Original Article

CHEMOPROTECTIVE ROLE OF BOVINE LACTOFERRICIN AGAINST 7, 12 DIMETHYLBENZ (A) ANTHRACENE INDUCED SKIN CANCER IN FEMALE SWISS ALBINO MICE

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ABSTRACT

Objective: To investigate the chemo-preventive effect of subcutaneous injection of bovine lactofericin (LfcinB) against 7, 12 dimethylbenz (a) anthracene (DMBA) induced carcinogenesis in swiss albino female mice.

Methods: 30 animals were divided into 3 groups. 200µl of DMBA (0.025µg/µl) per animal three times per week as topical was used to induce skin cancer in both groups II and III. In addition to DMBA, group III was protected with subcutaneous injection of LfcinB (20µg/µl) while group I served as control. At the end of 16th week lysates/blood/serum were subjected to various tests such as antioxidant, glucose, liver enzymes and kidney marker parameters.

Results: Topical application of 200µl DMBA (0.025µg/µl) per animal three times per week has produced 100% tumor incidence in DMBA treated group at the end of 16th week whereas only 20% developed tumors in the LfcinB+DMBA treated group. Antioxidants level were found to be significantly (P<0.01) depleted in reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and liver enzymes, Alaninine aminotransferase (ALT) and Alkaline phosphatase (ALP). Subcutaneous administration of 200µl of LfcinB (20µg/µl) significantly (P<0.01) normalized the antioxidants (GSH, GR, GPx and CAT) levels.

Conclusion: LfcinB exerts its chemoprotective effect through acting as an antioxidant, thereby inhibiting carcinogenesis, hepatocellular and renal damage. Lfcin B may act as a promising chemoprotective agent against DMBA induced skin cancer.

Keywords: Chemoprevention, Lfcin B, Antioxidants, Liver enzymes, Kidney function markers, Glucose

INTRODUCTION

Chemoprevention is a pharmacological process that involves the use of natural or synthetic product(s) to prevent, suppress, reverse or delay carcinogenesis [1]. The methods of possible chemoprevention include neutralization or detoxification of carcinogens, suppression of cell proliferation, induction of cell death, modulation of the immune system, suppression of genetic instability or mutation [2]. Many antioxidants have the capacity to inhibit cellular event associated with stages of carcinogenesis [3]. Chemoprevention may involve inhibition of various steps in tumor initiation, promotion and progression. Several potential mechanisms have been described and attempts have been made to classify agents broadly according to the effects they have on different stages of carcinogenesis. Compounds that inhibit cancer initiation are traditionally termed as "blocking agents". They may act by preventing the interaction between chemical carcinogens or endogenous free radicals and DNA, thereby reducing the level of damage and resulting mutations which contribute not only to cancer initiation but also progressive genomic instability and overall neoplastic transformation [4]. Protection may be achieved as a consequence of decreased cellular uptake and metabolic activation of procarcinogens and/or enhanced detoxification of reactive electrophiles and free radical scavenging, as well as induction of repair pathways [5]. Other protective processes include modulation of DNA methyltransferases to prevent or reverse the hyper methylation-induced inactivation of tumor suppressor genes. Inhibition of histone deacetylases has also been described among a variety of effects of blocking agents on epigenetic mechanisms of carcinogenesis [6].

Bovine lactoferricin (Lfcin B) is a peptide fragment of bovine produced from lactoferrin (LF), a peptide obtained from cow's milk by acid-pepsin hydrolysis of the lactoferrin [7]. Lfcin B consists of about 17 to 41 amino acid residues near to the NH2 end of bovine lactoferrin and has quite high proportion lop-sided group of basic amino acid residues. Lfcin is generated from the LF by gastric pepsin or by proteases *in vivo*. The extensive antimicrobial action of LF and LfcinB against microorganisms such as bacteria, fungi, parasites and viruses has been well documented. LF possess various antimicrobial properties which primarily acts by the chelation of iron. LF can bind to lipopolysaccharide (LPS) and DNA through an deficient iron mechanism. It has been proposed that a fragment of LF i.e. Lfcin B can cross the bacterial cell membrane resulting in the pushing of intact LF inside cytoplasm [8]. The intact LF or its proteases digest Lfcin B; it may then bind DNA and interfere with RNA synthesis. Considerable levels of LfcinB are established in the human stomach after intake of bovine lactoferrin, indicating that Lfcin B broken down naturally by means of proteases from cow's milk [8].

Due to its well established antimicrobial action, considerable attention has been paid to LfcinB [9-11]. Recent *in vivo* studies have shown LfcinB as a potent anticancer agent [12-13]. LfcinB suppresses azoxymethane-induced colon cancer up to 83% when given orally to rats. A similar result was also obtained by oral administration of intact bovine lactoferrin, which increases the chances that LfcinB resulting from nutritional bovine lactoferrin may protect against colon carcinogenesis. Consumption of milk and milk products has been recently identified to reduced risk of colorectal cancer in humans [14] and at the same time reduced tumor growth in mice induced by 1, 2 dimethylhydrazines [15].

Skin cancer is the most common form of cancer occurring in humans and proceeds through three distinct phases, initiation, promotion and progression. Skin cancer usually occurs more than that of lung, breasts, colorectal and prostate cancer; it is usually the most frequently diagnosed form of cancer. Skin cancer starts as precancerous lesions and environmental carcinogens play has been shown to play a role in the initiation of skin carcinogenesis [16, 17]. It represents the main and growing health problem in the public and of all new cancers diagnosed annually in the world, almost one-third was estimated to originate in the skin [18]. In the United States, almost 1.2 million new skin cancer cases are diagnosed each year [19]. The highest incidence of skin cancers were reported every year in South Africa and Australia than any other countries throughout

the world, due to the fact that populations of these countries receive high amounts of UV radiation [20, 21]. In India, skin cancer accounts for 1-2% of all cancers [22].

DMBA is a potent carcinogen and a polycyclic aromatic hydrocarbon (PAH) consisting of four aromatic rings structure [23]. Dihydrodiol epoxide is the active metabolite that is produced during the metabolic activation of DMBA and is responsible for DNA damage. Reactive oxygen species (ROS) generated in excess during metabolic activation of DMBA also contributes to the oxidative stress and the DNA damage. It is generally used to initiate and promote skin carcinogenesis in Swiss albino mice [24, 25]. The fact that DMBA induces carcinogenesis urges us to find chemopreventive agents either of natural or synthetic origin for therapy using experimental animals [26]. Oxidative stress takes place in the cells due to unbalance in oxidant and antioxidant status of the cells. Oxidative stress causes damaged to DNA, lipids and proteins and as well impair the structure and function of membrane bilayer [27]. Reactive oxygen species (ROS) mediated lipid peroxidation has been shown to be involved in causing many cancers including skin cancer. Mammalian cells have a number of antioxidant defense mechanisms that neutralizes the harmful effects of ROS and protect the cells. In spite of the role played by the skin antioxidants in maintaining the redox potential of the cell, premature aging of skin and tumor initiation occurs if ROS are excessively generated in the skin [28, 29]. In our previous research, we have demonstrated that Lfcin B inhibits GSTP1 activity in human placental and MDA-MB-231 cells, which may induce synergistic effects when used in combination with antineoplastic drugs that are substrates of GSTP1 enzyme. This combination will exert a double attack on cancers overexpressing GSTP1, first sensitizing them to anticancer drugs by preventing their metabolism and secondly by suppressing the GSTP1 sensitizing cells to anticancer drugs which are either substrates of GSTP1 or inducing programmed cell death by activating c-Jun N-terminal kinases (JNK) [30]. In our current study, we have investigated the *in vivo* chemopreventive activity of Lfcin B in DMBA induced mouse model of skin cancer. This type of role of Lfcin B as the chemopreventive agent has never been conducted before.

MATERIALS AND METHODS

Chemicals

7, 12-dimethylbenz (a) anthracene (DMBA) was purchased from Sigma-Aldrich India, Reduced glutathione was obtained from Calbiochem, India, 1-chloro-2, 4-dinitrobenzene (CDNB), 5,5' dithio (*bis*) nitro benzoic acid (DTNB), thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchase from Himedia India. AST, ALP, ALT, Uric acid, Urea and Creatinine kits were purchased from Span diagnostic limited (Surat) India.

Female, Swiss Albino mice 5-8 w old, weighing 20-35g were purchased from Luvas, Haryana, India. The animals were maintained in the Central Animal House, Sharda University. The animals were housed in polypropylene cages and provided standard pellet diet and water *ad libitum* and maintained under controlled conditions of temperature and humidity, with a 12 h light/dark cycle. The project was approved by the institutional animal ethics committee (Register number 1173/PO/Ere/S/08/CPCSE). The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Sharda University in accordance with Indian National Law on animal care and use.

Experimental design

The animals were randomly assorted into the following groups:

Group I (n=10); this group receive a normal diet and were treated with 200 µl of acetone on the skin thrice a week and serve a negative control

Group II (n=10); this group of animals received a normal diet and were treated thrice a week with 200 µl of DMBA only. This group serves as positive control.

Group III (n=10); this group also receive a normal diet and were treated thrice a week with 200 µl of DMBA topically along with 200 µl of lactoferricin B subcutaneously. This group serves as a test.

Experimental duration and animal sacrifice

The experiment was conducted for 16 w. At the end of the experiment, all the animals were sacrificed by cervical dislocation and blood was collected by heart puncture and centrifuged for glucose, AST, ALT and ALP estimations while tissues (liver and kidneys) were removed for various tests (reduce glutathione (GSH), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and lipid peroxides (LPO).

Preparation of tissue homogenate

Liver and kidneys were carefully excised from the animals, washed in a phosphate buffer saline (PBS) and weighed. Homogenate of the tissues was made in 100 mM Tris-HCl buffer (pH 7.4) and was used for the estimations of reduce glutathione (GSH), glutathione S-tranferase (GST), catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) and lipid peroxidation (LPO).

Estimations of anti-Oxidants and xenobiotic enzymes activities

Reduced glutathione (GSH)

GSH was estimated by the method described by Ellman [31]. About 500µl of tissue homogenate was precipitated with 2000µl of 5% trichloroacetic acid (TCA) and then centrifugation. 1000µl of the supernatant was taken out and added 500µl ml of 5, 5' dithi (*bis*) nitrobenzoic acid (19.8 mg of 5, 5' dithio (*bis*) nitrobenzoic acid in 100 ml of 1% sodium citrate) and 3000µl of phosphate buffer. The yellow color developed was read at 412 nm.

Glutathione-S-transferase (GST)

The activity of GST was measured by the method prescribed by Habig *et al.*, [32]. The reaction mixture containing 1000µl of the buffer, 100µl of 1-chloro-2, 4-dinitrobenzene (CDNB), 100µl of homogenate and 1700µl of distilled water was incubated at 37 °C for 5 min. The reaction was started on the addition of 1000µl of glutathione. The increase in absorbance was followed for 3 min at 340 nm.

Estimation of catalase (CAT)

Estimation of CAT was determined by the method described by Takahara *et al.*, [33]. Tissue homogenate (200µl) was mixed with 1200µl of 50 mM phosphate buffer pH 7.0. The reaction was initiated by the addition of 1000µl of 30 mM H_2O_2 solution. The decrease in absorbance was recorded at 240 nm after 30 seconds intervals for 3 min by a spectrophotometer. The enzyme activity was expressed as µ moles of H_2O_2 decomposed/min/mg protein.

Estimation of superoxide dismutase (SOD)

The activity of SOD was measured by the method of Misra and Fridovich [34]. Tissue homogenate (100µl) was mixed with ethanol (750µl) and chilled chloroform (150µl) and were centrifuged. To supernatant (500µl) equal amount of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer. The reaction was initiated by the addition of 500µl of 1.8 mM epinephrine (freshly prepared) and the increase in absorbance at 480 nm was measured in a spectrophotometer. One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto-oxidation.

Estimation of lipid peroxidation (LPO)

The homogenate was utilized for the determination of lipid peroxidation by the method described by Ohkawa *et al.*, [35]. Released Malondialdehyde (MDA) was used as the record for lipid peroxidation. In short, to 200µl of tissue homogenate, 200µl of 8.1% SDS, 1.5 ml of 20% acidic corrosive and 1500µl of 0.8% thiobarbituric acid (TBA) were included. The volume was made up to 4000µl with distilled water and was incubated in a water bath at 95.5 °C for 60 min. The above solution was then cooled and 100µl of water and 5000µl of n-butanol/pyridine blend were included and shaken vigorously. The constituents were centrifuged and the organic layer was isolated for estimation of absorbance at 532 nm.

Estimation of glutathione peroxidase (GPx)

GPx activity was measured by the method described by Rotruck *et al.*, [36]. To 0.2 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide

and 0.5 ml of tissue homogenate were included. Glutathione solution (0.2 ml) and hydrogen peroxide (0.1 ml) were included the mixture. The contents were blended well and were incubated at 37 °C for 10 min alongside the control tubes containing all the reagents, however, no enzyme. Following 10 min, the reaction was inhibited by the addition of 0.4 ml of 10% TCA. Tissue homogenate (0.2 ml) was added to the control tubes. The tubes were centrifuged and the supernatant was examined for glutathione content at 340 nm by the addition of Ellman's reagent.

Estimation of glutathione reductase (GR)

Glutathione reductase was evaluated by the method depicted by Carlberg and Mannervik [37]. The total reaction volume contained 1 ml of 0.2M sodium phosphate buffer (PH= 7.0), 2 mM EDTA, 1 mM EDTA, 0.2 mM NADPH. The reaction was initiated by including 25µl

of the homogenate (cytosol). The decrease in absorbance is measured after for 3 min at 340 nm. The decrease in absorbance is directly proportional to the activity of the enzyme.

Total protein

Total protein was estimated method described by Lowry *et al.*, [38] using bovine serum albumin as standard.

Estimations of biochemical parameters

Blood glucose estimation

Blood glucose was estimated by using glucose oxidase end point assay using a commercial kit (Span diagnostics Ltd, India) according to the manufacturer's protocol based on Trinder 1969 [39]. Table 1 shows the procedure for estimation of blood glucose.

Table 1: Procedure for estimation of blood glucose

Pipette into tube marked	Blank	Standard	Test
Plasma/serum	-	-	10µl
Reagent 3	-	10µl	-
Working reagent glucose	1000µl	1000µl	1000µl

Calculation

Serum/plasma glucose (mg/dl) = (absorbance of test/absorbance of standard) X 100

Estimation of aspartate aminotransferase (AST)

Aspartate aminotransferase activity was estimated by modified UV (IFCC), a kinetic assay using a commercial kit (Span diagnostics Ltd, India) according to the protocol provided by the manufacturers as recommended Schumann *et al.*, [40]. Table 2 shows the procedure for estimation of aspartate aminotransferase.

Calculation

AST activity = ΔA/minute × Kinetic factor

Where kinetic factor, 1768

Estimation of Alanine aminotransferase (ALT)

ALT modified UV (IFCC), the kinetic assay was performed using a commercial kit (Span diagnostics Ltd, India) according to manufacturer's protocol as recommended Schumann *et al.*, [41] table 3. Shows the procedure for estimation of alanine aminotransferase.

Table 2: Procedure for estimation of aspartate aminotransferase

Pipette into tube marked	Test
Serum/plasma	100µl
Working AST reagent	1000µl

Table 3: Procedure for estimation of alanine aminotransferase

Pipette into tube marked	Test
Serum/plasma	100µl
Working ALT reagent	1000 µl

Calculation

ALT activity = ΔA/minute × Kinetic factor

Where kinetic factor, 1768

Estimation of alkaline phosphatase

Alkaline Phosphatase is estimated by pNPP-AMP (IFCC), a kinetic assay using a commercial kit (Span diagnostics Ltd, India) according to the protocol provided by the manufacturers as by Tietz *et al.*, [42] table 4 shows the procedure for estimation of alkaline phosphatase.

Calculation

ALP activity = ΔA/minute × Kinetic factor

Where kinetic factor, 2712

Statistical analysis

The experimental results obtained are expressed as mean±standard deviation (SD). The data was subjected to one-way analysis of variance (ANOVA) and differences between samples were determined by Tukey multiple comparison tests using the SPSS 22 (Statistical program for Social Sciences) program. The level of significance was set at $p < 0.01$.

Table 4: Procedure for estimation of alkaline phosphatase

Pipette into marked tube	Test
Serum/plasma	20µl
Working ALP reagent	1000µl

RESULTS

The results of the study indicate that LfcinB possesses chemoprotective effect. Administration of DMBA has produced 100% tumor incidence in DMBA treated group at the end of 16th week whereas only 20% developed tumors in the LfcinB+DMBA treated group. The size of the tumors of the LfcinB+DMBA treated group was found to be significantly (P<0.01) decreased than those treated by DMBA only. The tumor volume of DMBA alone was found to significantly (P<0.01) higher than those LfcinB+DMBA treated group. A significantly decreased (P<0.001) in both liver and kidneys weight in DMBA treated animals was also noted. (table 5) shows initial and final weight of experimental animals.

Table 5: Weight parameters of experimental animals

Groups	Body weight (g)		Weight of liver (g)	Weight of kidneys (g)
	Initial	Final		
Control	31.825±2.22a	32.70±1.25a	1.61±0.15a	0.60±0.07a
DMBA only	31.81±1.75a	29.50±2.78b	1.49±0.26b	0.49±0.07b
DMBA+LfcinB	31.81±1.75a	32.70±1.41a	1.60±0.36a	0.59±0.12a

Values are expressed as mean±SD (n=10). Values that are not sharing common superscript in the same column differ significantly at p<0.01. Group 1(control) was compared against treated groups: Group II (DMBA alone) and Group III (LfcinB+DMBA) "The superscript "a" and "b" denotes statistical significance. For example, if groups I, II and III have "a" it means there is no statistical significance between them at p<0.01. On the other hand, if group I has "a" and either groups II, III have "b" it means there is statistical significance between them at p<0.01.

Administration of DMBA has produces 100% tumor incidence in DMBA treated group at the end of 16th week while only 20% developed tumors in the LfcinB+DMBA treated group. The size of the tumors on the LfcinB+DMBA treated group was found to be significantly (P<0.01) reduced than those treated by DMBA only (table 6). The tumor volume of DMBA alone was found to significantly (P<0.01) higher than those LfcinB+DMBA treated group. The tumor burden and a total number of the tumor were also found to be higher in DMBA alone than the LfcinB treated group. This has clearly indicated the effect of the LfcinB on the both tumor size and tumor volume in addition to its chemoprotective benefits.

Tumor volume was measured using the formula $v = 4/3\pi$ [D1/2] [D2/2] [D3/2] where D1, D2 and D3 are the three diameters of the tumors (cm). "The superscript "a" and "b" denotes statistical significance. For example, if group I and II both has "a" it means there is no statistical significance between them at p<0.01. On the other hand, if group I has "a" and group II has "b" it means there is statistical significance between them at p<0.01. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal. Number in parenthesis indicated a total number of animals bearing tumors.

Analysis of liver enzymes (AST, ALT ALP) has indicated that the group treated with DMBA alone exhibit elevated liver enzymes compared with control, and LfcinB+DMBA treated groups. Although there was no significant change observed in AST between DMBA and LfcinB+DMBA, there was a significant change in ALT and ALP in DMBA alone as compared to the control and LfcinB+DMBA.

Total protein was also found to be depleted. Liver is the major organ involved in detoxification of xenobiotics. Activation of pro-carcinogens like the DMBA also takes place in the liver. It is important that the liver function be tested to ascertain whether the action the DMBA causes changes in the liver enzymes. It is also important to check the effect of LfcinB on the liver enzymes. The results of the liver function tests are presented in table 7.

Table 6: Effect of Lfcin B on tumor incidence, tumor size, and tumor volume and tumor burden in DMBA treated mice

Groups	Tumor incidence	Tumor size(cm)	Tumor volume (cm³)	Tumor burden(cm³)	Total number of tumors
DMBA only	100% (10/10)	0.60±0.37a	0.36±0.40a	3.43±4.16a	7.70±3.89a
LfcinB+DMBA	20% (2/10)	0.12±0.15b	0.004±0.005b	0.005±0.007b	0.50±0.71b

Table 7: Hepatic marker enzymes

Groups	AST (UI/l)	ALT (UI/l)	ALP (UI/l)	Total protein (µg/ml)
Control	80.46±7.90a	40.12±2.22a	61.78±5.17a	465.44±68.17a
DMBA alone	91.68±8.04b	58.80±3.84b	81.76±9.69b	437.10±100.30b
DMBA+LfcinB	82.88±9.14a/b	42.39±3.89a	64.42±9.64a	458.85±22.39a/b

Values are expressed as mean±SD (n=10). Values that are not sharing common superscript in the same column differ significantly at p<0.01. Group 1(control) was compared against treated groups: Group II (DMBA alone) and Group III (LfcinB+DMBA) "The superscript "a" and "b" denotes statistical significance. For example, if groups I, II and III have "a" it means there is no statistical significance between them at p<0.01. On the other hand, if the group I has "a" and either groups II, III have "b" it means there is statistical significance between them at p<0.01.

At P<0.01 GSH was found to be significantly decreased in the group treated with DMBA only. A significant difference in the level of GSH has been observed between DMBA alone and other groups (control and LfcinB+DMBA). GST was also found to decrease in LfcinB treated group and DMBA alone treated group. This implies that GST is expressed in cancer-bearing mice compare to control, and LfcinB+DMBA treated groups. Glutathione peroxidase was also found to be decreased in DMBA alone treated groups. A significant difference was also observed in glutathione peroxidase content between the group treated with DMBA alone and other groups. In a similar manner, glutathione reductase and catalase were also found to differ between the groups. A significant difference was note in the level of catalase and glutathione reductase between DMBA and other groups while no differences were observed between LfcinB+DMBA and the control. Lipid peroxidation was found to be higher in the group treated with DMBA than other groups. Total protein content was also found to be depleted. The results of hepatic oxidative markers are presented in table 8.

Table 8: Oxidative stress markers in liver

Groups	GSH µmole/min/min	GST U/ml/min	GPx µmole/min/min	GR µmole/min/min	CAT µmole/min	SOD µmole/min/min	LPO µmole/min/min	Total protein (µg/ml)
Control	116.54[a] ± 7.25	17.86 ± 0.39[a]	109.12 ± 2.29[a]	117.42 ± 4.95[a]	103.17 ± 3.11[a]	50.95 ± 2.46[a]	109.86 ± 2.31[a]	465.44 ± 68.17[a]
DMBA alone	66.06 ± 3.94[b]	19.06 ± 3.27[b/a]	69.80 ± 3.90[b]	95.41 ± 4.53[b]	87.98 ± 6.19[b]	41.72 ± 1.60[b/a]	120.34 ± 4.81[b]	437.10 ±100.30[b]
DMBA+Lfcin B	110.40 ± 4.23[a]	15.60 ± 0.80[a]	104.87 ± 5.64[a]	111.83 ± 3.60[a]	105.17 ± 2.46[a]	46.80 ± 3.29[a]	110.67 ± 5.15[a]	458.85 ± 22.39[a/b]

Values are expressed as mean±SD (n=10). Values that are not sharing common superscript in the same column differ significantly at p<0.01. Group 1(control) was compared against treated groups: Group II (DMBA alone) and Group III (LfcinB+DMBA) "The superscript "a" and "b" denotes statistical significance. For example, if groups I, II and III have "a" it means there is no statistical significance between them at p<0.01. On the other hand, if the group I has "a" and either groups II, III have "b" it means there is statistical significance between them at p<0.01.

Renal GSH was found to be significantly depleted at p<0.01 in the group treated with DMBA only then in the other groups. However, no significant differences were observed between control and LfcinB+DMBA treated groups. At P<0.01, GST was also found to be significantly different in the group treated with DMBA alone than in the other groups. No significant difference was observed between control and DMBA alone treated group. In a similar situation, glutathione peroxidase, catalase and superoxide dismutase were found to be significantly depleted at P<0.01. A significant difference was observed in the level of glutathione peroxidase, catalase and superoxide dismutase between DMBA alone and the other groups (control and LfcinB+DMBA). The lipid peroxidation was also high in animals treated with DMBA alone. At P<0.01, significant different exist between DMBA alone and the other groups.

However, no significant differences exist between LfcinB+DMBA group and the control. The renal oxidative stress and xenobiotic enzymes are presented in table 9.

Table 9: Oxidative stress markers in kidney

Groups	GSH µmole/ml/min	GST U/ml/min	GPx µmole/ml/min	GR µmole/ml/min	CAT µmole/ml/min	SOD µmole/ml/min	LPO µmole/ml	Total protein (µg/ml)
Control	82.20 ± 6.09[a]	24.65 ±7.14[a/b]	87.13 ± 4.25[a]	87.64 ± 4.63[a]	140.82 ± 3.53[a]	62.74 ± 1.29[a]	63.60 ± 1.92[a]	333.35 ± 95.32[a]
DMBA alone	44.67 ± 3.87[b]	49.62 ±21.04[a]	60.78 ± 4.12[b]	67.24 ± 4.53[b]	126.81 ± 5.78[b]	53.13 ± 1.72[b]	75.56 ± 3.57[b]	295.60 ± 94.94[b]
DMBA+LfcinB	78.87 ± 6.34[a]	14.05 ± 2.0[b]	80.16 ± 2.95[a]	80.28 ± 4.51[a]	143.12 ± 3.53[a]	61.72 ± 3.72[a]	65.02 ± 2.58[a]	329.82 ± 62.01[a/b]

Values are expressed as mean±SD (n=10). Values that are not sharing common superscript in the same column differ significantly at p<0.01. Group 1(control) was compared against treated groups: Group II (DMBA alone) and Group III (LfcinB+DMBA) "The superscript "a" and "b" denotes statistical significance. For example, if groups I, II and III have "a" it means there is no statistical significance between them at p<0.01. On the other hand, if the group I has "a" and either groups II, III have "b" it means there is statistical significance between them at p<0.01.

Administration of 200µl of LfcinB subcutaneously has significantly lowered the blood glucose in group III compared to control group and DMBA alone treated groups. A significant changes has been observed in all the experimental groups at P<0.01. High glucose level was obtained in group II and differs significantly (P<0.01) than in all other groups. The decreased in blood glucose level associated with subcutaneous administration of LfcinB indicates the potential anti-diabetic abilities of bovine lactoferricin. The effect of administration of LfcinB is presented in table 10.

Table 10: Effect of administration of LfcinB on blood glucose

Groups	Blood glucose (mg/dl)
Control	81.92±5.97[a]
DMBA	92.12±4.63[b]
DMBA+LfcinB	70.71±4.93[c]

Values are expressed as mean±SD (n=10). Values that are not sharing common superscript in the same column differ significantly at p<0.01. Group 1(control) was compared against treated groups: Group II (DMBA alone) and Group III (LfcinB+DMBA) "The superscript "a", "b" and "c" denotes statistical significance. For example, if group I and II both has "a" it means there is no statistical significance between them at p<0.01. On the other hand, if group I has "a" and group II has "b" and group III has "c" it means there is statistical significance between them at p<0.01

The result of this study shows that the pro-carcinogen DMBA depleted the concentration of GSH while increasing the activity of GST. The activity of hepatic marker enzymes (AST, ALT and ALP) was found to have increased in DMBA treated animals significantly (P<0.01). This may be due to the activation of DMBA from pro-carcinogen to carcinogen followed by concomitant toxic to the liver.

The toxicity of DMBA has been confirmed by a significant depletion of GSH (P<0.01) in kidney and liver of DMBA treated animals compared with the control and LfcinB treated groups. The antioxidant status (GSH, GR, GPx, CAT and SOD) were also found to be depleted in group II. However subcutaneous injecting of 200µl of LfcinB three time per week for 16 w have significantly (P<0.01) increases the level of this antioxidants thereby preventing and reducing the tumor volume as well as the tumor size. This has clearly indicated the antioxidant benefits of LfcinB and its chemoprotective role in carcinogenesis.

Cancer chemoprevention as a strategy makes use of natural or synthetic products to prevent, delay, inhibitor reversed the development of cancer caused by a variety of agents. Chemoprotection has emerged as a means of cancer prevention for both individual under high risk and the general population. The potential role of cancer chemoprevention has been confirmed by several epidemiological and experimental studies [43] which emphasized the importance of the chemoprevention in both pre-neoplasm and neoplasm individuals. Molecules capable of acting as an antioxidant and Antioxidant enzymes as well has a potential role in providing protection against oxidative damage due to generated ROS as a result of DMBA treatment. Total protein was found to be decreased due to the free radical generated by DMBA in liver and kidney of DMBA treated mice. On the other hand, total protein in LfcinB treated group was found to be almost same with control animals indicating the carcinogenic reversal effect. DMBA may exert it inhibitory in protein synthesis, through reduced transcription of RNA and translation of DNA. It was described that the carcinogen, DMBA upsets intracellular calcium balance, thereby damaging endoplasmic reticulum which result in depletion of the protein [44].

GSH is a water-soluble antioxidant that plays a critical role in protecting the cell against oxidative damage and highly reactive oxygen species [45]. It makes a significant amount (about 90%) of the total non-protein in most cells and also plays an important role in detoxification of xenobiotics and peroxides into neutral or less toxic compounds through the catalysis by glutathione-s-transferase and glutathione peroxidase [46, 47]. The tripeptide participated in other cellular reactions, like the glyoxylase system, reduction reaction of ribonucleotides into corresponding deoxyribo-nucleotides, protein regulation and expression of gene through disulfide exchange reactions participates in the metabolism of estrogens, leukotrienes and prostaglandins, maturation of iron-sulfur clusters of diverse proteins and in the operation of certain transcription factors [48]. The tripeptide is present in the cells either as a reduced (GSH) or oxidized (GSSG) form. Keeping a balanced ratio between reduced and oxidized form of GSH is important to the cell survival and depletion of GSH increases the risk of cell for oxidative damage [49]. Its presence is a pre-requisite for protection against oxidative stress. Depletion of GSH has been associated with many pathophysiological processes and generates ROS and oxidative stress which affect the integrity of the cell and membranes of the organelles. The antioxidant effect of LfcinB has manifested in group III as LfcinB tries to restore the depleted GSH provoked by DMBA treatment. The low level of antioxidants (GSH, CAT, SOD, GPx, and GR) indicates poor antioxidant status. Studies have reported the decreased in antioxidant enzymes (CAT and SOD) in squamous and papilloma carcinogenesis which leads to a pro-antioxidant state of the cells, enabling tumorigenesis [50].

Oxidative stress leads to generation of MDA in the biological system. The MDA formed react with proteins and nucleic acid is thereby introducing cross linkages between nucleic acids and proteins, which result in changes in transcription and replication leading to the formation of tumors [51]. Elevated levels of MDA were observed in both kidney and liver of DMBA treated animals. Research has reported increased level of lipid peroxidation in the mouse skin model, which was decrease in response of flavonoids [52]. Significant decreased in MDA levels by LfcinB show reduce oxidative stress thus indicating it chemoprotective role against skin carcinogenesis. Oxidative stress causes injury to cells, induces gene mutation, and is involved in carcinogenesis by influencing intracellular signal transduction and transcription factors directly or indirectly via antioxidants. Oxidative stress is an indication of the imbalanced system between oxidants and antioxidant defenses. It occurs when oxidants overpower the antioxidant defense system and it is linked to many pathophysiologies including cancer [53].

GST is phase II detoxification enzymes which catalyzes the conjugation of reduced glutathione (GSH) with a variety of xenobiotics. The enzyme participates in detoxification reactions by catalyzing the conjugation of many electrophilic and hydrophobic compounds with reduced glutathione. In this study, GST was found to be significantly higher (P<0.01) in group II indicating that GST has been expressed in the cancer-bearing mice. Group III was found to have significantly (P<0.01) lower GST than in the other Groups indicating that LfcinB inhibits GST. An increased expression of GST pi has been reported in cancers of the breast, bladder, pancreas, colon, stomach, lung, neck and head, cervix, ovary, and, as well as soft tissue sarcoma, testicular embryonal carcinoma, glioma and meningioma [54-62]. In this scenario LfcinB has the ability of both acting as antitumor and inhibitor of GST found to be expressed higher in neoplasm cells than in normal cells.

Glutathione peroxidase is one of the essential antioxidant defense systems protecting the cells against oxidative stress. The glutathione peroxides reduce hydroperoxides by utilizing reduced glutathione (GSH). It carries out the reduction of hydroperoxides and hydrogen peroxide using GSH. The present study found significantly (P<0.01) reduction in glutathione peroxidase in Group II compared with the other groups. This clearly indicates poor antioxidants status in DMBA treated animal due to the generation of reactive oxygen species. However, no difference was observed between control and LfcinB treated group (group III). In this respect, LfcinB has clearly reversed the carcinogenic effect thereby restoring the antioxidants back to normal. Glutathione reductase is an important enzyme involved in the recycle of oxidized glutathione to reduced glutathione. The enzyme catalyzes NADPH-dependent reduction of oxidized glutathione to reduce glutathione thereby maintaining the concentration of reduced glutathione. The study found that the cancer-bearing mice have significant decreased (P<0.01) in the level of glutathione reductase. The decreased in the level of glutathione reductase implies that oxidized glutathione could not be converted into reduced glutathione. This exposes the DMBA treated mice to oxidative stress and as a result free radical mediated oxidative stress cause structural and functional abnormalities in the cells, making them susceptible to carcinogenesis [63]. Aminotransferases are a group of enzymes which carries out biotransformation of amino acids from α-amino acids to oxo acids. The enzymes are confined within the liver and are not a normal component of plasma. Their function outside organ of origin is unknown [64, 65]. The largest pool of ALT is found in the cytosol of hepatic parenchyma cells. AST is present in cytosol and mitochondria of the liver cells and also available in cardiac muscle, skeletal muscle, pancreas and kidney [66]. ALT is used for specifically to determine the hepatocellular damaged [67]. Although AST is being used to assess the liver function, it is considered to be as an indicator for mitochondrial damaged particularly centrilobular regions of the liver [68] ALP are a family of enzymes that hydrolyzed phosphate esters at alkaline pH and are used as markers for cholestasis liver functions [69].

CONCLUSION

The results from this study indicates that LfcinB exert it anticarcinogenic, chemoprotective effect through acting as an antioxidant thereby inhibiting carcinogenesis, hepatocellular and renocellular damage.

CONFLICT OF INTERESTS

Declared none

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