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Original article

Ameliorative effects of colostrum against DMBA hepatotoxicity in rats

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ABSTRACT

Colostrum, the sole diet for newborns, is an emerging nutraceutical. To date, the chemopreventive effect of Bovine Colostrum against liver injury induced by the potent carcinogen, 7,12-dimethyl-Benz[a] anthracene (DMBA) is unexplored. Humans are daily exposed to DMBA which is a highly lipophilic environmental organic pollutant. The study aimed to investigate the hepatoprotective role of Bovine Colostrum against DMBA-induced hepatotoxicity using a rat model. Fifty male rats were divided into five groups; GI (control), GII (olive oil, vehicle for DMBA), GIII (DMBA), GIV (DMBA + Bovine Colostrum), GV (Bovine Colostrum). After 12 weeks, body weight changes and mortality were calculated. Histological and ultrastructural examinations of liver tissue were performed. Expressions of p53, TGFβ2, TNF-α, S6K2, and c20orf20 were assessed by RT-PCR. Post-treatment with Bovine Colostrum increased both the body weight and the survival rate of rats treated with DMBA. In addition, remarkable protection against the pathological effect of DMBA was noted. Ultrastructurally, Bovine Colostrum ameliorated/prevented most of the toxic effects of DMBA on hepatocytes, including irregularities of nuclear envelope, clumping, and margination of heterochromatin aggregates, segregated nucleoli, and mitochondrial pleomorphism. Bovine Colostrum administration down-regulated p53, C20orf20, and S6K2 mRNA levels, and up-regulated TNF-α and TGFβ2. In conclusion, Bovine Colostrum have a protective effect against DMBA-induced toxicity on the liver of albino rats. Consequently, Bovine Colostrum may prevent polycyclic aromatic hydrocarbons-induced hepatotoxicity and may be useful in promoting human health if supplemented in the diet.

1. Introduction

Cancers share many features, such as their origins, their proliferation, loss of differentiation, and invasion of surrounding tissues (Lu and Shenoy, 2017). Liver cancer is the sixth most common malignancy and the second most common cause of death from cancer worldwide (Kang and Ahn, 2017). Cancer therapies include chemotherapy, surgery, hormonal therapy, and radiotherapy, which possess several side effects including toxicity to normal cells and drug resistance problems (Zhang et al., 2018). To overcome these problems; natural products were used as an alternative approach for cancer therapy (Salehi et al., 2018). Humans are daily exposed to polycyclic aromatic hydrocarbons (PAHs) which are a ubiquitous class of highly lipophilic environmental organic pollutants (Staal et al., 2008). PAHs are released into the environment

Abbreviations: BC, Bovine Colostrum; DMBA, 7,12-dimethyl-Benz[a]anthracene; PAHs, polycyclic aromatic hydrocarbons; TGFβ, transforming growth factor-beta; S6K, 40S ribosomal protein S6 kinase; CAM, Complementary and Alternative Medicine; IL-1β, cytokines including interleukin-1 beta; IL-6, interleukin-6; TNFα, tumor necrosis factor-alpha; INF-γ, interferon-gamma; IGF, insulin-like growth factor; ROS, reactive oxygen species.

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in large quantities mainly due to human activities (Sharma et al., 2012). PAHs have detrimental biological effects, toxicity, mutagenicity, and carcinogenicity effects (Haritash and Kaushik, 2009). Exposure to 7,12-dimethylbenz[a]anthracene (DMBA) which is a carcinogenic synthetic PAHs (Flint et al., 2011) underlie the development of many tumor types including; mammary gland, skin, liver, lung, hematopoietic system, and pancreatic tumors (Kerdelhué et al., 2016). DMBA requires metabolic activation in liver and extra-hepatic tissues to become an ultimate carcinogen (Girolami et al., 2008). The failure of repair mechanisms and constant exposure to PAHs induce mutagenesis in multiple genes including p53 (Muñoz and Albores, 2011). Inactivation of p53 and inhibition of transforming growth factor-beta (TGF-β) signaling are among the most common molecular events in human liver cancers (Morris et al., 2012). TGF-β is a potent anticancer agent that prohibits the uncontrolled proliferation of epithelial, endothelial, and hematopoietic cells (Tian and Schiemann, 2009). In the liver, TGF-β has been shown to play both tumor-suppressive and tumor promoting roles (Akhurst and Derynck, 2001; Tian and Schiemann, 2009). Pallardy et al. (1989) reported that treatment with DMBA can induce the release of cytokines by bone marrow stromal cells, such as TNF-α; leading to down-regulation of IL-2. TNF-α is being utilized as an antineoplastic agent for the treatment of patients with locally advanced solid tumors (Mocellin et al., 2005a), soft tissue sarcomas, and metastatic melanomas (Van Horssen et al., 2006). The 40S ribosomal protein S6 kinase (S6K) acts as downstream of mTOR pathway which plays a significant role in hepatocellular carcinoma progression by promoting neoangiogenesis (Khalil and Gout, 2012). Little is known about MRGBP role in liver cancer. It has been suggested that MRGBP influences the acetylation of histones and potentially transcription factors such as p53 (Gu and Roeder, 1997), MRGBP is upregulated in the majority of colorectal tumors, and the enhanced expression was associated with cell proliferation (Yamaguchi et al., 2010).

The use of Complementary and Alternative Medicine (CAM) that comprises the use of dietary medicine accompanied by a healthy lifestyle has overcome difficulties presented by drug resistance (Alwhibi et al., 2017; Ouhtit et al., 2013). Approximately 75% of the world's populations depend on CAM as reported by the world health organization reports (Grawish et al., 2010). Several natural products has been suggested to be beneficial for reducing DMBA toxicity including hops, rosemary, cat's claw (El Kholy et al., 2013) and bitter gourd and tomato (De et al., 2003). Bovine colostrum (BC) has been known and used for years in the treatment of infectious diseases in humans (Struff and Sprotte, 2008). Colostrum is the first milk produced by the mammary glands for the first 1–4 days postpartum. The following constituents are generally present in bovine colostrums: Immunoglobulin Ig (Kelly, 2003); lactoferrin (Lindner et al., 2011), cytokines including interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (INF-γ); growth factors including insulin-like growth factor (IGF) (Kelly, 2003), transforming growth factor (TGF-β) and epidermal growth factor; Prolin rich polypeptide (Rawal et al., 2008), lactoperoxidase; oligosaccharides, nucleosides (Pandey et al., 2011).

Colostrum plays a significant role in the protection and development of newborns, which may be due to its biologically active constituents (Lindner et al., 2011; Rawal et al., 2008). The biologically active constituents of bovine colostrum possess antimicrobial, anti-inflammatory, antioxidant, immune enhancing, and growth promotion activity (Liang et al., 2018; Lindner et al., 2011). Other constituents were extracted from colostrum and were found to possess an anticancer activity such as lactoferrin and β-lactoglobulin (Godhia and Patel, 2013). However, despite there are many studies concerning the properties of BC, there are sparse data on its anticancer activity. Hence, the aim of the present investigation was to explore the potential preventive effect of BC against the administration of the chemical carcinogen DMBA in rats.

2. Material and methods

2.1. Experimental animals

Adult male albino rats of the Wistar strain (*Rattus norvegicus*) weighing 138.5 ± 2.2 g, obtained from the Veterinary Science Institute, Cairo, Egypt, were used in this study. Fifty rats were housed in metal cages (5 rats/ cage) at room temperatures with relative humidity 60–70%, with free access to food and tap water. Rats were acclimated to the laboratory conditions for two weeks prior to the experiment.

2.2. Chemicals and routes of administrations

Bovine colostrum (BC) was obtained from the Milk Project of Faculty of Agriculture, Alexandria University. BC was harvested within the first few hours after calving, in the form of liquid. It was filtered by mesh and stored at 4°C until orally administrated at dosages equivalent to the human therapeutic dose (Playford et al., 2001). The dosage used was calculated and modified according to the table reported by Paget and Barnes (1964). DMBA (Cat. No. 57-97-6, Sigma-Aldrich) was administrated as a single oral dose of 178.6 mg/kg (Welsch, 1985).

2.3. Experimental design

Rats were randomly allocated into five groups (10 rats/group). Group I (GI) served as a control and did not receive any treatment. Group II (GII) received a single oral dose of 1 ml olive oil (vehicle for DMBA). Group III (GIII) was orally administered a single dose of DMBA (178.6 mg/kg). Group IV (GIV) was orally administered BC (11.15 ml/kg/day, 6 days/week) after 24 hrs of treatment as GIII. Group V (GV) was orally administered BC as mentioned in GIV. After 12 weeks, all rats were administered intravenously, 30–50 mg/kg pentobarbital to produces rapid anesthetic action then sacrificed by cervical dislocation and liver specimens were quickly collected at the autopsy.

2.4. Light microscopy

Liver specimens were fixed in 10% buffered formalin for 24 hrs. The specimens were dehydrated in ascending grades of ethanol, double cleared in xylene for 1 hr each, and embedded in paraffin wax. Tissue blocks were sectioned into 5–6 μm thickness and sections were stained with hematoxylin and eosin. A minimum of 10 fields for each slide/3 slide for each animal was examined and scored semi-quantitatively for the severity of changes. The scoring was done as none (−), mild (+), moderate (++), and severe (+++) changes (Suzuki and Suzuki, 1998).

2.5. Immunohistochemical detection for p53

For IHC, formalin-fixed wax sections of paraffin-embedded liver tissues were analyzed for the expression of p53 by immunohistochemistery. Tissue sections were incubated with the primary monoclonal anti-p53 (Labvision, USA). IHC staining was performed using streptavidin-biotin method by Histostain-plus kit (Zymed, USA) which contains 10% non-immune serum, biotinylated secondary antibody and streptavidinperoxidase. The peroxidase signal was developed in 0.05% diaminobenzidine and 0.01% hydrogen peroxide in PBS. The sections were lightly counter stained with haematoxylin before dehydration and mounting with permount.

The intensity of P53 expression in positively stained cells (5–7 microscopic fields from well labelled areas and not overlapping were randomly used at ×200 magnification) was quantified by using NIH Image J1047v software.

2.6. Electron microscopy

Liver specimens (1 mm^3) were fixed in $_4F_1G$ (40% formalin/50% glutaraldehyde) for 24 hrs at 4°C, post-fixed in 1% OsO_4 for 2 hrs at 4°C, then rinsed in 0.1 M phosphate buffer (pH = 7.2), dehydrated at 4°C in a graded series of ethanol. Infiltration was carried out using propylene oxide and Epon mixture. Specimens were embedded in Epon-Araldite mixture in labeled beam capsules. Ultrathin sections (50–70 nm thick) were cut with a glass knife on LKB ultratome, and double-stained with uranyl acetate and lead citrate, examined using Jeol 100 CX electron microscope.

2.7. RT-PCR

Total RNA was extracted from liver samples using Easy-RED™ total RNA extraction kit (Cat. No. 17063, INTRON Biotechnology) according to the manufacturer protocol. RT-PCR was performed using Maxim RT-premix kit (Cat. No. 25082, INTRON Biotechnology) and 2x PCR Master mix Solution (i-Taq) (Cat. No. 25027, INTRON Biotechnology). Primer sequences are shown in Table 1. PCR was performed at 94°C for 20 sec, 60°C for 20 sec, and 72°C for 1 min for 30 cycles and final extension at 72°C for 5 min. PCR products were separated in 1% agarose gel electrophoresis. Gels were stained with ethidium bromide and analyzed under UV. The densities of the bands were normalized to β-actin and controls using Quantity One (Bio-Rad). Primer design was done using the BLASTN program (Altschul et al., 1997).

2.8. Statistical analysis

The values are expressed as mean ± SE. The statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Significant difference with control group (GI) ($P < 0.05$)*, ($P < 0.01$)** and ($P < 0.001$)***, while ($P < 0.05$)#, ($P < 0.01$)## and ($P < 0.001$)### significantly difference with GIII (DMBA group), using SPSS version 20.

3. Results

3.1. Effect of BC on body weight change and survival percentage of DMBA-treated rats

DMBA administration resulted in clinical symptoms, including decreased activity, weakness, less food intake, yellow and soiled fur, reddish-brown secretions around eyes and noses, and diarrhea (Fig. 1). These symptoms were relieved to a certain extent by the BC administration. DMBA-treatment significantly decreased ($P < 0.01$) the body weight of rats while BC administration showed a significant increase ($P < 0.05$) in the final body weight when compared to controls (Fig. 2a). The body weight of the animals treated with DMBA and BC showed an insignificant difference to the controls and a significant difference ($P < 0.05$) with the DMBA-treated group suggesting the protective effect of colostrum. Furthermore, the survival curve of the DMBA and BC-treated animals shifted towards a better lifespan starting from the third week of the experiment in comparison to that of DMBA-treated animals (Fig. 2b). Since the first two weeks post treatment, DMBA and colostrum–administered group exhibited a total decrease in the survival percentage by 20%, while the DMBA- treated group showed 30% total mortality.

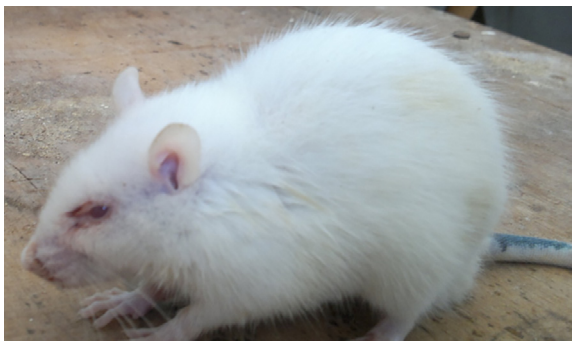

Fig. 1. A photograph illustrating the morphological appearance of DMBA-administrated rat (GIII). Notice, intense reddish-brown secretions surrounding the eye and nose, general animal weakness.

Table 1
RT-PCR Primers Sequence.

Primers	Sequences
p53	F: 5′-ACAGCGTGGTGGTACCGTAT-3′ R: 5′-GGAGCTGTTGCACATGTACT-3′
TGFβ2	F: 5′-CGGACTACTACGCCAAAGAAGT-3′ R: 5′-TGGTTTTGTCATAGATTGCGTT-3′
TNFα	F: 5′-TCTGTCTACTGAACTTCGGGGTGAT-3′ R: 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′
S6K2	F: 5′-GAGGACGTGAGCCAGTTTGA-3′ R: 5′-AGCCCTCTTTGATGCTGTCC-3′
C20orf20	F: 5′-GGGTCACTGACAAGGTCCTG-3′ R: 5′-ATCAGGCCACTGGCTTTTCA-3′
β -actin	F: 5′-AGAGCTATGAGCTGCCTGAC-3′ R: 5′-AATTGAATGTAGTTTCATGGATG-3′

3.2. BC reduced the DMBA-induced liver histopathological changes

Light microscopic preparations of liver from treated groups are shown in Fig. 3. Photomicrographs from the control and BC-administrated groups revealed normal liver architecture. The hepatocytes form anastomosing cords separated by the blood sinusoids, radiating from the central vein and extending to the portal areas. The hepatocytes appeared polygonal in shape; their nuclei were basophilic, each with a distinctly marked nucleolus and normal chromatin distribution. Moreover, few hepatocytes appeared binucleated. The blood sinusoids possess Kupffer and endothelial cells (Fig. 3a-c). Liver sections of DMBA-treated rats exhibited loss of hepatic cord regularity which might be due to hepatocellular swelling (Fig. 3d). The blood sinusoids were no longer visible except in few pericentral areas where some hepatocytes showed vacuolated cytoplasm. Also, necrotic changes were observed among periportal hepatocytes (Fig. 3d). In DMBA plus BC group, liver sections revealed a reduction of the DMBA-induced pathological changes. Hepatocytes appeared similar in many aspects to their corresponding control except for a few cells with pyknotic nuclei (Fig. 3e). The histopathological changes were graded and summarized in Table 2. Furthermore, in control and vehicle groups (Fig. 4a&b), it was observed that, portal areas containing elements of the hepatic triad that is a branch of the portal vein, a branch of the hepatic artery, and small bile ductules, along with lymphatic vessels and a minimal amount of connective tissue. However, liver sections of DMBA-treated rats showed an increased number of hepatic artery and bile ductules branches (Fig. 4c) in comparison to control groups. The wall of portal vein appeared thickened due to intimal hyperplasia and surrounded by leucocytic cellular infiltrations (Fig. 4C). There were also extensive proliferation and fibrosis of bile ductules in the portal triads (Fig. 4c). DMBA and

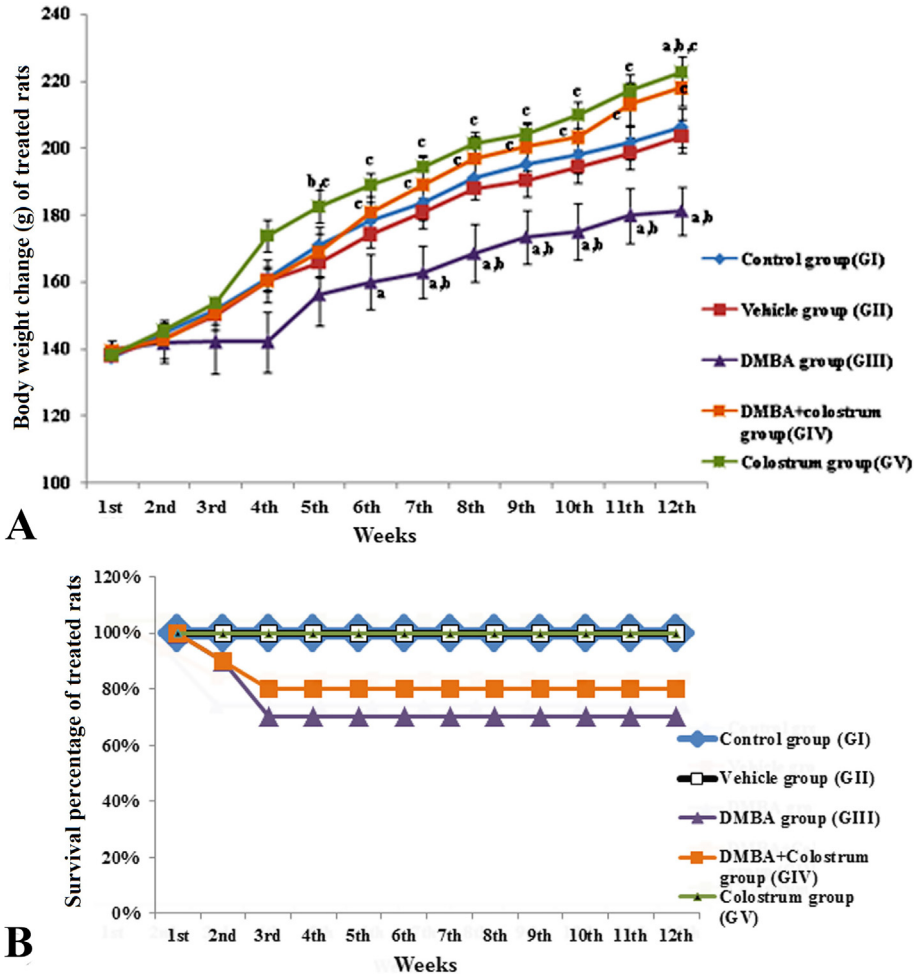

Fig. 2. Effect of BC on body weight change and survival percentage. a) Body weight change of treated animals. b) Survival curve of treated animals. The Y-axis represents the percentage of survived rats and the X-axis is the time course of the experiment (weeks). a, b, and c are significant differences with control, vehicle, and DMBA-treated groups respectively.

colostrum–administered rat, portal area elements appeared similar to their corresponding control normal (Fig. 4e).

3.3. BC ameliorated DMBA-induced toxic effects on hepatocytes

Ultrastructurally, the nuclei of control groups exhibited normal chromatin distribution. The hepatocytes showed granular cytoplasm with a profuse amount of RER around the nuclei and mitochondria (Fig. 5a). Few lysosomes, lipid droplets, and numerous glycogen granules were surrounded by SER. DMBA treatment resulted in ill-defined hepatocyte boundaries, widened intercellular spaces (Fig. 5c), fragmentation of the microvilli, distorted endothelial lining of sinusoids, and Kupffer cells. The nuclei exhibited dense chromatin granules, irregular membranes, and segregated nucleolus. The cytoplasm housed lipid vacuoles, numerous intensely stained pleomorphic mitochondria with unidentifiable cristae (Fig. 5c&d). The RER exhibited dilated cisternae and focal distribution in close relationship to mitochondria (Fig. 5d). The post-treatment with BC revealed significant resemblance to their controls. Most hepatocytes maintained their polygonal shape, normal cellular boundaries, narrow intercellular space, and intact tight junction at the periphery of the bile canaliculi. Kupffer cells occupied liver sinusoids and maintained regular outlines with a triangular nucleus and normal distributed heterochromatin (Fig. 6a). Moreover, the endothelial cells had regular outlines with oval hyperchromatic nuclei. Hepatocytes possessed large nuclei with single eccentric nucleoli and intact nuclear membrane with normal chromatin distribution. However, minor alterations (e.g., a number of pyknotic nuclei) were observed (Fig. 5e). The cytoplasmic vacuolization was decreased when compared to DMBA-treated group. Numerous ribosomes and glycogen granules were also observed. Furthermore, the fine structure of Kupffer cells indicated that cells were highly distorted with irregular altered nuclei with dilated nuclear pores (Fig. 6b). In addition, the sinusoids appeared normal filled with RBCs and normal kupffer cells with regular centric nuclei; and lined by regular spindle shaped endothelial cells (Fig. 6c). BC-administration did not result in any abnormalities. The hepatocytes exhibited normal appearance, normal boundaries, well-defined nuclear envelope, abundant mitochondria, scattered ER, and few lipid droplets (Fig. 5f). However, liver sections of the vehicle group possessed a few changes, including microvilli irregularity and an increased number of lipid droplets (Fig. 5b). The morphometric analysis revealed an insignificant decrease ($P < 0.05$) in hepatocytes dimensions and a significant decrease in their nuclear dimensions after DMBA administration. The post-treatment with BC partially suppressed the damage induced by DMBA in cellular dimensions. Hepatocyte dimensions were insignificantly increased in comparison to their corresponding controls. The diameters of the nuclei were insignificantly lower than that of control group and significantly higher ($P < 0.05$) than their

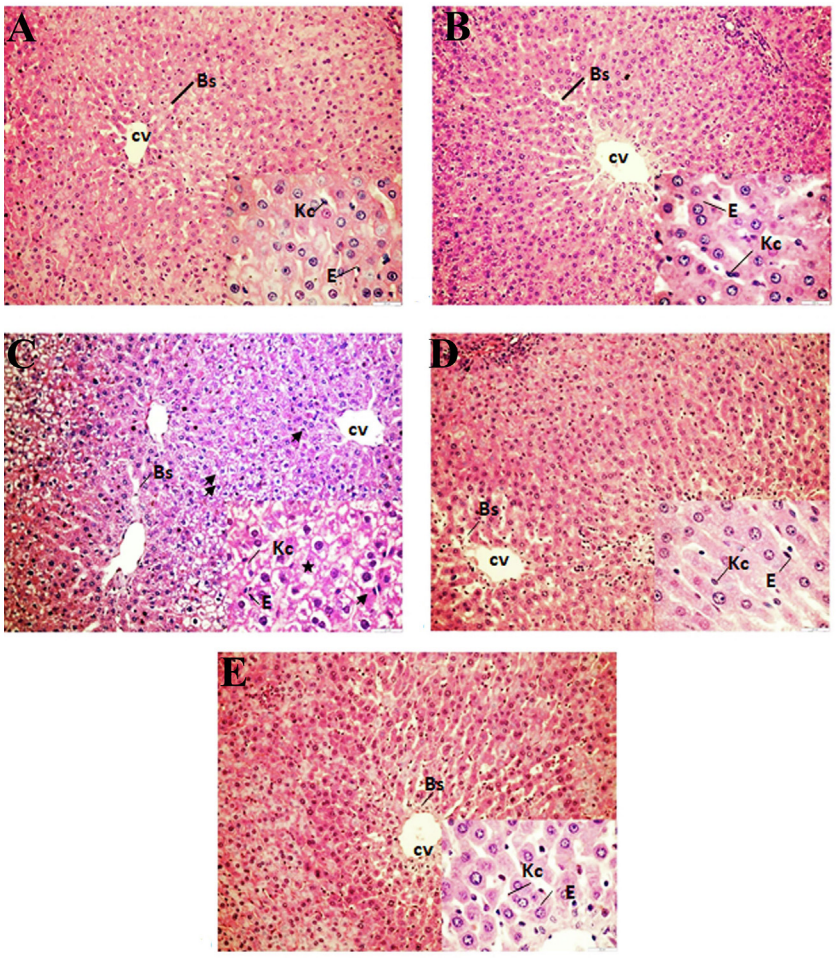

Fig. 3. Light micrograph showing that colostrum prevented the hazardous effect of DMBA. a-c) Liver sections of control (a), vehicle (b) and colostrum-administrated (c) rats showing normal histological hepatic architecture, including central vein (Cv), blood sinusoids (Bs), and Kupffer cells (Kc), endothelial cells (E), hepatocytes with centrally located nuclei (N). d) Section of rat liver administrated DMBA revealed considerable number of damaged hepatocytes, hepatocytes with high eosinophilic cytoplasm (arrow), vacuolated cytoplasm (double arrow), necrotizing hepatocytes (*), and binucleated cells (arrowhead). e) Liver section of rat treated with DMBA and post-treated with BC exhibited restoration of near normal hepatic architecture.

Table 2
The existence of some histopathological changes analysis in liver tissues of all experimental groups.

histopathological changes	control (GI)	vehicle (GII)	DMBA (GIII)	DMBA& Colostrum (GIV)	Colostrum (GV)
Irregular architecture	–	–	+++	–	–
Epithelium dislocation	–	–	++	–	–
Blood vessel dilation	–	–	++	–	–
Leukocytic infiltration	–	+	+++	–	+
Pyknotic nuclei	–	–	+++	+	+
necrotic changes	–	–	++	–	–
Vacuolar degeneration	–	–	+++	–	–
Portal vein dilation	–	–	+++	–	–
Hepatic artery branches	–	–	+++	–	–
bile ductules hyperplasia	–	–	+++	–	–

Severity of liver histological changes using scores on a scale of none (–), mild (+), moderate (++), and severe (+++) damage.

corresponding in DMBA administrated group (Fig. 7a). Furthermore, BC administration resulted in a significant decrease in the oval mitochondrial length and a significant increase ($P < 0.05$) in the round mitochondrial diameter compared to DMBA-treated animals. No significant differences were seen when compared to control group (Fig. 7b).

3.4. Bovine colostrum decreases the p53 expression level

The expression of the tumor suppressor gene p53 was examined by immunohistochemistry in the rat liver of all the groups. While, the immunostaining of p53 in the control, vehicle and colostrum groups showed weak expression manifested as brown col-

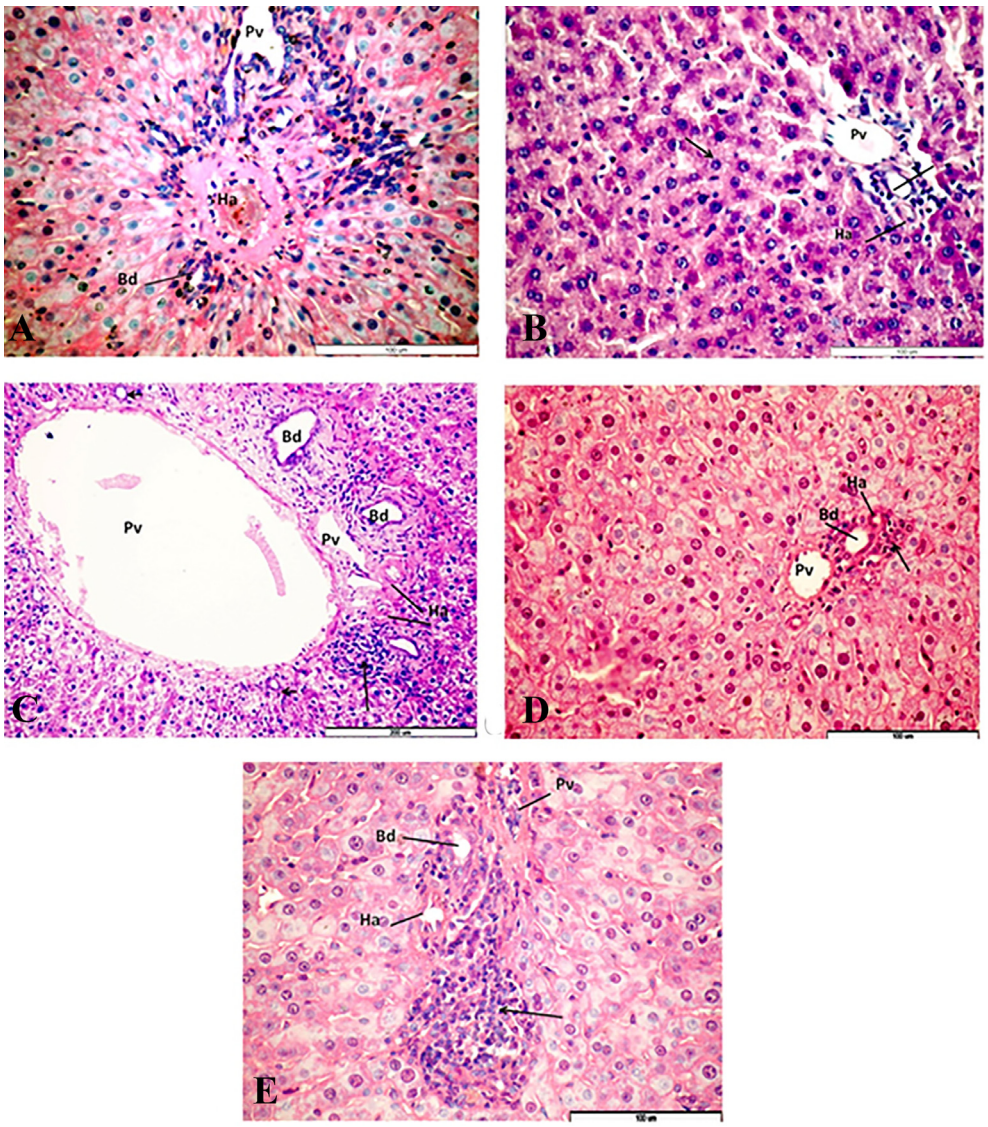

Fig 4. Light micrograph illustrating hepatic portal space. **a**. control group, notice, branch of portal vein (Pv), branch of hepatic artery (Ha), and a small bile ductule (Bd) with cuboidal cells and connective tissue containing fibroblast surround the portal space (white arrow). **b,** vehicle group. branch of portal vein (Pv), branch of hepatic artery (Ha), and a small bile ductule (Bd), large hepatocytes with foamy cytoplasm indicated by arrow. **c**, DMBA- group. Illustrating, bile ductules cell wall hyperplasia, bile ductile fibrosis (head arrow) and inflammatory cell infiltration (arrow). **d,** DMBA + Colostrum, notice branch of portal vein (Pv), branch of hepatic artery (Ha) and a small bile duct (Bd) surrounded by small amount of connective tissue (arrow), nuclei of various sizes and stainability. **e,** colostrum-treated, hepatic portal area including branch of hepatic artery (Ha), branch of bile duct (Bd) and branch of portal vein (Pv), infiltrated leucocytes (arrow).

oration (Fig. 8 a,b,d&f), DMBA-administration lead to increased expression of p53 evident by an insignificant increase in the nuclear p53 protein expression and a significant increase in the p53 cytoplasmic expression when compared to control groups (Fig. 8 c&f). While the post treatment with colostrum after DMBA administration significantly decreases the expressional level of the p53 protein in the cytoplasm level (Fig. 8 e&f).

3.5. Effect of BC on the transcriptional level of p53, TGFβ2, TNF-α, S6K2, and C20orf20

We examined the effect of BC treatment on the expression of the tumor suppressor gene, p53. In DMBA-treated animals, when compared to controls, the expression of p53 mRNA was significantly increased ($P < 0.001$). The post-treatment with BC resulted in a significant decrease ($P < 0.05$) in p53 mRNA versus DMBA-treated group and significantly increase ($P < 0.001$) compared to the controls (Fig. 9). Besides, the expression of TGFβ2 mRNA significantly decreased ($P < 0.005$) in DMBA-treated group and significantly increased ($P < 0.005$) by post-treatment with BC, when compared to controls. For TNF-α mRNA expression, it insignificantly increased in DMBA-treated group and significantly increased ($P < 0.05$) after treatment with BC. To understand the effect of colostrum on cellular proliferation, we analyzed the mRNA expression levels of the ribosomal S6 kinase 2 (S6K2) and C20orf20 (MRG-binding protein), a component of TRRAP/TIP60 histone acetyltransferase complex. The expressional level of S6K2 mRNA was significantly increased ($P < 0.05$) in DMBA treated group and significantly decreased ($P < 0.01$) by post treatment with colostrum after a single dose of DMBA. While, c20orf20 mRNA expressional level, it was found to be significantly increased ($P < 0.01$) in DMBA treated group and significantly decreased ($P < 0.001$) by post treatment with colostrum after a single dose of DMBA.

4. Discussion

Although it have been reported, 20 years ago, that Bovine Colostrum (BC) may be used for the treatment of liver malfunctions

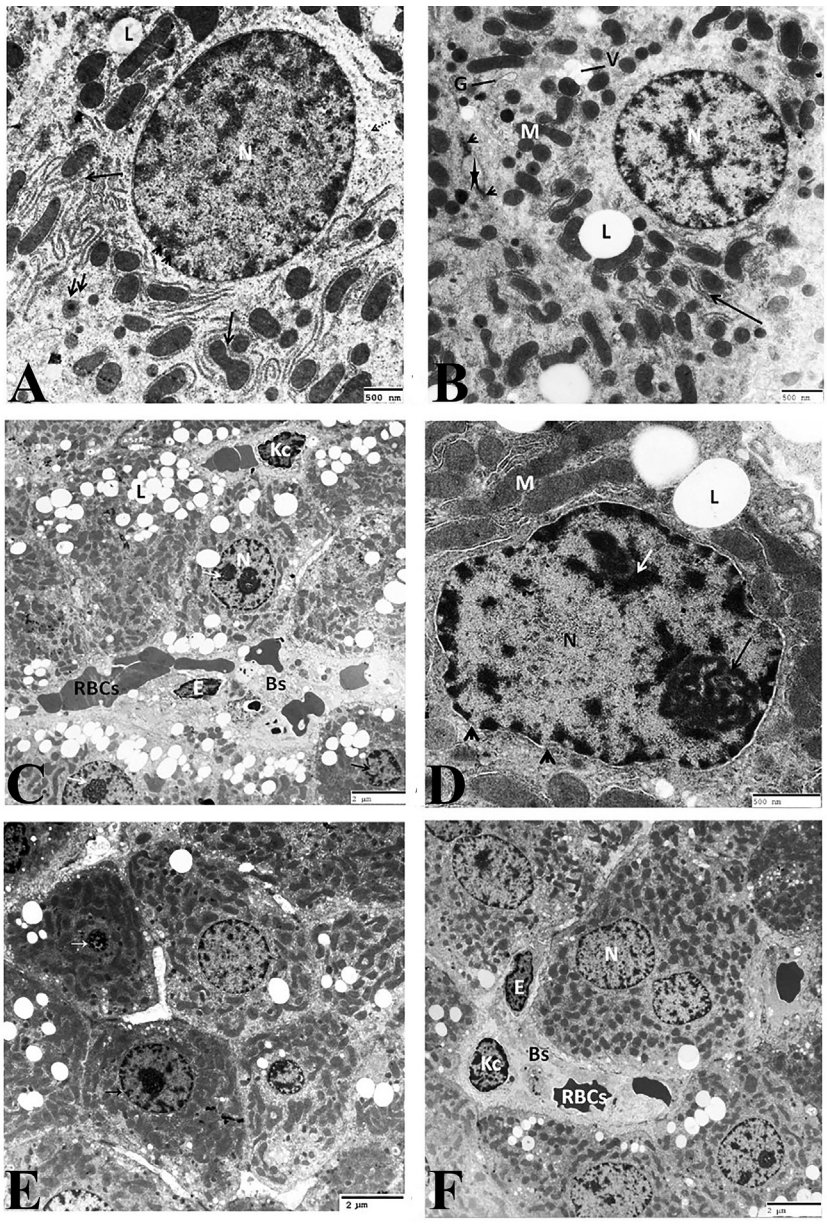

Fig. 5. TEM liver sections of rat. a) Control (GI) showing hepatocyte with a spherical nucleus (N), distinct nuclear membranes, few nuclear pores (arrow head), evenly distributed mitochondria encircled by RER (arrows), glycogen (g), lipid droplet (L), small peroxisome (double arrow) [X, 2500]. b) Olive oil-administrated rat liver. Hepatocyte nucleus (N), heterochromatin normally distributed, evenly-distributed mitochondria (M) with indistinct membrane and dense matrix associated with lipids (L), others associated with RER (arrow), Golgi areas (G), vacuoles (V), lipid droplets (L), and bile canaliculi (*) sealed with tight junction (arrowhead) [X, 3000]. C) Liver of rat administrated DMBA showing hepatocytes with ill- defined cell boundary, eccentric nucleus (N), segregated nucleolus (white arrow), nucleus with marginated heterochromatin (black arrow), lipid (L), blood sinusoids (Bs), endothelial cell (E), Kupffer cells (Kc), and (RBCs) [X, 1000]. d) Illustrating, nucleus (N) with decreased heterochromatin content, segregated nucleolus (black arrow), irregular nuclear membranes with dilated pores (arrow-head), mitochondria with dense matrix (M) surrounded by RER and lipid vacuoles (L) [X, 5000]. e) Liver of rat administrated DMBA and colostrum. Hepatocytes with regular outline, eccentric nucleus with normal distributed heterochromatin (N), dense nucleolus (black arrow), pyknotic nucleus (white arrow), numerous mitochondria [X, 1000]. f) Liver of rat administrated colostrum. Hepatocytes with polygonal outline, nucleus (N) with normal distributed heterochromatin, blood sinusoid (Bs), Kupffer cell (Kc), endothelial cell (E) [X, 1000].

(Lissner et al., 1998) and a satisfactory clinical trial have been completed in 2010 for the use of BC for patients with non-alcoholic fatty liver disease (Marinkovic et al., 2018), to our knowledge, the current study is the first report of the chemopreventive effect of BC against liver injury induced by DMBA. BC is the early milk produced by caws during the first days after parturition; it contains macronutrient, micronutrients, antimicrobial compounds, growth factors, and immune-regulating constituents in higher concentration than mature milk (Bagwe et al., 2015; Godhia and Patel, 2013; Maburutse et al., 2017). It also contains significant antioxidant activity (Lindner et al., 2011). Besides, it contains some potential anticancer constituents such as lactoferrin, conjugated linolenic acid (Godhia and Patel, 2013), and TNF-α (Van Horssen et al., 2006). The nutrient profile and immunological composition of early milk are different from those of mature milk. The major differences between BC and mature milk are that the former has higher levels of growth factors, immunoglobulin (Ig), cytokines, nucleosides, oligosaccharides, antimicrobials, and immune regulating factors (Kaducu et al., 2011; Przybylska et al., 2007). Colostrum contains higher concentrations of immunoglobulins than milk. IgA concentration in colostrum is almost a hundred-fold higher than in milk (Uruakpa et al., 2002). Human Colostrum and BC are rich in

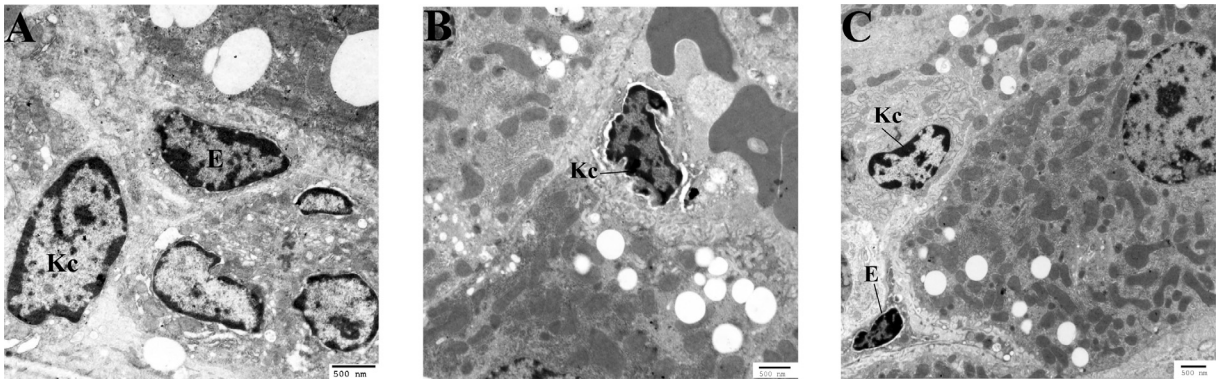

Fig. 6. TEM Liver section showing Kupffer cells. a) Illustrating, Kupffer cell (Kc) with normal distributed heterochromatin and dense nucleolus identified in control group [3000X]. b) altered Kupffer cell with highly irregular nuclear outline identified in DMBA-treated group [2500X] and c) Kupffer cell (Kc) with heterochromatic nucleus in rats treated with DMBA and colostrum [2000X].

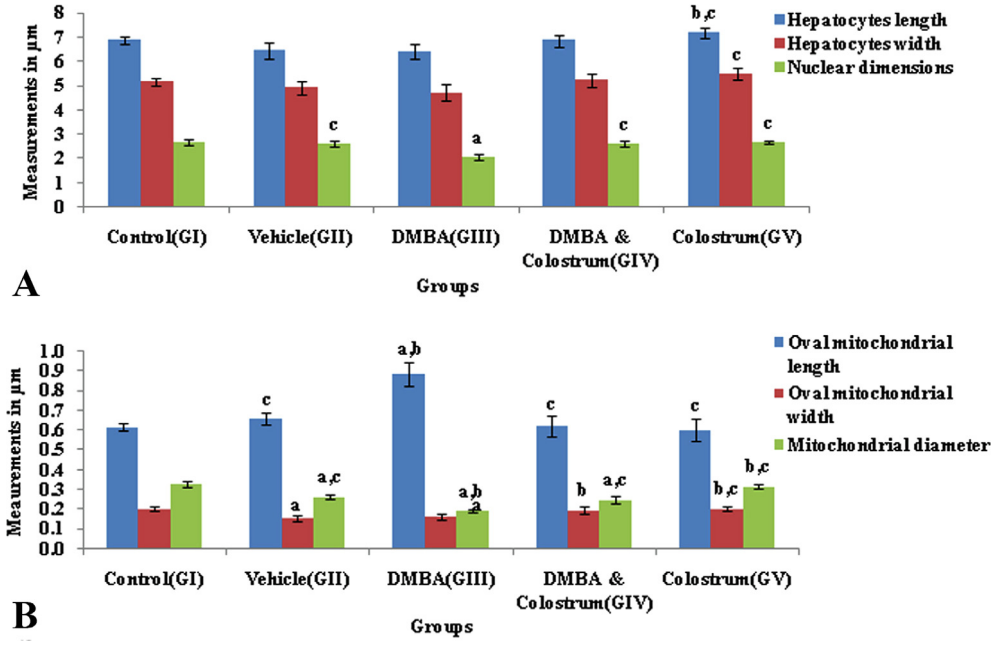

Fig. 7. Morphometric measurements of hepatocytes and mitochondria. a) Graphical representation of hepatocytes and nuclear dimensions (µm) in treated animals. b) Graphical representation of mitochondria (µm) in treated animals. Data are Means ± SE, n = 10, $P < 0.05$, a, b, and c are significant differences with control, vehicle, and DMBA-treated groups respectively.

growth factors, immunoglobulin, and lactoferrin (Godhia and Patel, 2013) but Cow colostrum is richer in IgG (20%) as compared to that in human colostrum (2%) (Stelwagen et al., 2009).

The ingestion of Colostrum can contribute to the improvement of wellness and health status in healthy humans and it helps the healing of subjects with an immune deficiency status. A low-dose of BC supplementation diminishes inflammatory indices following in soccer players (Kotsis et al., 2018). Several intact proteins that enriched in colostrum, such as immunoglobulins, lactoferrin, and growth factors, exert their effect directly in the intestine (Kindlein et al., 2018). Lactoferrin and immunoglobulins are partially resistant to digestive enzymes (Ontsouka et al., 2016) making direct effects feasible. Lactoferrin can interact with the intestinal epithelial cells via a receptor-mediated binding mechanism (Ontsouka et al., 2016). Studies in human infants and other animals suggest that the epidermal growth factor (EGF) and transforming growth factor-beta (TGF-b) can survive the gastrointestinal tract in neonatal animals (Bagwe et al., 2015; Hammon et al., 2013; Korhonen, 2013). All the previous characteristics of BC make it a good candidate as a potential anticancer therapy. Additionally, several studies have reported insignificant changes in transaminases liver enzymes (ALT and AST) levels after oral administration of colostrum (Karabacak et al., 2018; Maghraby et al., 2005). Here, BC did not cause any side effects, and was significantly effective in reducing liver injuries, suggesting its potential hepatoprotective effect which may be attributed to the antioxidant constituents of BC such as lactoperoxidase, catalase, superoxide dismutase, selenium-containing glutathione peroxidases, lactoferrin, vitamin E, carotenoids, vitamin C (Korhonen, 2013; Lindner et al., 2011).

Body status of experimental animals is generally considered as one of the essential parameters in studying the effects of toxic/carcinogenic chemical substances and their preventive effects as anticancer agents (Gangar and Koul, 2008). Our results indicated that rats administrated DMBA suffered from hair loss in accordance with the study on the effect of oral administration of DMBA in mice (Sharma and Paliwal, 2012). DMBA-administrated rats suffered from yellow and soiled fur, eyeball secretions, and diarrhea. The body weight changes occupy special attention since it is one of

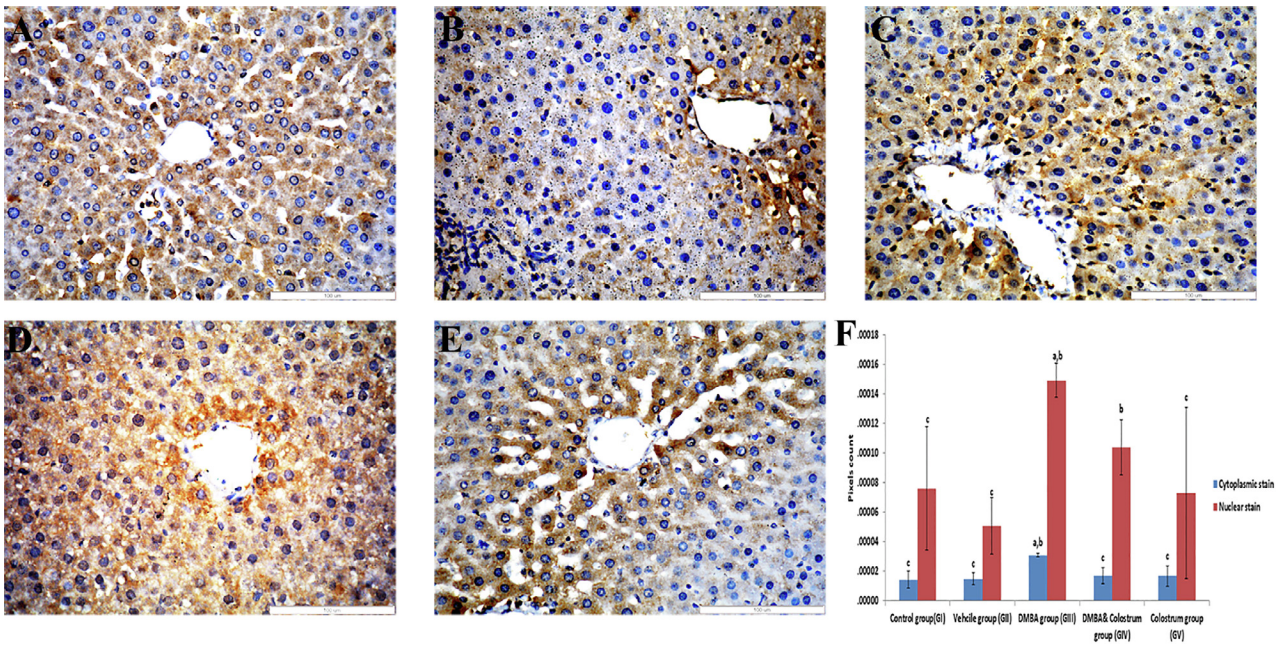

Fig. 8. A-E. Light micrograph showing Inhibition of p53 overexpression in DMBA-administrated rats as result of post treatment with bovine colostrum. a-b) Illustrating, few weakly stained hepatocytes of both control and vehicle-administrated rats liver sections. c) Illustrating, liver sections of rats administrated colostrum showed, weak p53 protein immunostaining similar to control group. d) Liver sections of rats treated with DMBA showed over expression of p53 protein in hepatocytes. e) Illustrating, decreased p53 due to administration of colostrum. f) Graphical representation of the p53 expression levels of n = 10 animals/group. Bars with different letters show significant differences between the groups.

the easiest parameters for assessment of how far the progression of the carcinogenesis process is (Gangar and Koul, 2008). In the present study, rats administrated DMBA showed a significant decrease in body weight gain. These results are in agreement with the findings reported previously (Sharma and Paliwal, 2012). The post-treatment with BC caused a significant increase in body weight in comparison to DMBA-treated animals. This may be due to the high antioxidant capacity of BC against oxidative damage induced by DMBA (Henkler et al., 2012; Lindner et al., 2011). Another possibility is the effect of colostrural growth factors which can help in building lean muscle mass and increasing the strength and endurance (Godhia and Patel, 2013). In the present study, DMBA administration caused a decrease in the survival rate in comparison to controls. Similar results were observed in rats as a result of DMBA treatment (Nisa et al., 2012).

Histologically, DMBA-treated rats showed injured hepatocytes displaying extensive cytoplasmic vacuolization, high eosinophilic cytoplasm, necrosis, compaction of blood sinusoids, and hyperchromatic nuclei. These alterations seem to follow the same pattern previously observed upon the effect of oral administration of toxins or carcinogens on the liver of rodents (Nandakumar et al., 2011; Sharma and Paliwal, 2012). These alterations were partially resolved by the BC administration. Ultrastructurally, the cytoplasm of hepatocytes exhibited different-sized vacuoles which may increase the permeability of cell membranes. This may result in intra-cytoplasmic lipid accumulation (Thoolen et al., 2010) and an increased number of pleomorphic mitochondria with dense matrix as reported previously (El-Mawla and Osman, 2012). This may explain the high eosinophilic cytoplasm observed here (Thoolen et al., 2010). In addition, the hepatocytes appeared swollen with dilated ER, distorted microvilli, and weak intercellular attachments. This may be due to mitochondrial dysfunction which can cause a reduction in the activity of the plasma membrane energy-dependent sodium pump resulting in intracellular accumulation of sodium and efflux of potassium (Kumar et al., 2013). The mitochondrial dysfunction was also observed when mouse hepatoma cells were treated with Benzo(a)pyrene (Ko et al., 2004). In addition, the treatment with DMBA led to the overexpression of p53 that may result in impairment in mitochondrial architecture and ER-mitochondria contact sites (Ottolini et al., 2013).

DMBA-administrated rats showed significant changes in the cell structure including highly irregular shape, hyperchromatic nuclei, and an increase in the number of bi-nucleated cells which is in accordance with previously mentioned data (Batcioglu et al., 2012; Poojari et al., 2010; Thapliyal et al., 2012). The increased number of bi-nucleated hepatocytes may be linked to the increase in mitotic activity (Poojari et al., 2010). Also, the nucleolus appeared irregular, large, and segregated similar to observations reported before (Bharati et al., 2012).

Inconsistence with the previous observation, blood sinusoids appeared ill-defined, compact, except in few pericentral areas, in DMBA-administrated group. Ultrastructural examinations revealed altered Kupffer cells with a large nucleus and many vacuoles, similar observations were reported (El-Mawla and Osman, 2012). The metabolism of DMBA may induce tumor formation through a primary step carried out by hepatocytes to yield detoxification and bioactivation products (Yardim et al., 2010). DMBA administration caused toxic, mutagenic, and carcinogenic effects due to the fact that its metabolites, as well as itself, could directly interact with DNA, and also can generate free oxygen radicals which are released during its metabolism (Batcioglu et al., 2012; Haritash and Kaushik, 2009), causing an increase in the oxidative stress that can exert a pathological influence.

The chemoprevention properties of BC may be attributed to its functional components which have been proved by in vitro studies to possess an antiproliferative activity by inducing apoptosis in cancer cells (Ebrahim et al., 2014; Godhia and Patel, 2013), inhabiting tumor progression, having antiangiogenic activity, and inducing tumor regression (Van Horssen et al., 2006). Additionally, it was reported that BC possessed an antioxidant activity that per-

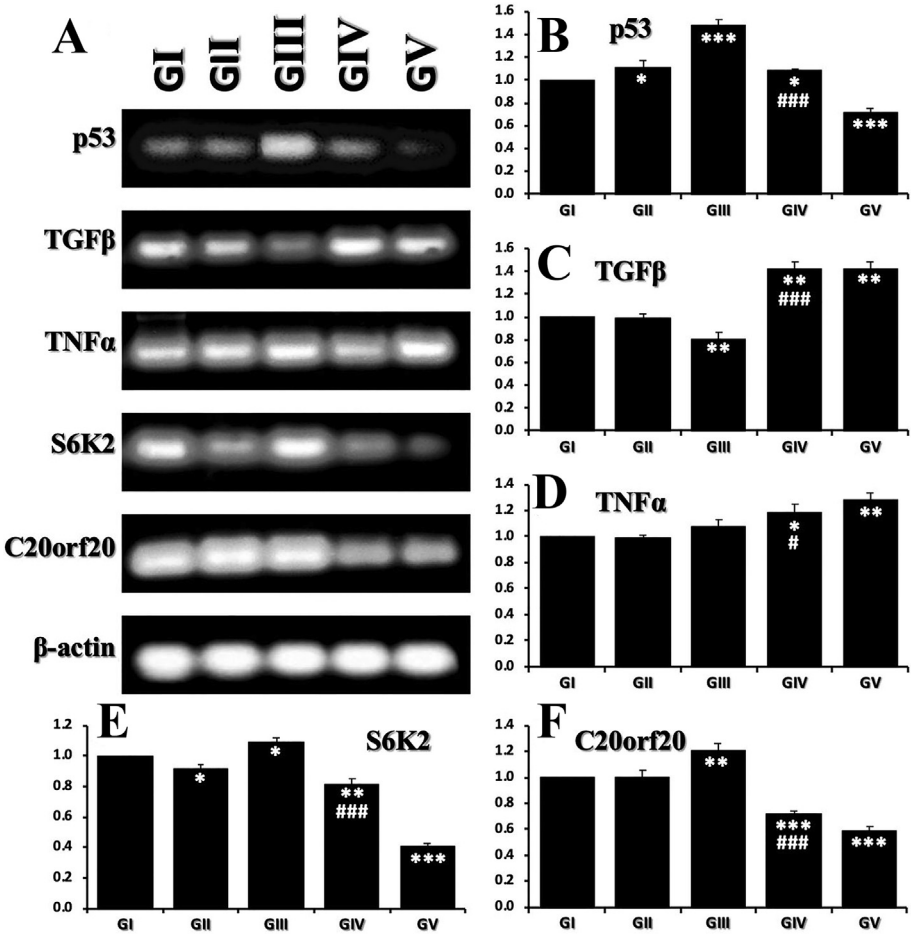

Fig. 9. Effect of bovine colostrum on the transcriptional level of p53, TGFβ, TNFα, S6K2 and C20orf20. a) The RT-PCR showed that Colostrum administration down-regulated p53, S6K2 and C20orf20 and up-regulated TGFβ and TNFα. b-f) Histograms represent semi-quantified expressions of genes after normalized to β actin and controls. * significant difference with Group I (*p < 0.05, ** p < 0.005, *** p < 0.001) # significant difference with Group III (DMBA group) (# p < 0.05, ## p < 0.005, ### p < 0.001).

mitted the scavenging of ROS (Lindner et al., 2011; Przybylska et al., 2007). Thereby, preventing oxidative damage to important biological macromolecules (Konopacka, 2004).

The p53 gene has been found mutated in more than 50% of all human tumors. It is a transcription factor that acts to restrict proliferation in response to DNA damage or induces apoptosis or senescence of altered cells (Zender et al., 2010). The failure of repair mechanisms and constant exposure to PAHs such as DMBA can induce p53 mutations (Muñoz and Albores, 2011). Moreover, in the present study, liver cells of control and BC exhibited weak p53 protein and mRNA expressional level. Since, in normal conditions p53 protein is not expected to be detected by conventional immunohistochemical technique as it cannot be accumulated in normal cells due to its short average half life time (de Freitas et al., 2014). However, DMBA treatment showed significant increase in p53 protein and mRNA expressional level, which was reduced by bovine colostrum administration. The stable mutant p53 protein accumulates in cells and can be easily detected (Rivlin et al., 2011). The high levels of functionally-inactivated p53 have been linked to HCC (Farazi et al., 2006). In the present study, DMBA treatment resulted in a significant increase in mRNA level of p53, which was decreased by BC treatment. Owing to the short half-life time of p53, it cannot accumulate in normal cells (de Freitas et al., 2014). The decrease in p53 levels after BC treatment might be attributed to the antimutagenic effect of BC through the antioxidant activity of its functional components which may reduce the intracellular levels of ROS induced by DMBA (Przybylska et al., 2007), hence preventing the DNA damage induced by ROS (Ozben, 2007).

TGFβ is a potent growth inhibitor for hepatocytes and has both tumor suppressor and oncogenic activities (de Caestecker et al., 2000). TGFβ-signaling was reported to be impaired during human hepatocarcinogenesis (Breuhahn et al., 2006). Mutational inactivation or dysregulated expression of TGFβ-signaling components help tumor cells to escape from the antiproliferative effects of TGFβ (de Caestecker et al., 2000). Exogenous TGF-βs have been shown to inhibit the growth and differentiation of mammary epithelial cells in cell culture, organ culture, and in vivo (Crowley et al., 2006). TGF-β overexpression inhibits tumorigenesis, and the abolition of TGF-β signaling accelerates tumorigenesis, suggesting that TGF-β acts as a tumor suppressor in mouse models of cancer (Law et al., 2002). TNF-α was the first cytokine to be employed for cancer biotherapy (Mocellin et al., 2005b). It can induce tumor regression in *in vivo* animal and human models by high-dose-shot TNF-α based regimens (Van Horssen et al., 2006). It can target the tumor-associated vasculature (TAV) inducing hyperpermeability and destruction of the vascular lining which results in an immediate effect of selective accumulation of cytostatic drugs inside the tumor and a late effect of the destruction of the tumor vasculature (Van Horssen et al., 2006). Here, BC treatment exhibited an increase in TGFβ and TNF-α mRNA levels. The up-regulation of TGFβ by BC treatment may aid to induce apoptosis as reported previously (Wiener et al., 2014). Also, it was reported that the mutant p53 achieves a gain of function by cooperating with TGF-β (Ji et al.,

2015). And the up-regulation of TNF-α may induce hyperpermeability of the vascular lining that may result in selective accumulation of cytostatic drugs and destruction of the tumor vasculature (Van Horssen et al., 2006).

The post-treatment with BC down-regulated C20orf20 and S6K2 mRNAs levels. S6K is represented by two homologous proteins, S6K1 and S6K2, both act downstream of mTOR and phosphorylate S6 ribosomal proteins (Khalil and Gout, 2012). Most of the studies have been focused on S6K1 and little is known about the function of S6K2 (Ismail et al., 2014). mTOR signaling pathway is a central regulator of protein synthesis, cell proliferation, and survival (Khalil and Gout, 2012). The increase in the expression of mTOR was reported in 5% of hepatocellular carcinoma (HCC), whereas overexpression of phospho-mTOR (the activated form of mTOR) was evident in 15% of HCC. Phospho-mTOR is positively correlated with increased expression of total S6K as reported in 45% of cases of primary liver neoplasms (Sahin et al., 2004). C20orf20 (MRGBP) is one of the ubiquitous MRG/MORF family of proteins that are involved in cell senescence and the terminal loss of proliferative potential (Bowman et al., 2006). Little is known about C20orf20 role in liver cancer. C20orf20 is up-regulated in the majority of colorectal tumors and its enhanced expression was associated with cell proliferation (Yamaguchi et al., 2010).

5. Conclusion

To the aim to investigate the effect of BC on DMBA-induced hepatic injury, our finding suggests that BC prevented DMBA-induced hepatotoxicity. The ultrastructural findings suggest that BC has a protective effect against DMBA evident in preserving hepatocyte integrity. On the transcriptional level, BC up-regulated TNF-α and TGFβ2, and down-regulated p53, S6K2, and C20orf20. Based on our results, BC supplementation may be helpful in the abrogation of DMBA-hepatotoxicity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

Approval from the institutional ethics committee was obtained. Animal experiments were done with strict accordance to the institutional ethical guidelines for care and use of animals in research. (Approval number: AU04200926301).

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