Biochemical and Toxicological Evaluation of Atorvastatin and Riboflavin in Diethylnitrosamine Induced Hepatocellular Carcinoma

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FA, AHA and FAAA designed and supervised the study. Authors KM, SS and TJ managed the analyses of the study and performed experiments. Authors KM, SS, AAO and SSR performed the statistical analysis, wrote the protocol and first draft of the manuscript. Authors AHA and KM managed the literature search. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: Atorvastatin, a commonly prescribed drug for the management of hyperlipidemia, acts as competitive inhibitors of HMG-CoA reductase—a rate-limiting enzyme in cholesterol synthesis. On the other hand, riboflavin is also a well-studied micronutrient known for its anti-proliferative, anti-metastatic and antioxidant properties. However, the synergistic or antagonistic effect of both drugs when administered together is not studied yet.
Method: This study was an attempt to evaluate the toxicity/efficacy of atorvastatin (30 mg/kg) in combination with riboflavin in hepatocarcinogenic rats when challenged by a single diethylnitrosamine DENA (160 mg/kg; I.P). Serum ALT, AST, creatine kinase, urea, uric acid, creatinine, bilirubin, albumin, LDL, FT3, FT4, calcium, phosphorus, and triglyceride levels were

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estimated. Histopathology was also performed to study the alterations in the cellular architecture of cardiac and hepatic cells.

Results: Result revealed that DENA significantly plummeted ($P < 0.001$) most of the parameters when compared with normal control. Atorvastatin+Riboflavin group significantly managed to restore the altered parameters like LDH, cholesterol, triglycerides and LDL-C. Nonetheless, this drug combination also caused mild hepatic damage by increasing the ALT, AST, total bilirubin and creatine kinase. The histopathology revealed that liver sample of DENA+ATS+B2 group exhibited severe necrosis with substantial fat depositions and binucleated cells, whereas the heart sample revealed partial detachment of cells with increased intracellular gaps.

Conclusion: It is suggested from current results that this combination therapy was not only unsupportive to hepatocellular carcinoma treatment but damaging to the hepatic and cardiac cells.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is widely spread across the globe. It is a serious health problem, especially in high prevalence regions. Currently, the global incidence of HCC is alarming, but with the new therapies in hepatitis C virus (HCV) and the improvement of hepatitis B vaccine, a gradual decline is observed, and cases of HCC are expected to decrease in the coming decades. Another important issue is the high mortality of the patients with this tumour. Despite, continuous observation of patients with chronic liver diseases, most tumours are diagnosed at the intermediate or advanced stage, due to which only palliative measures are taken. Many reports are available suggesting that cancer cells have developed the alternative mechanism or use cholesterol for cell synthesis, making scientist think in a manner that drugs affecting cholesterol utilization can prevent or slow down the cancer growth. [1,2].

Statins have gained tremendous importance as a drug in present-day life to control hypercholesterolemia, they inhibit HMG-CoA reductase, the main enzyme for cholesterol biosynthesis, with altered LDL receptors (LDLR) activity controlling the blood LDL levels [3]. They inhibit tumor growth through anti-invasive potential, anti-proliferative activity, and support the apoptotic process [4-7]. Further, there are several other cellular-based mechanisms which were tried to explain the anticancer mechanism by statin through cell cycle arrest *via* cyclin-dependent p21 and p27 kinase alteration along with inactivation of Rho A proteins responsible for cell migration with destabilization of actin molecules [8]. Several human studies on statins have proved the anticancer potential of these drugs, where there was a significant reduction in carcinomas be it breast, lungs, colon, prostrate or lymphomas compared to placebo-controlled studies [9,10]. Not all the statins can decrease the incidence of HCC; it is limited to some statins like atorvastatin [11] supported by Lai *et al.* where they have detailed the link between various statins and HCC [12]. Moreover, this drug was studied for multiple adverse effects on animals when given in therapeutic form where it prevented a significant decline in leukocytes count thus controlling the leukopenia in experimental animals. Furthermore, atorvastatin is well associated with protection from calcification, microvascular damage, antioxidant and its anti-apoptotic character [13].

In spite of all such proven records of statin from *in vivo* and *in vitro* studies to some clinical evidence, the effectiveness of these drugs are yet to pass the challenge of HCC; Especially when statins are combined with micronutrients like riboflavin, when there is a limited option for treatment of HCC. Riboflavin, also known as vitamin B2, is a member of vitamin B complex, responsible for the transfer of electrons in electron transport chain mechanism through redox intermediates coenzymes called FMN and FAD [14]. Riboflavin is well studied for its anti-proliferative, anti-metastatic [15] and apoptotic properties on various solid tumors [16], anti-inflammatory through alteration in TNF [17] with p53 alteration [18] have also been proved. Hence, this study attempts to treat DENA induced HCC with atorvastatin in combination with riboflavin and analyze the synergistic or antagonistic effect of this drug combination.

2. METHODOLOGY

2.1 Animals

Albino-Wistar Rats (n=51; weight=100–120 gms; male) were obtained from King Fahad Medical

Research Centre (KSA, Jeddah). They were acclimatized to three minutes of handling daily for initial seven days before the start of the experiment. Rats were accommodated at animal-house of Biochemistry Department; Faculty of Science, King Abdulaziz University at 21 ± 1ºC temperature, relative humidity (60 ± 10%) on a 12-hour light/dark cycle with free access to food and water. The experimental procedures were as per the King Abdulaziz University Research Ethics Committee guidelines which are in compliance with the National Commission on the Ethics of Scientific Research at King Abdulaziz City for Science and Technology.

2.2 Chemicals

Chemicals and reagents including atorvastatin and riboflavin were gifted by Jamjoom Pharmaceuticals Co., Jeddah, Saudi Arabia. Other chemicals and specific surgical was purchased from a local distributor.

2.2.1 Preparation of diethyl nitrosamine (DENA)

DENA solution for liver cancer induction: Hepatocarcinogenesis was induced by a single intraperitoneal injection of diethylnitrosamine (DENA) prepared in phosphate buffer.

2.2.2 Dose & duration of DENA administration

160 mg/kg, single administration.

2.3 Experimental Design

After one week of acclimatization, the fifty-one rats were randomly assigned to eight groups.

Animals in group I: (n = 6) served as normal controls. Rats received a daily gavage of 1 ml/kg of distilled water; hereafter referred as 'Normal control'.

Animals in group II: (n = 6+3) served as disease controls. They were treated with single intraperitoneal injections of (DENA) once at a dose of 160 mg/kg body weight; hereafter referred as 'DENA control'.

Three animals from this group were sacrificed at the termination of the initiation phase (i.e. in the eleventh week) to identify the histological alterations in the liver architecture.

Animals in group III: (n = 6) were subjected to atorvastatin only at a daily dose of 30 mg/kg body weight, for the whole period of the experiment (12 weeks); hereafter referred as 'ATS control'.

Animals in group IV: (n = 6) were subjected to riboflavin (50 mg/kg/day) mixed in water provided daily *ad libitum*, for the whole period of the experiment (12 weeks); hereafter referred as 'B2 control'.

Animals in group V: (n = 6) were subjected to atorvastatin and riboflavin at a daily dose as given to groups III and IV, for the whole period of the experiment (12 weeks); hereafter referred as 'ATS+B2 control'.

Animals in group VI: (n = 6) received DENA treatment as in group II and subsequently administered atorvastatin at a dose of 30 mg/kg of body weight/day for the whole experimental period; hereafter referred as 'DENA + ATS'.

Animals in group VII: (n = 6) were subjected to the same protocol as in group II and subsequently administered riboflavin as group IV for the whole experimental period; hereafter referred as 'DENA + B2'.

Animals in group VIII: (n = 6) were subjected with DENA i.p and followed by the treatment with atorvastatin and riboflavin in combination at a daily dose as given to group III & IV for the whole period of the experiment (12 weeks); hereafter referred as 'DENA+ ATS + B2'.

At the end of the dosing and treatment protocol, rats of all groups were sacrificed; blood-serum, heart and liver tissues were collected for the estimation of different biochemical parameters and histopathological examination, respectively.

2.4 Determination of Biochemical Parameters

On the 90th night, all the rats were kept for 12 hours fasting and next day under mild anesthesia. The blood samples were withdrawn from all the rats, one by one, *via* retro-orbital puncture technique and blood was collected in anticoagulant tubes. The collected blood was centrifuged at 4500 rpm and examined for the biochemical parameters. Triglyceride (TGL), cholesterol, low-density lipoprotein (LDL), aspartate transaminase (AST), alanine transaminase (ALT), uric acid, creatinine, bilirubin, albumin, LDL, FT3, FT4, calcium,

phosphorus etc. were determined using the reported method with minor modification [19,20]

2.5 Histopathology Study

After the blood collection, different organs (heart and liver) were isolated for histopathological analysis. The organs were fixed in 10% neutral buffered formalin, dehydrated by passing through a graded series of alcohol and paraffin infiltration. 5 μm sections were prepared using a semi-automated rotatory microtome and then it was dried at 37ºC overnight. Hematoxylin and eosin were used for staining and, 20x focus was used for images.

2.6 Statistical Analysis

Graph Pad Prism 6 was used for the estimation the statistical analysis. The whole data were expressed as the mean±SEM and analysis of variance (ANOVA). T-test was performed as follows: Normal control versus DENA control, ATS control and B2 control; DENA control versus DENA+ATS, DENA+B2 and DENA+ATS+B2. *P* values of different biochemical parameters were considered to be increasingly significant in the following order <0.05(*), < 0.01(**) and < 0.001(***).

3. RESULTS AND DISCUSSION

3.1 Biochemical Test Reports

3.1.1 Alanine aminotransferase (ALT)

ALT constitutes the essential part of liver function tests and any alteration in ALT is directly implicated in liver function. ATS and B2 controls significantly increased the ALT levels, with *P* values < 0.0001 and < 0.01, respectively. Among the treated groups DENA+ATS+B2 group was found elevated 2X to normal control group (56 ± 1.9 U/L).

3.1.2 Aspartate aminotransferase (AST)

DENA control was significantly ($P < 0.05$) decreased, whereas DENA+ATS and DENA+B2+ATS therapeutic groups significantly ($P < 0.0001$) increased the level when compared to the DENA control. The ALP levels in B2 control and DENA+B2 was observed to be lowered, though this decrease was non-significant when compared to normal and DENA controls, respectively.

3.1.3 Total bilirubin

Despite the narrow range of total bilirubin levels in different groups, there was clear demarcation expressing significant alterations among controls and therapeutic. DENA control was significantly decreased ($P < 0.01$) as compared to normal control (0.064 ± 0.0042 mg/dL). All the three therapeutics groups showed highly significant alteration in the TB level when compared to DENA control.

3.1.4 Blood urea nitrogen (BUN)

The alteration in normal BUN levels could mean that either the kidneys or the liver may not be working properly. The blood urea nitrogen levels in DENA control was slightly increased, but surprisingly all the other control and therapeutic groups revealed significantly low levels of BUN. The B2+ATS control group was observed with the lowest level of blood urea nitrogen (16 ± 0.089 mg/dL). Among all, B2 control and therapeutic groups showed levels which were near to normal levels.

3.1.5 Creatinine

Due to the low standard mean error in all the groups, we can easily make out the variations in the levels of creatinine in different groups, though the range is apparently narrow. The DENA control elevated the creatinine level with high significance ($P < 0.0001$). The combinational therapeutic group (DENA+B2+ATS) significantly normalized the effect of DENA and brought the creatinine level to almost normal.

3.1.6 Uric acid

Uric acid is the end product of purine metabolism. The pathological alteration s of uric acid in blood serum in most patients results for serious clinical implications. It is useful in diagnosing for most of purine metabolic disorders. The effect of atorvastatin on the uric acid is clearly seen in uric acid levels. ATS control group was highly increased as compared to normal ($P < 0.0001$). B2+ATS control also exhibited the highest level (1.5 ± 0.022 mg/dL) of uric acid, unlike all the other groups.

3.1.7 Creatine kinase

Creatine kinase catalyzes the reversible transfer of phosphate from ATP to creatine and also facilitates the energy transfer function. All the
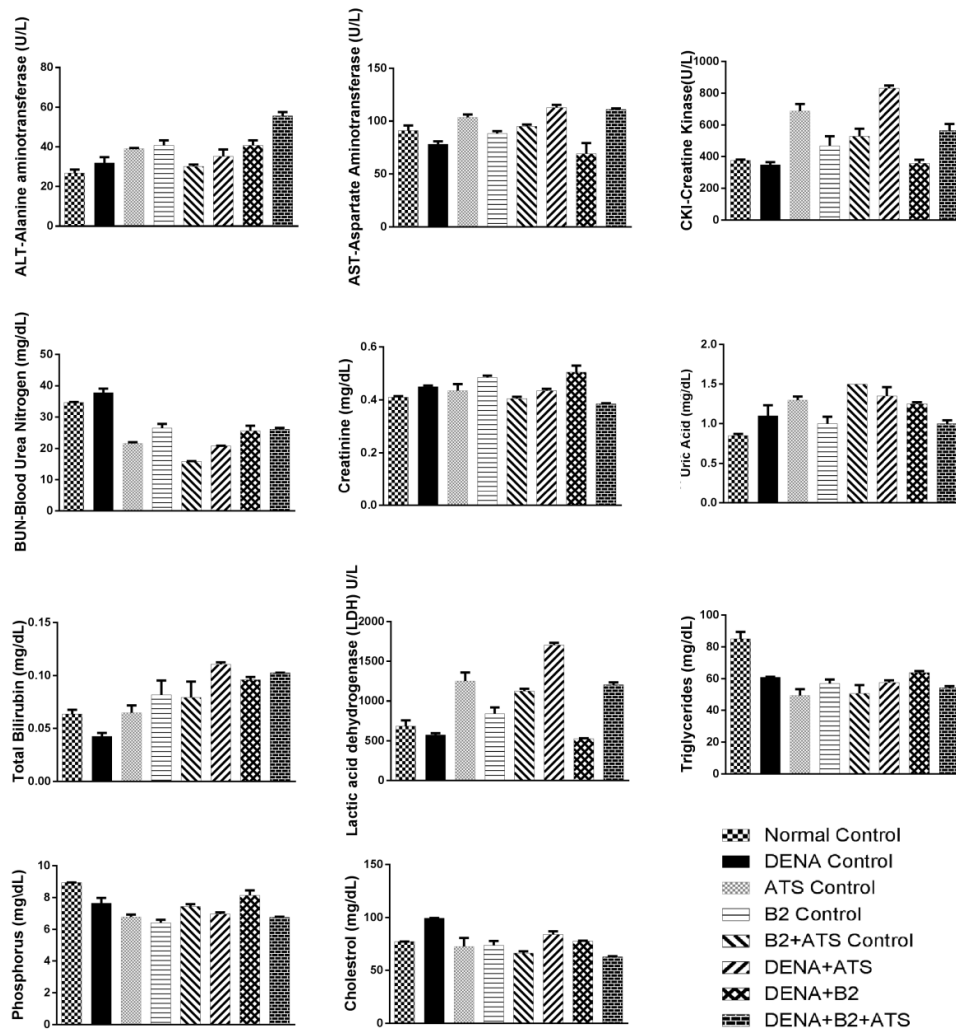
Fig. 1. Effect of atorvastatin and riboflavin therapy on ALT, AST, CK, BUN, creatinine, uric acid, bilirubin, phosphorus, LDH, triglyceride, and cholesterol levels of different groups; DENA, ATS, B2 controls were compared to normal control group; DENA+ATS, DENA+B2 and DENA+ATS+B2 were compared to DENA control group; values are mean ± SEM; n = 6

groups associated with atorvastatin have a significant increase in the level of creatine kinase ($P < 0.0001$). DENA control was observed with near to normal level (349 ± 17 U/L). The ATS control group CK level was almost two times higher (688 ± 44 U/L) and DENA+ATS was found to be with highest level of CK (832 ± 18 U/L) which is approximately three times to that of normal CK level in normal control group (377 ± 5 U/L).

3.1.8 Lactic acid dehydrogenase (LDH)

Lactate dehydrogenase is an enzyme required by cells during the process of turning sugar into

energy. Like CK, LDH levels were also elevated in all the atorvastatin groups. Though DENA alone was not found to elevate the levels of LDH but DENA+ATS was observed with the highest level of LDH (1706 ± 29 U/L; $P < 0.0001$) when compared to other groups in the protocol followed by DENA+B2+ATS (1206 ± 30 U/L), ATS control (1253 ± 109 U/L) and B2+ATS control (1127 ± 29 U/L).

3.1.9 Cholesterol

Atorvastatin as an established cholesterol-lowering drug has significantly affected the cholesterol levels in all the ATS control and

therapeutic groups. The DENA control group was found to be with the highest level of cholesterol (99 ± 0.34 mg/dL) whereas the therapeutic groups lowered the levels significantly (DENA+ATS: 84 ± 3.0 mg/dL, DENA+B2+ATS: 63 ± 0.58 mg/dL)

3.1.10 Triglycerides

Triglycerides level was significantly (P < 0.001) lowered by the DENA control group as compared to the normal control group. Similarly, ATS control also lowered the TG level. Among the therapeutic groups, the combination therapy i.e DENA+B2+ATS was found with the lowest level of TG (54 ± 1.1 mg/dL).

3.1.11 Phosphorus

All the drug controls, individually and in combination, exhibited significant increase (P < 0.001) in the serum phosphorus levels. Though the phosphorus levels were altered in the therapeutic groups as well, none of them turned out efficient in bringing the phosphorus to normal levels.

3.2 Histopathological Study

3.2.1 Liver histopathology analysis

Liver sections from the normal control group exhibited normal liver histology with no evidence of hepatocyte injury or dysplasia or malignancy or fibrosis noticed. DENA control animals revealed central veins surrounded by inflammatory infiltrate and extensive necrosis, clusters of hepatocyte necrosis and the portal tract with marked atypia and bile duct proliferation. The tumor cells resembling hepatocytes show pleomorphism and are seen as 2–8 cell wide trabeculae that are separated by endothelium-lined sinusoidal spaces. The ATS and B2 control groups exhibited mild necrosis and binucleated cellular architecture with very less fat depositions. Moreover, the therapeutics groups barely absorbed the stain due to DENA. The DENA+ATS+B2 group exhibited severe necrosis with substantial fat depositions and binucleated cells (Fig. 2).

3.2.2 Heart histopathology analysis

Fig. 3 reveals the randomly selected heart samples of various controls and therapeutics groups of current protocol. A normal heart cellular architecture with regular muscle tone is observed. No intracellular gaps are seen and a regular pattern of cell arrangement is noticed with minimum fat deposition; DENA Control: The muscle tone is lost with high intracellular gaps; the cells are not connected with each other. Though the fat deposition is less, the intracellular spaces seem to be highly occupied with fat. The heart seems to be in the state of remodeling with eosinophilic infiltration. The ATS and B2 control groups exhibited regular stretches of cells with slight eosinophilic infiltration. The DENA+ATS+B2 group reveals partial detachment of cells with increased intracellular gaps filled with fat.
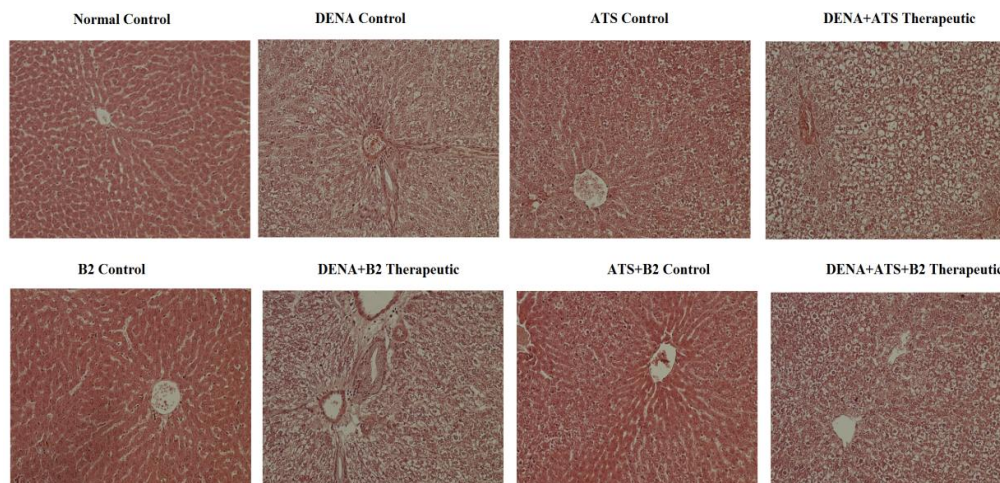

Fig. 2. Liver histopathology samples of all the control and therapeutic groups (randomly selected; H&E stained; 20X)
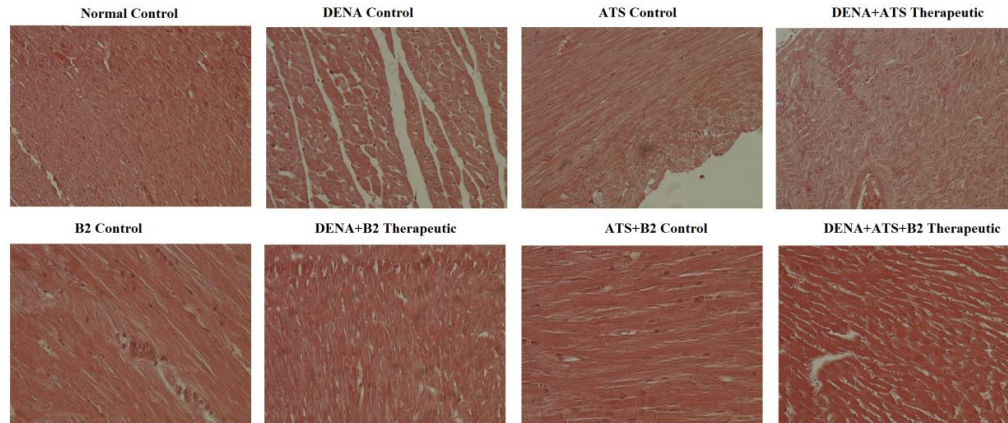
Fig. 3. Heart histopathology samples of all the control and therapeutic groups (randomly selected; H&E stained; 20X)

4. DISCUSSION

The concept of drug-repositioning can reveal potential therapeutic horizons which are promising and are expected to pass the clinical approvals at a faster pace [21]. Recently, atorvastatin—a renowned cholesterol-lowering drug has attracted the attention of many researchers to explore its anti-cancer potential [22-25]. In fact, significant attempts have been made to treat liver cancer and hepatic damage with atorvastatin. In a recent study, Braeuning A et al. have treated DENA induced HCC in C3H/He mice with atorvastatin, but found no positive influence on the tumor growth in the liver [26]. In another study, thioacetamide-induced liver cirrhosis treated with atorvastatin has also revealed similar negative results [27]. In the current study, initially it was hypothesized that atorvastatin in combination with riboflavin might treat HCC due to their cholesterol-lowering and antioxidant properties respectively. Cholesterol is essential for the formation of the cell membrane and cell-division couldn't be accomplished in the absence of cholesterol. Considering the above-mentioned hypothesis, several biochemical tests were performed and histopathology samples of liver and heart were taken to analyze the biochemical variation and cellular morphology of DENA-induced HCC rats. Unexpectedly, the results suggested that this combinatorial therapy was not only unsupportive but detrimental to the hepatic and cardiac cells.

As noted in previous independent studies, both statins and water-soluble vitamins are known to elevate the liver enzymes [28-30] in our study the atorvastatin and riboflavin combination also elevated the ALT levels significantly. Atorvastatin is often neglected in this regard as this elevation in ALT (even up to 3times) is asymptomatic [31]. The riboflavin, though its water soluble and excess of it flow out as free riboflavin, is causing toxicity as it can react with light, resulting in adverse cellular effects [29,30]. Aspartate aminotransferase also known as SGOT is one of the commonly used markers for the evaluation of any toxicity that has been induced in the liver. This enzyme generally elevates and squeeze out of the liver cells during necrosis or hepatitis whether chronic hepatitis or cholecystitis hepatitis [32]. In contrast, this study revealed mild reduction level of AST in the DENA control group this is a unique observation which is not evidenced in earlier studies.

Total bilirubin level commonly elevates after DENA exposure in the experimental animals [32]. Lower bilirubin levels are usually not a health concern, but higher levels are. All the three therapeutics groups showed a highly significant increase in the TB level when compared to DENA control; this expresses the combined toxicity of all the drugs together on hepatic cells. In all the groups the fall of urea corresponds to the excess discharge of nitrogen, indicating the overwork of kidneys or load on kidneys plus disturbance in metabolism of nitrogen. This may relate to alteration in parathyroid hormone [33] by influence by atorvastatin. The fall may be due to nephrotoxic effect in this dose where inhibition of p38-mitogen-activated protein kinase (MAPK), as well as nuclear factor kappa-β (NF-κβ) signaling pathways, and inducible nitric oxide synthase (NOS) expression is altered [34].

The preclinical studies unfold that statins may help in the prevention of certain type of cancers such as prostate, but the population and epidemiological studies gave contradictory results [35]. The molecular mechanism underlying this phenomenon has not been clearly understood, except one hypothesis that relates ATP alteration in cells to the detrimental effects of statin. The modulation of ATP level is a biomarker in the diagnosis of muscular myopathy in animal tissues which is generally associated with statin therapy [36]. Any alteration in the ATP content will disturb the metabolic processes of normal or cancer cells with the disruption in the respiratory chain of mitochondria [37]. Additionally, Knauer et al. have also reported a noticeable decrease in the ATP content of both cell lines and animal tissue are found when atorvastatin is administered with some adverse cardiac condition [36].

Currently, the micronutrients like vitamins have fascinated researchers in designing effective therapeutic regimens in chemoprevention of many tumors; in particular, vitamin B9 (also known as folic acid or folate) antimetabolites can target the dihydrofolate reductase [38]. It is well documented by *in-vivo* and *in-vitro* studies that folate is an important co-factor required for the biosynthesis of methyltransferase to obtain purine bases to meet the demands of rapidly proliferating tumor cells [39]. Riboflavin is a biochemical precursor of FAD and FMN that are involved in the electron transport chain to generate ATP. Presently, the mitochondrial enzymes NDUFV1, NDUFV2 and NDUFV3 [40] of complex I associated with vitamin B2 are promising targets to inhibit cell division. Many FDA approved drug molecules are being tested currently to explore this mechanism [41]. Furthermore, it is important to mention that deficiency of riboflavin may significantly inhibit the proliferation of breast and colon cancers in mice models [42]. On the contrary, the excess of riboflavin may enhance the cell division of cancer cells as vitamins cause a manifold increase in the biosynthesis of nucleotides, replication of DNA, the supply of methyl-groups, growth and repair of the cells. Aberrant dysregulation in all these biological processes have been implicated in the development of carcinogenesis [43].

Altogether, these evidences suggest that intake of riboflavin could enhance the level of ATP synthesis and subsequently promote the cell division of tumor cells in DENA-induced HCC rat model. Furthermore, this excess ATP inhibits the statin activity and initiate the de novo synthesis of the fatty acid and cholesterol by the cancer cells by an alternate pathway [44]. However, the precise molecular pathways remain elusive which can be explored by the application of latest technologies by 'omics' approach.

5. CONCLUSION

Ironically, this study was an attempt to treat HCC with the atorvastatin and riboflavin combination; however, the results obtained, infer otherwise. The significant increase in the serum parameters like ALT, AST, total bilirubin and creatine kinase by the ATS+B2 combination therapy has led us to conclude it to be liver damaging supported by the histopathology results.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental procedures were as per the King Abdulaziz University Research Ethics Committee guidelines which are in compliance with the National Commission on the Ethics of Scientific Research at King Abdulaziz City for Science and Technology.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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