International Journal of Basic & Clinical Pharmacology
Hewedy WA et al. Int J Basic Clin Pharmacol. 2023 Jan;12(1):1-9
http://www.ijbcp.com
pISSN 2319-2003 | eISSN 2279-0780

DOI: https://dx.doi.org/10.18203/2319-2003.ijbcp20223348

Original Research Article

Oncogenic challenge of bromocriptine and L-arginine versus conventional antidiabetics on diethyl nitrosamine-induced liver tumorigenesis in diabetic rats: focus on AMPK activation

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Received: 15 October 2022
Accepted: 04 November 2022

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ABSTRACT

Background: Diabetes mellitus (DM) is associated with a spectrum of cancers where the metabolic antecedents, consequences, and therapy might affect cancer risk. The association between hepatocellular carcinoma (HCC) and DM had been confirmed. Approaches to HCC prevention focus on the molecular regulators of the disease process defined as the inflammation-fibrosis-cancer axis. The AMP-activated protein kinase (AMPK) is an interesting metabolic tumor suppressor and a promising target for cancer prevention and therapy. This study aimed to investigate the effects of bromocriptine mesylate and L-arginine on hepatic carcinogenesis on a rat model of hepatic neoplasia induced by diethyl nitrosamine (DENA) and promoted by type-2 DM in contrast to the conventional antidiabetics.
Methods: One hundred male Wistar rats were randomly assigned into two sets; control set (normal, HCC, DM, and combined HCC/DM) and treated set where rats received one of the following drugs for another 5 weeks: insulin glargine, glimepiride, metformin, pioglitazone, bromocriptine mesylate, or L-arginine. Bodyweight changes, blood glucose level, liver functions tests, serum C-peptide and alpha-fetoprotein (AFP), and hepatic activated AMPK were assessed beside the hepatic histopathological changes.
Results: Equivalent to metformin, bromocriptine and L-arginine treatment significantly reduced AFP, despite their minor glycemic control. L-arginine induced AMPK activation, yet less than metformin. Histopathologic examination revealed a reduction in hepatic intra-lobular chronic inflammatory cell infiltration, steatosis and necrosis by metformin, bromocriptine, and L-arginine. Hepatic necro-inflammatory changes were most prominent in insulin-treated rats.
Conclusions: L-arginine and bromocriptine mesylate prevent early neoplastic changes almost equivalent to metformin at least partially via hepatic AMPK activation.

Keywords: Hepatocellular carcinoma, Insulin, Metformin, T2DM

INTRODUCTION

Diabetes mellitus (DM) is a globally endemic disease with a large economic impact on the health care system. In addition to its known complications, DM has been recognized as a key factor contributing to the development of solid organ malignancies; most notably those of the liver and pancreas.[1] Observational clinical studies have confirmed a higher prevalence of hepatocellular carcinoma (HCC) in diabetic patients and an increased incidence of diabetes in patients with HCC.[1,2] The pathogenic sequence of events leading to HCC in diabetic state is thought to be related to the detrimental effects of insulin resistance, and oncogenic

effects of hyperglycemia.[3] Insulin resistance promotes lipolysis and release of free fatty acids (FFAs) from adipose tissues, which are accumulated in hepatocytes producing hepatic steatosis. Additionally, mitochondrial oxidative stress increases, which in turn stimulates inflammation and cellular necrosis. Reactive oxygen species (ROS) contribute to the development and activation of pathways involved in both fibrotic and oncogenic processes. Kupffer cells-derived ROS initiate pro-inflammatory effects and sensitize hepatocytes to undergo apoptosis. On the other hand, ROS-derived from the non-phagocytic form of NADPH oxidase participate in the activation of the fibrogenic actions of hepatic stellate cells (HSCs).[4] The abovementioned processes are orchestrated by the known diabetic inflammatory milieu that upregulates proinflammatory cytokines and downregulates the anti-inflammatory ones.[5] The interplay of these variables leads to cell damage followed by fibrosis, proliferation, and finally the development and progression of HCC. This process is known as the inflammation-fibrosis-cancer (IFC) axis, which links inflammation with cancer via promoting the progression of the inflamed liver to various stages of fibrosis and finally HCC.[1,6] The adenosine monophosphate-activated protein kinase (AMPK) constitutes an important target for metabolic disorders and cancer. It is a principal regulator of energy homeostasis at cellular levels. AMPK activation stimulates fatty acid oxidation, enhances insulin sensitivity, alleviates hyperglycemia and hyperlipidemia, and inhibits pro-inflammatory changes.[7]

Pertinently, the effect of AMPK on glucose uptake occurs independently of insulin and could circumvent the insulin-stimulated glucose uptake defects in insulin resistant individuals. Additionally, AMPK is considered, among others, an important downstream signalling pathway of insulin growth factor-1 receptor (IGF-1R).[8] Beyond its critical roles in re-programming metabolism, AMPK has been reported to regulate cell growth and proliferation.[7] AMPK down regulates mTORC1 which is known to play key role in the regulation of cell growth and division, and act as a negative regulator of autophagy.[9,10] Hence, hepatic AMPK activation may represent a pharmacological tool in cancer prevention and treatment.[11] It is still a matter of controversy whether conventional antidiabetic drugs can affect the risk and the prognosis of HCC. The liver cancer risk is reported to be higher among patients using insulin than those using metformin.[1,12] Bromocriptine mesylate is a D_2-dopamine agonist that was approved for the treatment of type-2 DM.[13] It modulates central glucose and energy metabolism pathways, thus reducing plasma glucose, triglycerides and FFA levels, and possibly cardiovascular events.[14] Interestingly, bromocriptine seems to act also by a dopamine receptor-independent mechanism as it inhibits glucose-stimulated insulin secretion through direct activation of the pancreatic α_2-adrenergic receptors.[15] This action adds an advantage in preserving pancreatic β-cell mass, thereby preventing long-lasting insulin hypersecretion and subsequent β-cell failure. L-arginine is a nitric oxide precursor that stimulates β-cell glucose consumption and intermediary metabolism. It exerts antioxidant and protective responses on β-cells challenged with proinflammatory cytokines.[16] Moreover, its chronic oral administration is capable of promoting NO production that ameliorates endothelial dysfunction and insulin sensitivity in type-2 diabetic patients.[17] In this context, we aimed to evaluate the effect of bromocriptine and L-arginine, in reference to conventional oral antidiabetic drugs or insulin glargine, on hepatic AMPK and AFP levels, hepatic function and structure changes in a rat model of hepatic neoplasia induced by diethyl nitrosamine and promoted by type-2 DM.

METHODS

Animals

A total of one hundred male Wistar rats (150-180 g) were adopted from the animal experiment centre of Alexandria university. Animals were maintained in a 12h light/dark cycle with free access to standard rat chow and water. Animals received care in compliance with the ARRIVE guidelines for laboratory animal care, and protocol was approved by the local ethical committee, Faculty of Medicine, Alexandria University.

Chemicals and drugs

Diethyl nitrosamine (DENA), streptozotocin (STZ), bromocriptine mesylate and protease inhibitor cocktail were obtained from Sigma, USA (N0258, S0130, B1150000, and P2714, respectively). For drug administration, L-arginine (General Nutrition Corporation, GNC), Metformin (Glucophage® 500 mg/tablet, Bristol-Myers Squibb), Pioglitazone (Actos® 15 mg/tablet, Abbott - Egypt), Glimepiride (Amaryl® 4 mg/tablet - Aventis), and Insulin glargine (Lantus® 100 IU/ml vial - Aventis) were used. All other chemicals were analytical graded commercial products.

Experimental design

After being acclimatized for two weeks, animals were randomly divided into two sets. The first control set (N=40) was further subdivided into four groups (N=10); normal control and DENA control groups that were fed on standard diet (SD) throughout the study and received a single intraperitoneal (IP) injection of citrate buffer (0.1 M, pH 4.5) at the end of 5th week. DENA control group received a single IP injection of DENA; 125 mg/kg at the end of 2nd week.[18] DM control group was fed on high-fat diet (HFD) (7.74 KJ /g diet as fat) for 5 weeks and then they received a single IP injection of STZ; 50 mg/kg after an overnight fasting.[18] Similarly, DENA/DM control group was fed on HFD for 5 weeks then received both DENA and STZ IP injection in same dose and schedule as the 2 previous groups. The four groups received 2 % gum acacia 1 ml/day orally for another 5 weeks. The second treated set of animals (N=60) were fed on HFD.

Hepatic neoplastic changes and type-2 DM were induced by injection of DENA and STZ at the end of 2nd and 5th weeks, respectively, as previously mentioned. For the next 5 weeks, rats were randomly subdivided into 6 groups (N=10) according to the drug treatment: insulin glargine 12.5 U/kg/day; subcutaneously (SC), glimepiride 0.5 mg/kg/day per oral (PO), metformin 500 mg/kg/day po, pioglitazone 10 mg/kg/day po, bromocriptine mesylate 10 mg/kg/day po, or L-arginine 500 mg/kg/day po. The selected doses were chosen based on previous literatures.

Experimental procedure and tissue sampling

Forty-eight hours post-STZ injection, glycemic levels were assessed by tail-vein sampling using a glucometer device (One Touch, Johnson & Johnson, USA). Rats with fasting blood glucose (FBG) of >180 mg/dl were included in the study. Individual body weights were recorded weekly, and animals were closely surveyed for pre-set humane endpoints including weight loss, abnormal motility or behavior, and diarrhoea. By the end of the 10th week, animals were weighed, anesthetized with thiopental sodium (45 mg/kg) after overnight fasting and blood samples were collected by cardiac puncture in serum gel containing tubes. Blood samples were then centrifuged at 3000 rpm for 10 min. to separate serum and stored at -20˚C for further biochemical analysis. Livers were rapidly dissected, rinsed with ice-cold phosphate buffered saline (PBS, pH 7.4), weighed, and divided into two parts. The first part was homogenized in PBS containing protease inhibitor cocktail at 4°C, sonicated for 10 min., centrifuged at 3000 rpm for 15 min., and aliquots of supernatant were stored at -80°C for measurement of the activated hepatic AMPK. The other part of the liver was kept in buffered formol-saline (10%) for histopathological examination.

Biochemical estimates

Fasting blood glucose and glycosylated hemoglobin: FBG was measured according to the glucose oxidase peroxidase method using a colorimetric kit (Diamond Diagnostic, USA). Glycosylated hemoglobin (HbA1c) determination was measured based on the turbidimetric inhibition immunoassay (TINIA) method for hemolyzed whole blood.[19]

Liver function tests: as biomarkers of liver injury, serum aminotransferases; alanine amino-transferase (ALT) and aspartate aminotransferase (AST), were assayed using colorimetric kits (Diamond Diagnostic, USA) according to the methods described by Reitman and Frankel.[20] Results are expressed as units per liter (U/l). Alkaline phosphatase (ALP) activity was also measured using colorimetric kit (Biodiagnostic), where the ALP enzyme catalyzes the conversion of alkaline phosphate into phosphate and phenol.[21] The results are expressed as units per liter (mU/ml). Serum C-peptide and alpha-fetoprotein: serum C-Peptide was determined based on the principle of competitive enzyme immunoassay (EIA) using commercially available kit (BioVision, USA) and serum Alpha-Fetoprotein (AFP) was estimated using rat ELISA kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. Results were expressed as pg/mL and ng/mL for serum C-peptide and AFP, respectively.

Hepatic AMPK assay: hepatic AMPK was measured in the supernatants of hepatic homogenates using ELIZA kit (Invitrogen™ AMPK alpha-1,2 (Phospho)-pT172 ELISA Kit, USA). The AMPK concentration was expressed as pg/mg total protein. Protein concentration was determined using purified bovine serum albumin as standard, according to Lowry et al.[22]

Histopathological assessment of hepatic tissue: paraffin blocks of hepatic tissues were sectioned at 5 µm thick, de-paraffinized and stained with hematoxylin and eosin (H&E) for histopathological assessment.

Statistical analysis

Results were processed using Graph Pad Prism (version 7.0) software and data were expressed as means ± S.E.M. Shapiro-Wilk test was used to investigate normality within groups. Being normally distributed, data were analyzed with One-way analysis of variance test (ANOVA), followed by post-test Tukey's multiple comparison to assess the significance among groups. The percent change in mean rat body weight was analyzed using Bland-Altman % difference test. Correlation analysis was done using Pearson's correlation analysis, $p<0.05$ was considered significant difference.

RESULTS

Whole body and liver weight changes

By the end of 10th week, both normal and DENA control groups showed a significant increase in their mean body weight, while the DM and DENA+DM control groups showed a significant decrease in their mean body weight compared to their basal values ($p<0.05$). Treatment with insulin, glimepiride, pioglitazone, and bromocriptine for 10 weeks was associated with a significant increase in the mean body weight for their corresponding groups. Their percent weight differences were significantly higher compared to DENA+DM control rats ($p<0.001$). Despite the significant change in the mean body weight of rats treated with metformin and L-arginine by the end of 10th week, they showed less significant increase in the percent weight difference compared to DENA+DM control rats ($p<0.05$) (Table 1). The mean liver/body weight ratio was significantly increased in DM and DENA+DM control rats compared to normal or DENA control groups ($p<0.05$). Treatment with glimepiride, metformin, pioglitazone, bromocriptine, and L-arginine was associated with a significant reduction in the liver/body weight ratio versus DENA+DM control group ($p<0.05$).

Moreover, both metformin and L-arginine treated rats showed a significant decrease in their liver/body weight ratio compared to glimepiride, pioglitazone, and bromocriptine treated groups ($p<0.05$). Conversely, a non-significant change in liver/body weight ratio was associated with insulin treatment ($p>0.05$) (Table 1).

Table 1: Effects of bromocriptine and L-arginine versus insulin and oral anti-diabetic drugs on physical and metabolic parameters.

Parameters	Body weight	Liver/body weight ratio	FBG (mg/dl)	HbA1C level %	C-peptide (pg/ml)
Normal					
0 week	181.4±2.583	-	-	-	-
5th week	-	-	72.70±0.869	-	-
10th week	281.0±3.130*	2.733±0.084	73.48±0.668	3.490±0.139	247.4±1.447
DENA					
0 week	183.8±1.788	-	-	-	-
5th week	-	-	76.40±1.416	-	-
10th week	245.5±2.535*	3.121 ±0.091	75.60±0.893	2.510±0.13*	248.7±1.585
DM					
0 week	180.5±0.969	-	-	-	-
5th week	-	-	351.0±1.28*	-	-
10th week	141.9±1.337*	4.016±0.15*	240.4±1.739*	11.12±0.12*	159.1±1.50*
DENA+DM					
0 week	187.3±2.324	-	-	-	-
5th week	-	-	302.5±2.94*	-	-
10th week	136±1.317*	6.108±0.14*	252.2±0.98*	8.170±0.25*	166.4±0.83*
Insulin					
0 week	178.9±1.552	-	-	-	-
10th week	252.3±1.21#ϕ	6.417±0.10ϕ	121.9±1.34#ϕ	4.790 ±0.18#	167.1±0.72
Glimepiride					
0 week	172.2±1.692	-	-	-	-
10th week	229.1±1.426#ϕ	5.441±0.10#ϕ	111.2±1.42#ϕ	5.310±0.19#ϕ	228.9±1.41#ϕ
Metformin					
0 week	173.2±1.162	-	-	-	-
10th week	139.5±2.045#	2.811±0.10#	98.33±0.77#	4.480± 0.17#	167.2±0.904
Pioglitazone					
0 week	174.1±1.320	-	-	-	-
10th week	240±1.033#ϕ	4.054±0.08#ϕ	132.0±1.42#ϕ	7.010±0.23#ϕ	169.5±2.540
Bromocriptine					
0 week	181.3±0.966	-	-	-	-
10th week	230.9±1.581#ϕ	3.680±0.12#ϕ	121.4±1.06#ϕ	6.850±0.13#ϕ	164.4±0.81
L-arginine					
0 week	179.4±1.002	-	-	-	-
10th week	200.3±1.165#	2.946±0.14#	131.3±1.56#ϕ	7.610 ± 0.13ϕ	165.3±0.68

*Statistically significant at $p\leq0.05$ compared to normal control, #Statistically significant at $p\leq0.05$ compared to DENA+DM, ϕ: Statistically significant at $p\leq0.05$ compared to metformin.

Effect of antidiabetic drugs on metabolic parameters

By the end of the second week, there was no significant difference in the mean FBG among all groups (data not shown). At the end of the 5th week, there was a significant increase in FBG in DM and DENA+DM control groups versus normal or DENA control groups ($p<0.05$) and remained significantly increased up to the end of the experimental period ($p<0.05$) (Table 1). Similarly, the mean HbA1c % of DM and DENA+DM control groups was significantly increased in comparison to normal control ($p<0.001$) at the end of 10th week, however, the mean HbA1C% of DENA group was significantly decreased versus other control groups ($p< 0.05$). The FBG of all treated groups showed non-significant changes compared to DENA+DM by the end of 2nd and 5th weeks (data not shown). However, by the end of the 10th week, all treated groups showed a significant FBG reduction compared to DENA+DM control group being lowest with metformin treatment ($p<0.001$). The HbA1c % of all treated groups showed significant reduction compared to DENA+DM control

(p<0.05), in exception to L-arginine treated group (p>0.05) (Table 1). Serum C-peptide was significantly reduced in DM and DENA+DM control groups as compared to normal control (p<0.05). Nevertheless, only glimepiride treatment resulted in a significant increase in serum C-peptide compared to DENA+DM control (p<0.05) (Table 1).

Table 2: Effects of bromocriptine and L-arginine versus insulin and oral anti-diabetic drugs on liver function parameters and serum AFP levels.

Groups	ALT (U/ml)	AST (U/ml)	ALP (U/l)	AFP ng/ml
Normal	43.53±0.541	38.78±0.866	93.69±2.751	0.600±0.057
DENA	55.63±1.45*	57.22±1.070	118.4±1.62*	4.910±0.53*
DM	50.23±0.98*	50.44±1.331	136.8±0.66*	0.770±0.052
DENA+DM	128.8±1.50*	126.2±1.36*	240.6±1.15*	24.18±1.22*
Insulin	123.3±1.34*ϕ	120.9±0.69*ϕ	136.4±1.29#ϕ	13.58±0.80#ϕ
Glimepiride	131.6±1.89*ϕ	119.7±0.81*ϕ	148.4±1.31#ϕ	3.17±0.27#
Metformin	47.66±0.64#	38.7±0.37#	104.8±2.80#	0.93±0.233#
Pioglitazone	55.63±1.45#ϕ	48.9±1.67#ϕ	126.1±1.19#ϕ	1.84±0.323#
Bro9mocriptine	54.10±1.05#ϕ	38.9±0.68#	119.9±1.65#ϕ	0.76±0.11#
L-arginine	50.23±0.98#	44.4±0.92#ϕ	105.9±1.85#ϕ	1.17±0.18#

*Statistically significant at p≤0.05 compared to normal control, #Statistically significant at p≤0.05 compared to DENA+DM, ϕ: Statistically significant at p≤0.05 compared to metformin.

Effect of antidiabetic drugs on liver function tests

There was a significant increase in serum ALT and ALP activity of both DENA control group and DM control group (p<0.05) in comparison to the normal control group, while a highly significant increase was noticed in DENA+DM control group (p<0.01) as depicted in (Table 2). The serum AST activity, on the other hand, was significantly increased in DENA+DM control group only in comparison to the normal control (p<0.01). Treatment with metformin, pioglitazone, bromocriptine, and L-arginine was associated with a significant reduction in serum ALT and AST activity in comparison to DENA+DM group (p<0.05). On the other hand, all treatment exhibited a significant reduction in serum ALP activity in comparison to DENA+DM group (p<0.05). It is worthy to note that there was no statistical difference in the serum ALT between the metformin-treated group and L-arginine-treated group, and in serum AST between metformin-treated group and the bromocriptine-treated group (p<0.05).

Effect of antidiabetic drugs on serum AFP

As a marker of hepatic damage or oncogenesis, serum AFP was significantly increased in both DENA and DENA+DM control groups as compared to normal control (p<0.001). All treated groups revealed a significant reduction in AFP compared to DENA+DM control group (p<0.001). Nevertheless, treatment with insulin showed a significantly increased level of AFP compared to metformin (p<0.05) (Table 2).

Effect of antidiabetic drugs on activated AMPK

Hepatic phospho-Thr-172 AMPK concentration (AMPK) was significantly decreased in DENA, DM, and DENA+DM groups versus normal control (p<0.01). A significant increase in the phosphorylated AMPK concentration in all drug-treated groups was observed versus DENA+DM control group, (p<0.05) being significantly higher in metformin treated group versus all other treated group (p<0.05) (Figure 1).

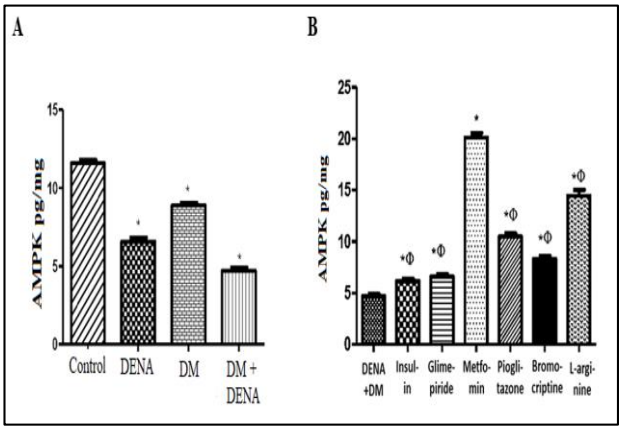

Figure 1: Effect of antidiabetic drugs on hepatic phospho-Thr-172 AMPK concentration. A) The hepatic phospho-Thr-172 AMPK concentration of normal control group (control), Diethyl nitrosamine control group (DENA), diabetes mellitus control group (DM), and DENA+DM control group; *statistically significant at p≤0.05 compared to normal control group. B) the hepatic phospho-Thr-172 AMPK concentration of the DENA+DM drug-treated groups. *Significant difference versus disease control (DENA+DM), Φ: significant difference versus Metformin-treated group. Data are analyzed by ANOVA followed by Tukey criterion as a post-hoc test and expressed as means±SD.

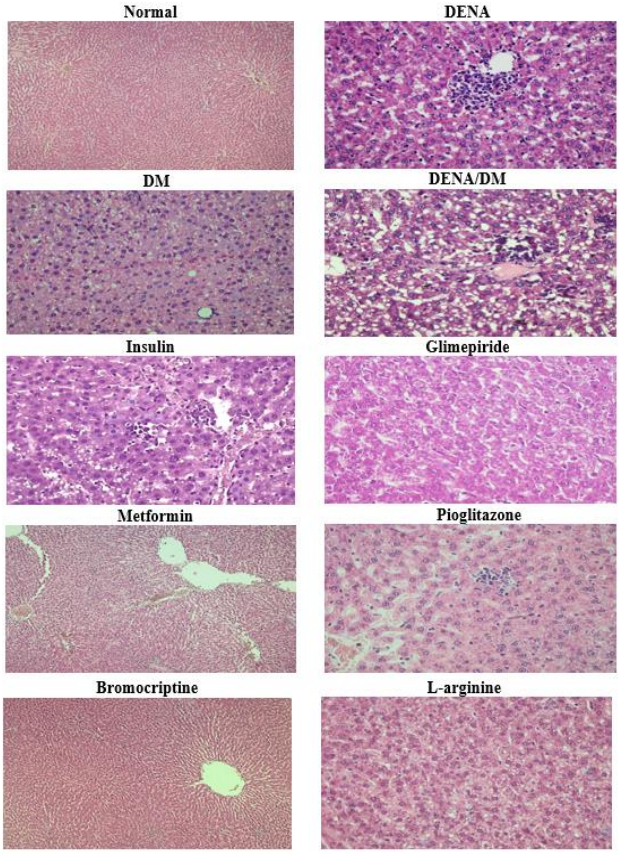

Figure 2: Photomicrographs of hematoxylin and eosin-stained liver sections.

Images shows normal liver architecture in the normal control group (H&E×100), intra-lobular lytic necrosis, peri-central chronic inflammatory infiltrate, and congested sinusoids in DENA control group (H&E×400), micro- and macro-vesicular steatosis and hepatocellular ballooning in DM control group (H&E×400), and macro-vesicular steatosis and multiple foci of lobular lytic necrosis in DENA+DM control group (H&E×400). Treatment of DENA+DM animals with insulin glargine (12.5 U/kg/day) shows foci of intra-lobular and portal inflammation, and areas of macro-vesicular steatosis and sinusoidal congestion. Treatment with glimepiride (0.5 mg/kg/day orally), metformin (500 mg/kg/day orally), pioglitazone (10 mg/kg/day orally), bromocriptine (10 mg/kg/day orally), and L-arginine (500 mg/kg/day orally) show preservation of hepatic lobular architecture with mild chronic inflammatory infiltrate in glimepiride treated group (H&E×400), congestion of central veins and foci of intra-lobular lytic necrosis in metformin-treated group (H&E×100), chronic inflammatory infiltrate in pioglitazone-treated group, diffuse micro-vesicular steatosis and mild peri-central and portal chronic inflammatory infiltrate in L-arginine-treated group (H&E×400), and more apparent preservation of lobular architecture in bromocriptine-treated group (H&E×100). DENA; Diethyl nitrosamine (125 mg/kg, IP at the end of 2nd week), DM; diabetes mellitus induced by STZ (50 mg/kg, IP) at the end of 5th weeks and high fat diet for 5 weeks after STZ injection.

Liver histopathological examination

In contrast to the normal hepatic architecture, microscopic examination of liver sections from the disease control groups revealed evidence of chronic inflammation and pre-neoplastic changes. Hepatic sections from DENA control rats showed intra-lobular lymphocytic infiltration, intra-lobular lytic necrosis, focal macro- and micro-vesicular steatosis, and mild sinusoidal congestion (Figure 2). Sections from DM control showed micro- and macro-vesicular steatosis, and hepatocellular ballooning with areas of intra-lobular necrosis that varied from spotty necrosis to areas of confluent necrosis. Likewise, sections from DENA+DM control revealed more distortion of hepatic architecture evidenced by moderate macro-vesicular steatosis, marked congestion and dilatation of hepatic sinusoids, and multiple foci of lytic necrosis. Marked intra-lobular inflammations as well as portal infiltration by chronic inflammatory cells were also seen (Figure 2).

Insulin treated rats showed mild macro-vesicular steatosis and sinusoidal congestion, and areas of marked intra-lobular and portal inflammation were detected. However, sections from glimepiride, metformin, pioglitazone, bromocriptine, or L-arginine treated rats showed preservation of lobular architecture, with a reduction in central veins congestion and chronic inflammatory infiltrate, in exception to pioglitazone sections that showed focal lobular necrosis with few foci of confluent necrosis (Figure 2).

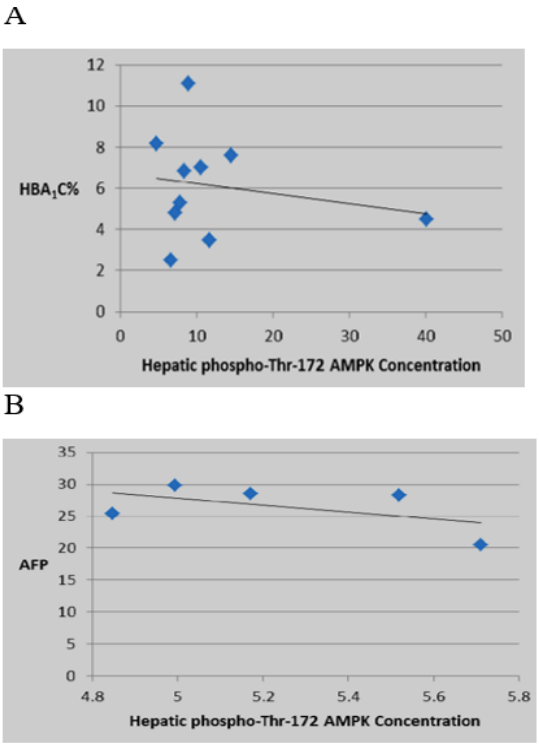

Figure 3: Correlation between hepatic phospho-Thr-172 A) AMPK concentration and HBA1C% and B) serum AFP concentrations.

Correlations studies

The correlation between hepatic phospho-Thr-172 AMPK concentration and all studied variables in all

groups is depicted in (Figure 3). The hepatic phosphor-AMPK concentration was found to be negatively correlated with HbA1c% ($r = -0.479$, $N=100$, $p<0.01$) and serum AFP ($r = -0.322$, $N=100$, $p<0.001$).

DISCUSSION

Hepatocellular carcinoma (HCC) is a serious healthcare problem by virtue of its increasing morbidity and mortality. Epidemiological studies point to the correlation between DM and increased incidence of malignancies, particularly HCC. Moreover, the presence of DM in a HCC patient is associated with a worse prognosis.[2,23] The use of chemical-induced HCC models allows a broader understanding of different pathways in HCC development.[24] Accordingly, in the present study we adopted the modified DENA model for HCC in which DENA (an initiator) is combined with type-2 DM (as a promotor) to allow exploring the early events involved in cancer formation and the role of the studied drugs on such events. Our results showed a significant increase in AFP serum level in DENA control group, being highly significant in DENA+DM group. A fluctuation of rat serum AFP according to the degree of tissue injury or drug-induced healing potential was previously reported.[18] However, AFP level was non-significantly changed in DM group, which could be explained by the absence of the initiator, where DM alone cannot induce a high liver cell turnover or hepatocellular epithelial to mesenchymal transition.

In accordance to previous studies, we reported a significant reduction in the hepatic AMPK in DENA, DM, and DENA+DM control groups. Upon exposure to chronic hyperglycemia, AMPK activity is down-regulated by glucose-dependent activation of ATP synthesis and consequent changes in AMP/ATP ratio.[25] The downstream components of AMPK pathways contribute to the stabilization of mitochondrial membrane potential essential for hepatocyte survival and the suppression of hepatocyte apoptosis induced by hyperglycemia and oxidative stress.[26,27] Moreover, the inhibition of AMPK has been shown to enhance the transforming growth factor (TGF) β-SMAD3 signaling, as well as the platelet derived growth factor (PDGF)-stimulated proliferation and migration of HSC.[28] The observed minimal reduction in serum AFP and liver functions in the insulin-treated group could be attributed to the prompt insulin-induced glycemic control.[29] Insulin glargine is known to exert metabolic effects comparable to that of human insulin, but it displays a greater affinity toward IGF-1 receptor.[12] High IGF-1 and insulin levels in the microenvironment are known to mediate anti-apoptotic signaling and metabolic reprogramming by activation of many intracellular signaling pathways such as PI3K-AKT-mTORC1 and Ras/MAPK/ ERK-1/2 that could exert important roles in induction of carcinogenesis.[30]

Theoretically, sulfonylureas were hypothesized to have possible pro-cancerous effects through increasing insulin level and activating the IGF-1/IGF-1R pathway.[31] Nevertheless, an anti-cancerous rather than a pro-cancerous effect of sulfonylureas has been previously suggested.[32] Notably, modulation of K_{ATP} channels by sulfonylureas could regulate proliferation and migration of tumor cells through resting potential depolarization.[33] The present study showed a significant elevation of the hepatic AMPK concentration accompanied with a significant reduction in liver enzymes and serum AFP, achieving near normal control values, in metformin- and pioglitazone-treated rats. The histological results further supported the favorable effects of both drugs. Metformin and PPAR-γ agonists are documented to indirectly activate AMPK via inhibition of the mitochondrial respiratory chain.[34] The latter effect promotes a switch from aerobic to anaerobic glycolysis, increasing the AMP/ATP ratio and promoting AMPK activation. In turn, AMPK activation exerts a metabolic tumor suppressant effect through the regulation of glucose, lipid, and protein metabolism. AMPK also modulates the activity of a tuberous sclerosis complex-2 (TSC2) that forms a tumor suppressor complex with TSC1 to inhibit mTORC1.[35] The latter regulates protein synthesis and cell survival by directly activating two important targets involved in this process, S6 kinase and translation initiation factor 4E binding protein.[7] Additionally, activation of AMPK by energy shortage reprograms cellular metabolism and enforces a metabolic checkpoint on the cell cycle. Loss of such checkpoint could lead to unrestrained cell growth.[36] The present study showed a fair hepatoprotective effect of bromocriptine as evidenced by the significant reduction in serum AFP, liver enzymes, and liver/body weight ratio, as well as the spectacular amelioration of histopathological features. Bromocriptine improves the metabolic alterations associated with insulin resistance by resetting hypothalamic circadian control on monoamines.[37] Moreover, bromocriptine reduces gluconeogenesis resulting from cortisol secretion-induced by low dopamine, as well as it decreases FFAs and TGs levels.[13,37,38] The effect of bromocriptine on AMPK activation is scarce in literature. Previous studies addressed the involvement of central AMPK in modulating several central regions including the thalamus and hypothalamus. However, it has not yet been determined whether bromocriptine controls circadian and seasonal neuroendocrine rhythm control on metabolism and body weight via AMPK activation.[39,40] de Leeuw van Weenen et al reported a lack of activation of hepatic AMPK by bromocriptine in an in-vitro study.[15] Herein, we observed an increase in hepatic phospho-Thr-172 AMPK concentration by bromocriptine although less than that of metformin. Hence, further studies are needed to elucidate bromocriptine effect on AMPK in different conditions.

Regarding L-arginine, its glycemic benefit as monotherapy was manifested by a reduction in FBS without any significant decrease in HbA1C% level and it

was associated with a significant increase in hepatic AMPK. In line, Barbosa et al reported that chronic arginine treatment increases glucose uptake and metabolism in vitro, and these metabolic changes are coincident with increased NO/c-GMP levels and signal transduction via Akt and AMPK.[41] Moreover, the AMPK has been recently identified as a fundamental modulator of short- and long-term arginine responses in cells, counter regulating β-cell glucolipotoxicity and contributing to β-cell functional integrity.[16,42] The observed L-arginine ameliorative effect on serum AFP could be explained by its documented anti-fibrotic, anti-inflammatory and antioxidant effect mediated through inhibition of iNOS and chemotaxis.[42] Additionally, histological results showed preservation of hepatic lobular architecture with mild intra-lobular, and portal necro-inflammatory activity and sinusoidal congestion compared to all other groups.

CONCLUSION

In conclusion, the precise molecular mechanisms connecting DM and HCC remain elusive although metabolic abnormalities, insulin resistance, and inflammation are significant risk factors. Anti-diabetic drugs could affect the development and/or progression by different mechanisms. L-arginine, as an AMPK activator, may prevent the early pre-neoplastic changes following the IFC axis almost equivalent to metformin. Bromocriptine did not activate AMPK to the same extent, yet it reduced the progress of the IFC axis. Thus, both drugs could be helpful in type-2 diabetic patients at high risk of liver cancer. Cautious use of insulin in diabetic patients with HCC is postulated as it may cause progression of neoplastic changes.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Hewedy WA, Anwar EI, Sharara GM, Hadi MA, Darwish SA, Ismail CA. Oncogenic challenge of bromocriptine and L-arginine versus conventional antidiabetics on diethyl nitrosamine-induced liver tumorigenesis in diabetic rats: focus on AMPK activation. Int J Basic Clin Pharmacol 2023;12:1-9.