats, thus confirming the detrimental effect of both ACN and DM2 in producing acute hepatotoxicity. Seemingly, diabetic animals exposed to ACN have shown a marked increase in all the parameters of liver injury in comparison to ACN alone treated rats and untreated drug induced diabetic rats, thus confirming the finding so for the relevant studies[22-26].

In addition, a remarkable feature in DM2 is enhancement of oxidative stress which is in turn responsible for several chronic degenerative complications. The prime objective of the treatment of DM is to prevent several chronic degenerative complications which is basically due to the phenomenon of oxidative stress which in turn occurs as a result of excessive formation of free radicals sufficient enough to overcome the detoxification capacity of the cellular antioxidant system[27,28]. A well-established fact in DM is the vulnerability of hyperglycemia as a driving force for the development of well-known diabetic complications[29]. This study remarkably revealed the substantial effect of ACN as well as drug induced DM2 on the parameters of both enzymatic antioxidants which includes depressant activity on the hepatic content of GSH as an antioxidant marker and significant amplification of lipid peroxidation MDA and similar a depressant effect on non-enzymatic antioxidants such as SOD enzymatic activity and CAT activity on both hepatic tissue homogenate of normal and STZ-induced diabetic rats.. Furthermore, these observations were enforced by a marked increase in MDA as lipid peroxidation marker. However, a concomitant effect was revealed on non-enzymatic antioxidants *i.e.,* SOD and CAT. These results are fairly consistent with the observations revealed in several studies[27,28].

Several studies demonstrated significant elevation of CYP2E1 enzymes in different liver disorders and DM[14,30,31]. In addition it was also demonstrated that the expression of CYP1A2 and CYP2E1 was observed to be significantly higher in diabetic rats in comparison to normal rats[32,33].

In in this current study it was noteworthy that CYP2E1 activity was appreciably augmented in both ACN-exposed nondiabetic and diabetic groups, respectively, compared to the control group. However, exposure to ACN in diabetic rats markedly amplified the levels of CYP2E1 enzyme, which has directed the observation of potentiation of harmful effect of ACN in DM.

Finally, it is pertinent to emphasize the consequence of ACN on the hepatic homogenates in this present study, which has revealed negligible formation of cyanide in the absence of ACN in both the control and diabetic groups. However, in nondiabetic

rats significant enhancement of cyanide was observed in comparison to the control group. Likewise, exposure to ACN in diabetic rats obviously caused further augmentation in cyanide production, compared to the control group, an effect that was also significant in comparison to ACN-exposed nondiabetic rats. These findings are splendidly sustained by the findings of several studies illustrating the oxidation of ACN by CYP2E1, which subsequently gets converted to 2-cyanoethyleneoxide (CEO), which is well known as a reactive and a relatively long-lived epoxide[34]. CEO gets in turn metabolized leading to the release of CN- reactive and a relatively long-lived epoxide[38].

Interestingly, the present study results of augmentation of cyanide production by ACN is further authenticated by the studies demonstrating that CN might enhance ACN induced oxidative stress by increasing hydroperoxide generation and lipid peroxidation[35].

Conclusion

Acrylonitrile, a well-known industrial pollutant, is capable of producing acute hepatic injury as well as induction of DM2. In addition, it can also saturation of the CYP2E1 enzyme which sequentially leads to generation of oxidative stress and its metabolic product, cyanide, that poses additional deleterious effects due to potentiation of the oxidative stress.

Conflict of Interest

The author has no conflict of interest regarding the publication of this paper.

Disclosure

The author did not receive any type of commercial support for this study.

Ethical Approval

Obtained by the Bioethical Research Unit of Faculty of Medicine, King Abdulaziz University.

Acknowledgement

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تعزيز سمية الكبد في داء السكري عن طريق ملوث كيميائي عالمي (أكريلونيتريل) في الفئران

سمير عيضة الحارثي
قسم علم الأدوية، كلية الطب
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المستخلص. تم تصميم هذه الدراسة للتحقيق في أضرار الكبد المحتملة بسبب الأكريلونيتريل (CAN) في مرض السكري الناجم عن مركب ستربتوزوسين في الفئران حيث تم تقسيم فئران التجارب الى أربعة وعشرين مجموعة. المجموعة ١: الفئران غير السكرية التي تتلقى الماء المقطر، المجموعة ٢: الفئران غير السكرية التي تتلقى المحلول المائي اكرايلونيترايل (١٠ mg / كغ / يوم)، المجموعة ٣: الفئران التي تتحكم في مرض السكري والتي تتلقى الماء المقطر والمجموعة ٤: الفئران التي لا تتلقى السكري، وتستقبل المحلول المائي اكرايلونيترايل (١٠ mg / كغ / يوم). تلقت جميع المجموعات العلاج لمدة ٤ أسابيع بعد ذلك تم تقييم الحيوانات لمعرفة علامات تسمم الكبد في مصل الدم (AST، ALT، Bilirubin، الدهون الثلاثية والكوليسترول الكلي)، علامات الأكسدة (GSH، MDA، SOD، Catalase)، نشاط CYP2E1 وتشكيل السيانيد في الأنسجة. أكريلونيتريل المصل كانت مرتفعة بشكل كبير AST، ALT، ومستويات البيليروبين الكلي، الدهون الثلاثية في الدم والكوليسترول الكلي في مجموعات السكري بالمقارنة مع مجموعة التحكم الطبيعية. تظهر علامات مضادات الأكسدة مثل GSH في أنسجة الكبد المتجانس انخفاضًا كبيرًا في حين أن هناك زيادة كبيرة في MDA و SOD و CAT في الفئران المصابة بمرض السكري التي عولجت بـ ACN. تم زيادة نشاط CYP2E1 بشكل كبير بنسبة ٢٩٪ و ٢٠٪ في المجموعات غير المصابة بداء السكري والسكري المعرضة لـ ACN بالمقارنة مع السيطرة. تمت زيادة تكوين السيانيد في كلتا المجموعتين غير المصابتين بمرض السكري والسكري بنسبة ٢٠٪ و ٣٠٪ على التوالي بالمقارنة مع المجموعة الضابطة. ACN أحد الملوثات الصناعية المعروفة قادر على إنتاج إصابة كبدية حادة بالإضافة إلى تحريض DM٢، بالإضافة إلى أنه يمكنه أيضًا تنشيط إنزيم CYP2E1 الذي يؤدي بشكل متتالي إلى توليد إجهاد مؤكسد ضار.