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Research Article
Protective Effect of Glucose-6-Phosphate Dehydrogenase and Dihydrofolate Reductase Against Diethylnitrosamine-Induced Hepatocellular Carcinoma in Rats

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Abstract

Background and Objective: Antineoplastic acts by numerous mechanisms and have variability in action on healthy and cancerous cells. To examine the protective effect of Glucose-6-Phosphate Dehydrogenase (G6PD) and Dihydrofolate Reductase (DHFR) in Diethylnitrosamine (DENA) induced hepatocellular carcinoma in rats. **Materials and Methods:** A total of 30 healthy male divided into 5 groups (n = 6): Group 1 rats entitled normal controls, rats of Group 2 was serving as disease controls and exposed to a single dose of DENA (200 mg kg^{-1}, IP). Group 3, 4 and 5 were treatment groups that were subjected to DENA administration as scheduled in group-2 and treated with primaquine (PQ) (0.21 mg kg^{-1} per day, administered p.o.), methotrexate (MTX) (7.5 mg kg^{-1} per 3 doses per week) and low dose PQ+MTX (0.12 mg kg^{-1} per day p.o.+7.5 mg kg^{-1} per 3 doses per week) respectively for three weeks. The serum Aspartate Transaminase (AST), Lactate Dehydrogenase (LDH), Alanine Aminotransferase (ALT), α-fetoprotein levels were estimated. In liver tissue, levels of Catalase (CAT), Glutathione (GSH) and Malondialdehyde (MDA) were estimated. The levels of NF-κB, Bcl-2 and IkB-α were measured using western blot. **Results:** Serum levels of AST, ALT, LDH and α-fetoprotein elevated significantly in group 2 animals which were found maintained in treatment animals. The levels of antioxidant enzymes level of NF-κB, Bcl-2 and I κB-α were also altered in disease control group animals which were restored in treated animals. Results of group 5 were more consistent and satisfactory. **Conclusion:** The lower dose combination therapy with an inhibitor of G6PD and DHFR can successfully manage toxicities associated with methotrexate and may reduce the dose of methotrexate to a safer level.

Key words: Diethyl nitrosamine, dihydrofolate reductase, glucose-6-phosphate dehydrogenase, intracellular proteins, proto-oncogenes, cisplatin, leukaemia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Liver cancer or hepatoma are cancers that arise in the liver. Cancer that has spread to the liver from somewhere else is called secondary liver cancer[1]. Proto-oncogenes sleeping cancer-causing genes are present in every cell. Numerous triggering agents (chemical, physical or biological) mutate and can awake these proto-oncogenes into oncogenes, these triggering agents are called carcinogens[2]. Primary Liver Cancer (PLC) or Hepatocellular Carcinoma (HCC) is at the sixth position of most commonly occurring cancers globally and is considered third in the merit to cause deaths from cancers[3]. The economic burden of HCC per patient has been declared $32,907 annually in the United States alone. Hence the average yearly HCC treatment cost is valued at about $454.9 million[4].

Platinum-based (cisplatin, carboplatin), DNA synthesis inhibitors (gemcitabine, pemetrexed), drugs targeting tubulin (docetaxel, paclitaxel), or inhibitors of DNA gyrase (irinotecan, topotecan), anthracycline (doxorubicin) and 5-FU are recommended for liver cancer and other cancers. Various novel techniques such as epidermal growth factor receptors [gefitinib, erlotinib], antibodies targeting Haemoglobin-derived Growth Factor (HGF) {bevacizumab} and target tyrosine kinase used for the treatment of liver cancer. Further, multiple drug therapies use platinum-based compounds and are the mainstay of therapy. Sorafenib is common in the targeted delivery of liver Cancer. But these drugs possess some severe side effects[5,6]. Due to the potential side effects and economic burden of currently available drugs, there is a need for researchers to develop a new anticancer agent which can treat cancer with minimum risk of toxicity[7].

The X-chromosome linked enzyme G6PD is responsible to catalyze the initial most rate-limiting reaction of the Pentose Phosphate Pathway (PPP)[8]. Researchers confirmed its involvement in cell suicide, new blood vessels formation, development of neoplasms and a potential target of anti-cancer therapy[9]. Several studies revealed the elevated levels of G6PD in different tumours like leukemia[10], skin cancer[11], uterine tumours in females[12], cancers of mammary glands[13], cancers of the intestine[11], cervical cancers[14], epidermoid carcinomas of head and neck[15].

Primaquine inhibits G6PD and it has been confirmed using primaquine in G6PD deficient patients and an elevated vulnerability to acute intravascular haemolysis[16]. Methotrexate an inhibitor of the DHFRase enzyme, DHFrase is responsible for the reduction of dihydrofolates to tetrahydro-folates later tetrahydro-folates enter the reactions to synthesize purine nucleotides and thymidylate[17]. Hence, methotrexate affects nucleic material synthesis, cellular overhaul and replication. Enthusiastically multiplying tissues are in common more sensitive to these effects of methotrexate. As cellular growth in cancers is greater than normal tissues, methotrexate may diminish uncontrolled and unwanted growth, deprived of irreversible harm to normal tissues[18].

Hence it can be concluded that current anticancer drugs still lacking specificity and are associated with toxicities more if we are comparing their risk-benefit ratios. Hence it is required further to discover new pharmacological strategies with limited toxicities to humans. This research protocol intended to study the effect of two enzymes 'G6PD and DHFRse' in cancer cell progression.

MATERIALS AND METHODS

Study area: All experimental procedures were carried out at the Department of Pharmacology, Pharmacology and Toxicology Laboratory, College of Pharmacy, Jouf University, Aljouf, KSA from December, 2019-January, 2020. The total duration of the treatment protocol was of 3 weeks, animals were acclimatized for one week before commencement of experiments.

Chemicals: Primaquine and methotrexate were purchased from Carbosynth limited, Berkshire, UK, DENA purchased from Sigma Aldrich, USA. Reverse transcription kit (High-capacity cDNA) and PCR Master Mix (SYBR1 Green), (Applied Biosystems, UK), reagent TRIzol, (Life Technologies, Grand Island, USA). Antibodies (Primary and secondary) (Santa Cruz Biotechnology, TX, USA). LV PVDF (ImmunoBlot-1) membrane and filter paper (Bio-Rad Laboratories, USA). Chemiluminescent HRP from (EMD Millipore, USA).

Animals: Total 30 Albino Wistar rats (6-7 weeks) were acquired from the animal care unit of College of Pharmacy, Jouf University, Saudi Arabia. All animals were maintained at optimum conditions of humidity 45-55%, light/dark cycle 12:12 hrs and 24±2°C temperature and fed with standard pellet diet and water *ad libitum* during the research protocol. All experiments were conducted by strictly adhering to the standard guidelines of the Local Committee of Bioethics (LCBE) Jouf University at Pharmacology Department of College of Pharmacy.

Experimental design: Rats were arbitrarily divided into 5 groups (n = 6): Group 1 was the normal control group and served Normal Saline (NS) for 21 days. Group 2, designated toxic group and exposed to DENA (200 mg kg^{-1}, intraperitoneally [i.p.], single dose). Group 3, 4 and 5 served as

the treatment groups and were subjected to administration of DENA as schedule in group 2 and treated with primaquine (0.21 mg kg^{-1} per day, administered orally), methotrexate (7.5 mg kg^{-1} per 3 doses per week) and with primaquine (0.21 mg kg^{-1} per day, administered p.o.) with methotrexate (7.5 mg kg^{-1} per 3 doses per week) combination for 21 days.

On the termination day of the research blood samples were collected using light ether anaesthesia and animals were sacrificed after blood collection by cervical dislocation method. Livers were isolated and subjected to washings with icy saline and used for the valuation of intracellular oxidative stress and western blot analysis of inflammatory markers.

Biochemical estimations: The serum AST, ALT, LDH and α-fetoprotein levels were valued using a fully automated biochemistry analyzer, Erba XL Mannheim EM 200.

Estimation of free radical scavenging enzymes

Estimating lipid peroxidation as malondialdehyde levels: Lipid Peroxidation (LPO) is articulated as the content of Malondialdehyde (MDA) in the tissue, aliquotes of hepatocytes homogenized using potassium chloride (KCl) 1.15% (w/v). Formerly, 100 µL of homogenate added to chemical mixture for reaction Sodium Dodecyl Sulfate (SDS) 8.1% (w/v), 200 µL+1.5 mL Acetic acid 20% (v/v) pH 3.5+1.5 mL, thiobarbituric acid 0.8% (w/v) and distilled water 700 µL[19]. Well, ahead the samples were heated to 95°C for the next 1 hr, condensed utilizing glass balls and brought to normal temperature using tap water, centrifuged another time for 10 min at 4,000×g. Finally supernatant separated and the absorbance of the supernatant was measured with a UV spectrophotometer (UV 1280, Shimadzu, Tokyo, Japan) at 650 nm.

Quantification of glutathione content: Quantification of GSH was done in liver tissue according to Dhadde *et al.*[20] protocol. Dithiobis-2-nitrobenzoic acid admixed to reaction blend and absorbance documented instantly (within 5 min) at a wavelength of 412 nm contrary to blank.

Determination of catalase (CAT) activity: CAT commotion was examined in PMS which was attained after homogenization of liver tissue[21]. The reaction mix [0.1 M phosphate buffer 1.95 mL (pH 7.4)+H_2O_2 (0.019 M, 1.0 mL)] added with PMS (0.05 mL) and final quantity achieved 3 mL. Alteration in absorbance was documented each minute for the next 5 min at 240 nm. Enzymatic activity expressed as moles of H_2O_2 consumed/min/mg protein after calculations.

Protein extraction and expression analysis using western blotting: For protein expression analysis, tissue samples were lysed in protein lysis buffer containing (RIPA buffer, protease inhibitor cocktail, DTT and triton-X-100). Well after the Lysates were centrifuged for 20 min at 4°C (12000×g). The quantification of protein content was done using the Bradford reagent. For the assay, an equal amount (50 µg) of protein samples were subjected to 10% SDS-polyacrylamide gels and transferred by trans-blot turbo (BioRad, USA) on to PVDF membrane. The membranes were choked with 5% nonfat dried milk (BioRad, USA) and washed away thrice with PBST for 10 min each. Samples were probed overnight at 4°C against primary antibodies including β-Tubulin, Iκβ, NF-κβ (Santa Cruz, USA). Probed samples were washed thrice with PBST for 10 min each and then incubated with HRP conjugated anti-mouse and anti-rabbit secondary antibodies (dilution 1:1000; BioRad) for 1 hr at room temperature. After probing, membranes were again washed thrice with PBST and subsequently, the bands were detected in the presence of ECL substrate (BioRad) using Azure ECL imaging instrument. (Bio-Rad Laboratories, USA) with ECL imager (Azure Biosystems, USA).

Statistical analysis: Results are expressed as the Mean±SEM, (n = 6). One-way ANOVA followed by the Tukey-Kramer post-test for statistical analysis. Results considered significant when $p<0.001$ (most significant), $p<0.01$ (significant), $p<0.05$ (less significant) and ns = non significant. DENA control was compared with Normal Control and the rest of the treatment groups were compared with DENA Control. Graph pad prism version 5.0 software system was used for Statistical analysis.

RESULTS

Biochemical estimations

Effects of primaquine and methotrexate combination and DENA on serum AST, ALT, LDH and α-fetoprotein: Serum analysis of DENA control animals (Group II) exhibited a substantial elevation ($p<0.01$, $p<0.001$) in AST, ALT, LDH and α-fetoprotein in contrast to Group I (normal controls). The altered level of these parameters was less significantly ($p<0.05$, $p<0.01$) restored by primaquine alone (Group III). Methotrexate alone (Group IV) also restored altered biochemical parameters meaningfully ($p<0.05$, $p<0.01$, $p<0.001$) when data compared with that of group II animals. Surprisingly, administration of primaquine and methotrexate in combination significantly lowered ($p<0.001$) these elevated of AST, ALT, LDH and α-fetoprotein in Group V when compared to Group II animals (Table 1).

Effects of primaquine and methotrexate combination and DENA on free radical neutralizing enzymes: MDA represents LPO, DENA exposure caused significant alterations ($p<0.001$) in MDA levels and CAT activity in Group II when results compared to Group I animals. Primaquine treatment (Group III) resulted in less significant restoration ($p<0.05$) of the intracellular levels of MDA, CAT, GPx and GST when findings compared with the toxic group (Group II). Further, methotrexate administration (Group IV) leaded significant restoration ($p<0.05$, $p<0.01$, $p<0.001$) in the same manner as primaquine alone when results compared with Group II animals. Results of Group V exhibited very significant restorations ($p<0.001$) in MDA, CAT, GPx and GST activity up to the normal levels when compared with Group II animals. Primaquine and methotrexate combination maintained back these levels significantly (Table 2).

Effects of primaquine and methotrexate combination and DENA on NF-κB, Ik-Bα and Bcl-2 protein expressions: Western blot scrutiny exhibited that DENA administration to investigational rats significantly upregulated the expressions of ($p<0.001$) NF-κB and Bcl-2 while Iκ-Bα indices recorded significantly reduced ($p<0.01$) in Group II animals. Contrary to this treatment with primaquine and methotrexate alone and in combinations exhibited significantly lower intracellular levels of NF-κB, Bcl-2 and significantly upregulated levels of Ik-Bα. Result analysis depicted significantly ($p<0.05$, $p<0.001$) results in the combination treatment group as compared to disease controls (Fig. 1-3).

Table 1: Effects of primaquine and methotrexate combination on serum AST, ALT, LDH and AFP of animals

Treatments	AST (IU L^{-1})	ALT (IU L^{-1})	LDH (IU L^{-1})	AFP (ng dL^{-1})
Normal control	38.78±1.56	51.79±1.02	188.45±1.79	28.33±0.48
DENA control	79.37±1.35###	88.89±0.81##	259.10±1.98###	291.21±5.99###
Primaquine treatment	65.25±1.69*	69.89±1.09*	201.99±3.46*	195.13±1.49**
Methotrexate treatment	71.35±1.29*	60.28±1.27**	211.25±3.39*	65.36±1.89***
Primaquine+methotrexate	46.39±0.91***	55.01±1.92***	176.29±1.39***	32.78±0.78***

Values are expressed as Mean±SEM, (n = 6) ***p<0.001, **p<0.01, *p<0.05, ###Negative control, AST: Aspartate transaminase, LDH: Lactate dehydrogenase, ALT: Alanine aminotransferase and AFP: α-fetoprotein levels

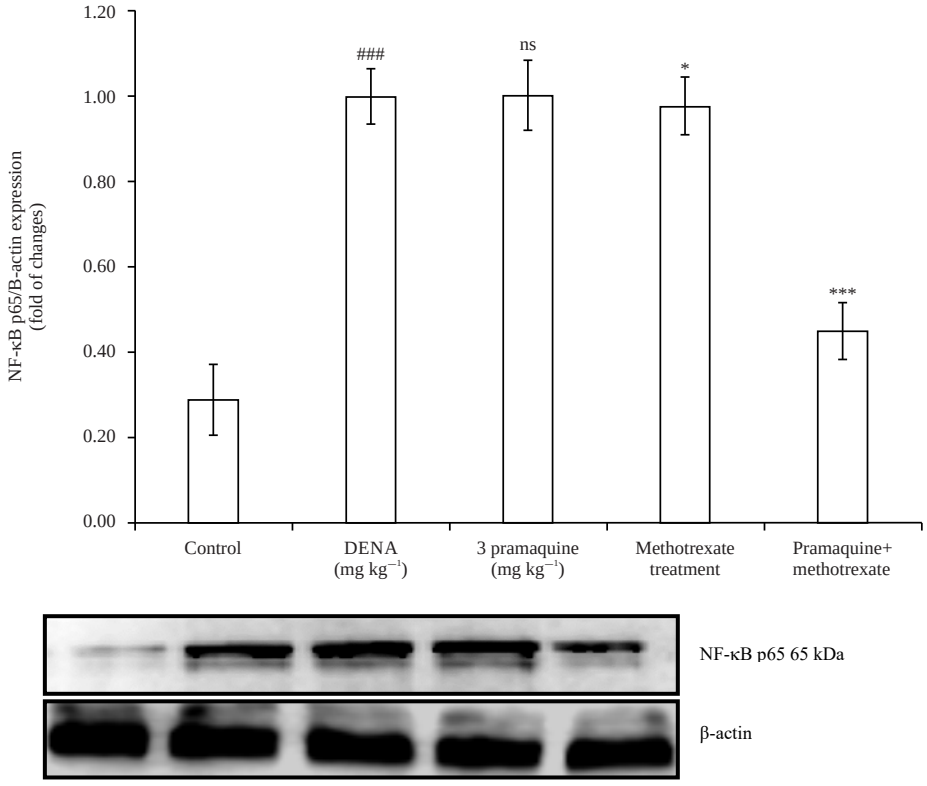

Fig. 1: Effects of primaquine and methotrexate combination and DENA on NF-κB expression
Values are expressed as Mean±SEM, (n = 6) ***p<0.001, *p<0.05, ns: Non significant and ###Negative control

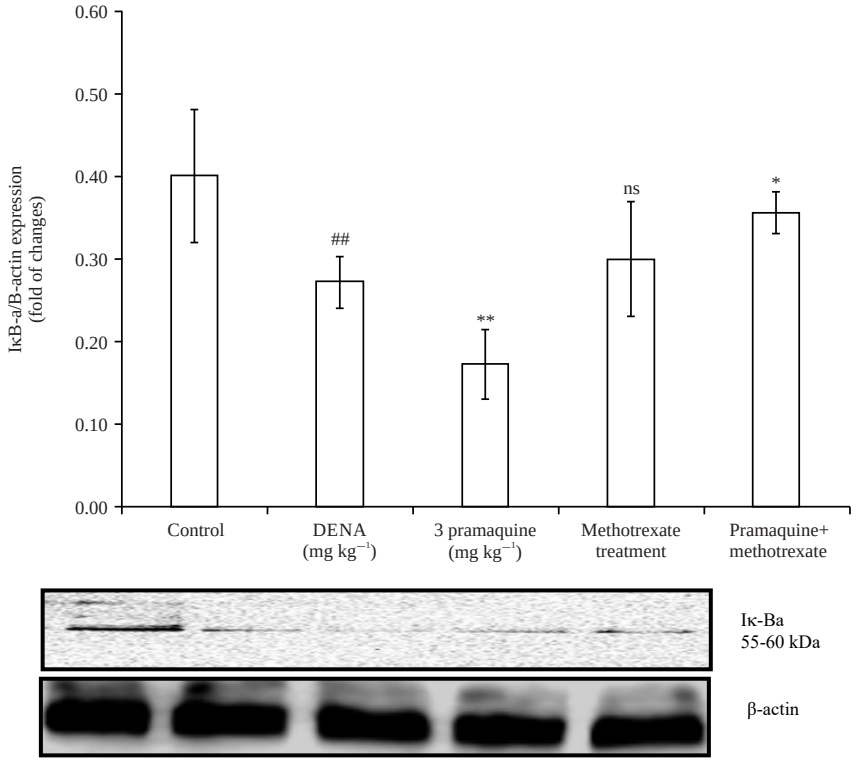

Fig. 2: Effects of primaquine and methotrexate combination and DENA on Ik-Bα expression
Values are expressed as Mean±SEM, (n = 6), **p<0.01, *p<0.05, ns: Non significant and ##Negative control

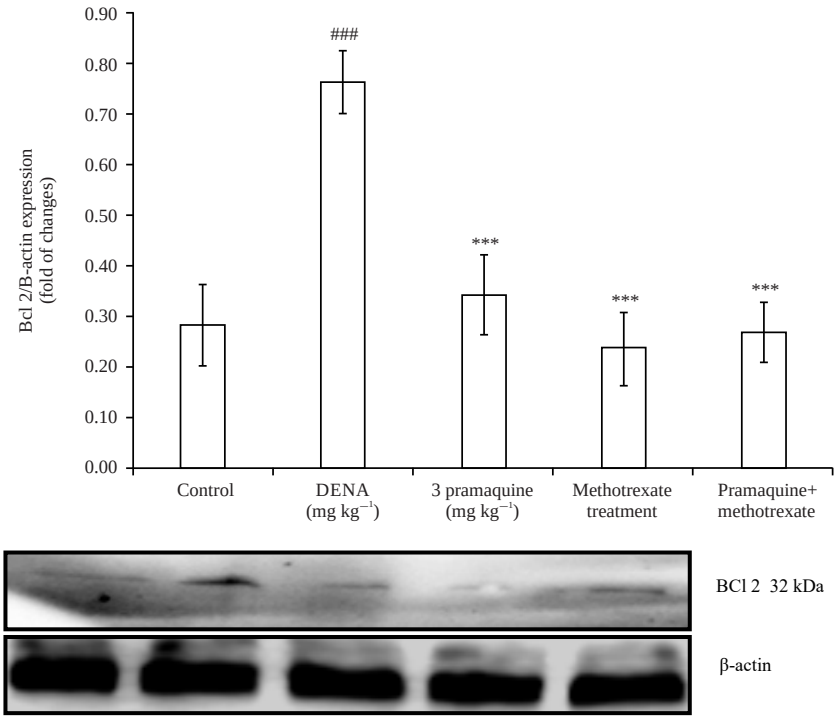

Fig. 3: Effects of primaquine and methotrexate combination and DENA on BCL/B-action expression
Values are expressed as Mean±SEM, (n = 6) ***p<0.001 and ###Negative control

Table 2: Effects of primaquine and methotrexate combination on antioxidant enzymes in DENA induced hepatocellular carcinoma in rats

Groups	MDA (μM mg^{-1} protein)	CAT (nmol $min^{-1}mL^{-1}$)	GPx (μmol)	GST (U $min^{-1}mg^{-1}$ protein)
Normal control	7.24±0.82	1.12±0.59	9.18±0.49	0.55±0.06
DENA control	14.24±0.78###	0.07±0.14###	4.02±0.66###	0.07±0.01###
Primaquine treatment	12.25±0.69*	0.52±0.07*	5.35±0.51*	0.18±0.05*
Methotrexate treatment	8.81±0.88***	0.71±0.09**	6.56±0.64**	0.15±0.04*
Primaquine+methotrexate	7.32±0.61***	1.02±0.44***	8.05±0.83***	0.49±0.09***

Values are expressed as Mean±SEM, (n = 6) ***p<0.001, **p<0.01, *p<0.05, ###Negative control, CAT: Catalase, MDA: Malondialdehyde, GPX: Glutathione peroxidase and GST: Glutathione-s-transferase

DISCUSSION

The current study investigated the effect of G6PD and DHFR in DENA induced hepatocarcinogenesis in rats. We found that combination therapy with G6PD and DHFR inhibitor (Primaquine+Methotrexate) produce consistent and satisfactory effects in DENA induced carcinoma in rats. HCC ranked third most predictable foundation of mortality owe to cancer[22-24]. DENA is used as hepatotoxin in animal models and is known to potentially damage the hepatocytes[25-28]. Injury to hepatocytes was established by gauging the serum indicators (α-fetoprotein, ALP, ALT and AST) in the DENA-exposed rodents. Elevated serum α-fetoprotein is embodying cancerous state[5] which is further confirmed by western blot analysis of inflammation-induced proteins[29-33]. Results of present research implicated that DENA administration causes hepatocyte damage which progress to HCC that can be easily correlated with considerably up-regulated serum liver enzymes and α-fetoprotein levels. Researchers established well that an increase in serum liver enzymes is directly proportional to hepatocellular damage[34-38]. DENA-induced hepatocarcinogenesis has been well established in different experimental models[39,40]. Researchers have firmly documented elevated levels of AST, ALT, LDH and α-fetoprotein in serum of DENA administered animals due to hepatocytic injuries which at later stage progress to carcinogenesis[41]. Results of our research protocol are in agreement with these, which deduce that DENA administration to experimental rats causes a significant increase in serum AST, ALT, LDH and α-fetoprotein levels. Treatment with methotrexate and primaquine alone and in combination reverted these elevated levels towards normal value and the maximum efficacy was observed in animals that were treated with methotrexate and primaquine combination. DENA administration is known to cause cellular damage via the generation and release of oxidative species, these reactive radicals have been created during the biotransformation process by liver cytochrome P450 enzymes[42]. Researchers also concluded that DENA down-regulates the gene expressions of antioxidant enzymes GSH, GRs and CAT[43] and unregulated the intracellular levels of MDA[44]. Existing literature also substantiates that excessive free radical generation and their diminished neutralization contribute to carcinogenicity[45]. The findings of our research attest to these alterations in intracellular oxidative free radicals and diminished antioxidant defence. Exposure of animals to DENA significantly altered the intracellular levels of GSH, GRs and CAT, which were significantly reduced in methotrexate and primaquine-treated animals. The most significant changes were recorded in animals treated with methotrexate and primaquine combination. Earlier findings also penlight to the roles of reactive oxygen species and weak antioxidant defence on the generation of pro-inflammatory mediators such as necrosis factor-α, inducible Nitric Oxide Synthase (iNOS), IL-1β, IL-6 and IL-12[46]. Others reported that oxidative stresses up-regulate of expression of NF-κB and Bcl-2, which is considered one of the prominent mediators of inflammatory cascade[38]. NF-κB in inactivated form resides in the cytosol as NF-κB: Ik-Bα complex. NF-κB is considered as the first responder to detrimental cellular incitements, numerous factors found to trigger NFκB activation (Reactive free radicals, tumour necrosis factor-alpha, lipopolysaccharides, etc.)[47]. The triggering factors via the intermediacy of transmembrane receptors stimulate IkB Kinase enzyme, which in turn phosphorylate the Ik-Bα and the NF-κB: Ik-Bα complex dissociates, leading to free active NF-κB, which on activation translocate into the nucleus and finally leads to mRNA expressions of proteins involved in inflammation, immunity, cell survival and proliferation[48]. NF-κB is gradually more renowned as a critical performer in various footsteps of cancer origination and advancement[49,50]. Our research findings confirm the earlier hypothesis and the results obtained from western blot assay conclude that DENA administration in animals caused an increase in NF-κB and Bcl-2 protein expressions and reduced its inhibitory protein IkB-α, while treatment with methotrexate and primaquine alone and combination of both of these drugs significantly reduced the elevated levels of NF-κB, Bcl-2 and restored the inhibitory protein IkB-α, the combination exhibited most prominent action among all three treatment groups.

CONCLUSION

The overall economic burden of HCC and its prevalence has alarmed the scientific community to search for new

therapeutics for its treatment. The unavailability of selective drugs for the treatment of HCC and potential adverse effects of existing combination therapy penlights' the way to search for more selective and efficacious pharmacotherapeutic regimens for HCC. Findings of the present research pointed out that therapeutic regimens including methotrexate (a dihydrofolate reductase inhibitor) and primaquine (glucose-6-phosphate dehydrogenase inhibitor) can be proved more efficacious which are required for the de-novo synthesis of DNA nucleotides. Combination with a low dose will be more efficacious and adverse effects will be minimized.

SIGNIFICANCE STATEMENT

Findings of this research protocol reveal that sequential blockage of consecutive two steps which are involved in cell division (Glucose-6-phosphate dehydrogenase (G6PD) and dihydrofolate reductase (DHFR))offers more beneficial effects as compared to methotrexate (DHFRase inhibitor) alone. Further administration of G6PD inhibitor (Primaquine) will help to reduce the dose of methotrexate and this will minimize the adverse effects of methotrexate.

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