Original Research Article

Dexamethasone induced hepatic and renal damage in mice: Protective potential of Lycopene enriched tomato extract

ABSTRACT

Aim: The present study was carried out to determine the potential of Lycopene enriched tomato extract (LycT) in ameliorating hepatic and renal damage in mice caused by dexamethasone administration.

Study design: Group I (control) animals served as control and were orally administered with olive oil (vehicle). Group II (DEX) animals were intraperitoneally administered with dexamethasone at a dose of 5mg/kg b.w. on alternate days for three weeks. Group III (LycT) animals were orally administered with LycT at a dose of 5mg/kg b.w. on alternate days for five weeks. Group IV (LycT+DEX) animals were co-administered with LycT and dexamethasone as explained above.

Results: Dexamethasone caused hepatic and renal damage as evident from disturbed histoarchitecture, deranged levels of organ function markers and enhanced level of cell damage and oxidative stress markers. Increased blood glucose levels and decreased hepatic glycogen levels along with inhibited activities of enzymes involved in glycolysis (hexokinase, phosphoglucoisomerase) were also observed. Mitigation in histoarchitecture alterations, cell damage and oxidative stress markers, improved levels of organ function markers, blood glucose levels along with ramped up antioxidant defense system indicated the protective potential of LycT against dexamethasone induced ill effects.

Conclusions: These results point towards beneficial effects of LycT against dexamethasone induced damage to hepatic and renal tissues in mice.

Keywords: dexamethasone, glucocorticoids, carotenoids, lycopene, oxidative stress

1. INTRODUCTION

Corticosteroids include steroid hormones that are produced by the adrenal cortex and the synthetic analogues of these hormones which are used as drugs. They are broadly classified into mineralocorticoids and glucocorticoids and exhibit a wide range of physiological functions such as stress response, immune response, electrolyte balance, metabolism, etc (Ramamoorthy and Cidlowski, 2016). This 'steroid' class of drugs is used to treat and manage a variety of pathological conditions including inflammatory bowel diseases, auto-immune disorders, joint and muscle diseases, allergies, cancer etc. They are also prescribed during 'hormone replacement therapy' when the body is incapable of making endogenous steroids (Shaikh et al., 2012). Despite their immense clinical value, the use of corticosteroids is associated with many short- and long-term side effects (Rice et al., 2017). The range and severity of side effects experienced with use of corticosteroids is dependent upon dosage and duration of treatment (Yasir et al., 2020). Many of the side effects are reversible after the termination of steroid treatment while other side effects may be permanent. When steroids are used for long periods of time, or taken on multiple occasions, more serious side effects may occur.

Dexamethasone is a synthetic corticosteroid that has appreciable anti-inflammatory and immunosuppressive actions. It exhibits strong glucocorticoid effects and has minimal mineralocorticoid effects. Dexamethasone is considered as one of the most important drugs required in a basic health system and World Health Organization (WHO) has included it in its list of essential medicines (WHO, 2019). For several decades, dexamethasone is being used in various ailments including management of inflammatory conditions. Unfortunately, the use of dexamethasone has been linked to adverse effects including hypertension, hyperglycemia, muscle breakdown, hepatic steatosis, electrolyte imbalance, delayed healing, increased susceptibility to infections and even psychiatric disturbances (Bernal-Mizrachi et al., 2003; Vegiopoulos et al., 2007; Drozdowicz and Bostwick, 2014; Yasir et al., 2020).

It has been reported that long term use of glucocorticoids causes damage to liver and kidneys (Yasir et al., 2020). Studies have demonstrated that prenatal and neonatal dexamethasone exposure in rodents may lead to renal damage and failure in later life (Kamphuis et al., 2007; Liu et al., 2008; de Vries et al., 2010). Dexamethasone leads to impaired glucose and lipid metabolism, increased hepatic gluconeogenesis, adiposity (central and visceral), fat accumulation in liver which can contribute to insulin resistance resulting in hyperglycemia induced disorders (Ferris and Kahn, 2012). High levels of circulating glucocorticoids accelerate metabolic rates, and elevate free radical levels leading to oxidative stress. Dexamethasone administration led to enhancement in the levels of oxidative stress indicators in liver and kidneys of rats and mice (Kakali et al., 2004; Tayade et al., 2012; Hasona et al., 2017; Safaeian et al., 2018; Hasona and Morsi, 2019). Increased production of ROS and suppression of antioxidant enzyme activities has been reported after dexamethasone administration (Eid et al., 2007; Yi et al., 2016).

Carotenoids are powerful antioxidants that confer yellow, orange and red color to fruits and vegetables. Among the carotenoids, lycopene has the highest antioxidant activity which has been attributed to the presence of maximum number of double bonds in its structure. Lycopene is the most abundant carotenoid found in tomatoes (*Lycopersicon esculentum L*.). It is also present in rosehips, watermelon, papaya, pink grapefruit, guava, apricot etc (Mangels et al, 1993). The strong radical scavenging and antioxidant ability of lycopene is responsible for its several health benefiting effects. Studies have demonstrated the beneficial effects of lycopene against xenobiotic induced toxicities (Karahan et al., 2005; Moawad et al., 2007; Jamshizadeh et al., 2008). The cancer chemopreventive properties of carotenoids has been very widely reviewed and several epidemiological and experimental

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studies demonstrate the same (Tanaka et al., 2012). We have previously reported the protective effects of lycopene enriched tomato extract (LycT) against doxorubicin nephrotoxicity in mice (Koul et al., 2013). This was evident from improved urea and creatinine levels, reduced histopathological damage and lipid peroxidation level, and upregulation of antioxidant defense system. Studies conducted in our laboratory have shown considerable cancer chemopreventive action of LycT against hepatic and skin cancer in mice. LycT modulated several dysregulated pathways such as carcinogen biotransformation, DNA damage, cell proliferation, cell death, angiogenesis, metastasis, cell-cell communication etc (Gupta et al., 2013a; Gupta et al., 2013b; Bhatia et al., 2015; Gupta et al., 2016; Koul et al., 2020).

Considering the clinical usefulness of glucocorticoids, it becomes essential to minimize their associated adverse effects. Natural products or dietary components are appreciated for their suitability in counteracting drug induced organ toxicities and chronic diseases owing to their easy availability and acceptability (Arora and Koul, 2014; Madrigal-Santillán et al., 2014; Simon and Prince, 2016). Worldwide, tomatoes are commonly used for their flavor and nutritive values. The diversified biological effects of tomato and tomato-based products and the recognized health potential of carotenoids makes it worthwhile to examine the effects of LycT (carotenoid enriched extract) against dexamethasone induced hepatic and renal damage in mice.

2. MATERIAL AND METHODS

2.1 Chemicals

Dexamethasone was obtained from Sigma Chemical Co. (St Louis, MO, USA). Bovine serum albumin (BSA), 1-Chloro-2,4-dinitrobenzene (CDNB), 5,5-Dithiobis-2-nitrobenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide (NADH), oxidized glutathione (GSSG), reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), sodium acetate, thiobarbituric acid (TBA) etc were obtained from local reputed firms (Sisco Research Laboratory, Central Drug House, HiMedia). Other chemicals used for reagent preparation were obtained from above mentioned local firms and were of highest purity/analytical grade. Kits for estimation of kidney and liver function markers were obtained from Reckon Diagnostics Private Limited (Gujarat, India). Fresh tomatoes for the preparation of LycT were obtained from the local market.

2.2 Preparation of lycopene enriched tomato extract (LycT)

LycT was prepared in our laboratory from red tomatoes by following the method described previously (Gupta et al., 2013a; Gupta et al., 2013b). Tomato extracts including the one prepared for the present study have been previously characterised using spectroscopic techniques (FAO/WHO, 2009; Gupta et al., 2013a). NMR and FT-IR spectroscopy demonstrated the presence of groups characteristic of lycopene (=CH, $=CHCH_2$, $=CCH_3$, $=CCH_2$, trans C=C, carbon-carbon double bond stretching, $–CH_2$ bending, CH_3/CH_2 stretch, $–CH_3$ group etc). UV-Vis spectroscopy revealed that this extract exhibited absorbance maxima at 444, 470 and 503nm. In order to avoid interference from other carotenoids the content of lycopene in the extract was evaluated at 503nm using 1.72×10^5 L mol^{-1} cm^{-1} as the extinction coefficient (Gupta et al., 2013a; Gupta et al., 2013b; Bhatia et al., 2015; Gupta et al., 2016; Koul et al., 2020). This extract contained an average lycopene content of 12-14 mg/kg of tomato. It has been reported that use of dietary fats enhances lycopene absorption into the intestinal mucosa (Shi and Maguer, 2000). Considering this, olive oil was used to reconstitute LycT before its oral administration to animals. Pilot studies carried out in our laboratory have indicated no consequential alterations between control (untreated) and olive oil administered animals (data not included).

2.3 Animal model and experimental conditions

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Random bred male LACA mice (25–30 g each) procured from Central Animal House, Panjab University, Chandigarh (India) were housed in polypropylene cages. The cages were bedded with clean rice husk and the animals were provided *ad libitum* drinking water and standard animal pellet diet (Ashirwad Industries Ltd., Tirpari, Distt. Ropar, Punjab, India). The animal room was maintained at a temperature of 21±1ºC and humidity of 50-60%. All the experimental protocols were initially approved by the Institutional Ethics Committee of Panjab University, Chandigarh (India) and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals. The animals were acclimatized for one week before the commencement of the treatment regimen.

After acclimatization, the animals were randomly divided into four treatment groups (n=6-7 animals per group) depending upon the treatment they received (Figure 1). Group I (control) animals served as control and were administered with olive oil (vehicle) orally. Group II (DEX) animals were intraperitoneally administered with dexamethasone at a dose of 5mg/kg b.w. on alternate days for three weeks. Group III (LycT) animals were orally administered with LycT at a dose of 5mg/kg b.w. on alternate days for five weeks. Group IV (LycT+DEX) comanimals were co-administered with LycT and dexamethasone as explained above. The dose for dexamethasone administration was selected from literature reports (Poggioli et al., 2013). The dose for LycT administration was standardized in our laboratory and selected based on our previous reports (Gupta et al., 2013a; Gupta et al., 2013b; Bhatia et al., 2015; Gupta et al., 2016; Koul et al., 2020).

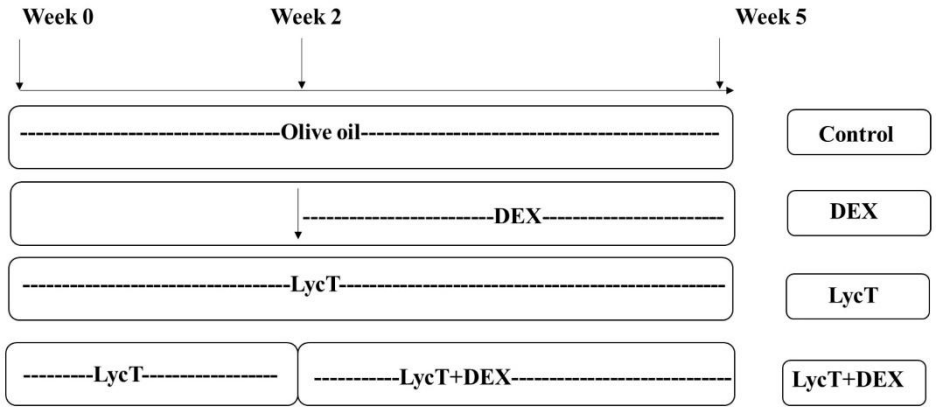

Figure 1: Treatment regimen of mice

2.4 Sample preparation

The animals were kept on overnight fasting before obtaining blood for serum preparation and excising out liver and kidney tissues for biochemical and histoarchitectural studies. 0.5% proparacaine hydrochloride ophthalmic solution was used to anaesthetize the mouse eye and whole blood was obtained from the retro-orbital plexus using a glass capillary. Blood was collected in a plain (without anti-coagulant) microcentrifuge tube and left undisturbed (allowing clot formation) for 4h at 37℃. After this, the tubes were kept at 4℃ for 30 min and then centrifuged at 3000 x g for 15min. The supernatant (serum) thus obtained was stored at -20°C until use. Tissue perfusion was done with cold 0.9% NaCl. The tissues were then blot dried, weighed and processed. The tissues were homogenized in 50mM Tris buffer (pH 7.4) to obtain 10 % homogenate (w/v). Aliquots of 10% homogenate were kept at 4°C for analysis of reduced glutathione and lipid peroxidation levels and the remaining homogenate was subjected to cold centrifuge at 10,000 x g for 30 min. The pellet was discarded and the

supernatant (post mitochondrial fraction) was used for the biochemical analysis of antioxidant defense system and carbohydrate metabolizing enzymes. A part of liver tissue was processed for estimation of glycogen content.

2.5 Glucose Metabolism

2.5.1 Glucose

Serum glucose level was analyzed using a commercially available assay kit. The kit is based on the procedure described by Trinder (1969). Glucose oxidase oxidizes β-D-glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide thus produced is acted upon by peroxidase and oxygen is liberated. The liberated oxygen is transferred to chromogen system consisting of 4-aminoantipyrene and phenolic compound to produce a red colored quinoneimine dye whose absorbance is read at 505 nm. The glucose concentration is proportional to the absorbance of the colored product and is expressed as mg/dL.

2.5.2 Glycogen

The glycogen content in the hepatic tissue was estimated using the protocol described by Seifter al., (1949). This method involved a series of steps including alkali digestion of tissue, ethanolic precipitation of glycogen, hydrolysis of the precipitate and determination of glucose using anthrone. A standard of glucose was plotted to determine the glucose content in the precipitate. A conversion factor of 1.11 (Morris factor) was used to convert glucose values into glycogen content (Morris, 1948). The glycogen content is expressed as mg glycogen/100mg liver.

2.5.3 Glucose metabolizing enzymes

2.5.3.1 Hexokinase

The hexokinase activity in hepatic and kidney tissues was estimated according to the method described by Crane and Sols, (1955) and Brandstrup et al., (1957). Hexokinase catalyzes the ATP dependent reaction of glucose into glucose-6-phosphate. The inorganic phosphorous (Pi) is reacted with ammonium molybdate to form phosphomolybdate which upon reduction with 1-amino-2-napthol-4-sulphonic acid forms a blue colored product whose absorbance is measured at 660 nm. The increase in the absorbance is directly proportional to the hexokinase activity. A standard curve using Pi was plotted and the amount of Pi in the test samples was calculated from the standard curve. The enzyme activity is expressed as nanomoles of Pi liberated/min/mg protein.

2.5.3.1 Phosphoglucoisomerase

The phosphoglucoisomerase activity in hepatic and kidney tissues was estimated according to the procedure described by Horrocks et al., (1963). This enzyme catalyzes the isomerization conversion of glucose-6-phosphate to fructose-6-phosphate. Fructose-6-phosphate in the presence of concentrated HCl gets dehydrated and converts to hydroxyl methyl furfural. This upon further condensation with resorcinol forms a cherry colored complex whose absorbance is measured at 470nm. The increase in absorbance is proportional to phosphoglucoisomerase activity and is expressed as nanomoles of fructose formed/min/mg protein.

2.6 Liver Function Markers

2.6.1 Serum glutamate oxaloacetate transaminase (SGOT)

SGOT activity was analyzed using a commercially available enzyme assay kit. The kit is designed according to the procedure described by Karmen et al., (1955). L-aspartate and α-ketoglutarate react in the presence of SGOT to yield oxaloacetate and L-glutamate. Oxaloacetate is reduced by malate dehydrogenase to yield L-malate accompanied by the oxidation of NADH to NAD. The decrease in the absorbance of NADH at 340nm is proportional to SGOT activity and is expressed as International Units/L (IU/L).

2.6.2 Serum glutamate pyruvate transaminase (SGPT)

SGPT activity was analyzed using a commercially available enzyme assay kit. The kit is designed according to the procedure described by Henry et al., (1960). L-alanine and α-ketoglutarate react in the presence of SGPT to yield pyruvate and L-glutamate. Pyruvate is reduced by lactate dehydrogenase to yield lactate accompanied by the oxidation of NADH to

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NAD+. The decrease in the absorbance of NADH at 340 nm was proportional to SGPT activity and is expressed as IU/L

2.6.3 Alkaline Phosphatase (ALP)

ALP activity in serum was analyzed using a commercially available enzyme assay kit. The kit was designed according to the procedure recommended by German Society for Clinical Chemistry (1972). ALP in the presence of magnesium ions hydrolyses p-nitrophenyl phosphate into p-nitrophenol and phosphate. The increase in absorbance at 405 nm due to generation of p-nitrophenol is proportional to ALP activity and is expressed as IU/L.

2.6.4 Bilirubin

Total and direct bilirubin in serum were analyzed using a commercially available kit. The kit is based on the procedure described by Jendrassik and Grof (1938). Bilirubin reacts with diazotized sulphanilic acid to form a colored azocompound whose absorbance is measured at 546 nm and reflects the concentration of bilirubin. Bilirubin concentration is expressed as mg/dL.

2.7 Cell Damage Marker-Lactate dehydrogenase (LDH)

LDH activity in serum was estimated according to the procedure described by Bergmeyer (1965). Pyruvate is reduced by LDH to yield lactate accompanied by the oxidation of NADH to NAD+. The rate of decrease in absorbance at 340nm due to the formation of NAD+ is indicative of LDH activity and is expressed as nanomoles of NADH oxidized/min/mg protein.

2.8 Kidney Function Markers

2.8.1 Urea

Serum urea level was analyzed using a commercially available kit. The kit is based on the modified Berthelot method as described by Chaney and Marbach (1962). Urease breaks down urea into ammonia and carbon dioxide. In the presence of sodium nitroprusside, ammonia reacts with hypochlorite and salicylate to form dicarboxyindophenol, a colored compound whose absorbance is read at 578 nm which is proportional to the urea content. The concentration of urea in the serum is expressed as mg/dL.

2.8.2 Creatinine

Serum creatinine level was analyzed using a commercially available kit. The kit is based on the method described by Folin (1904). In alkaline medium, creatinine reacts with picric acid leading to the formation of a red creatinine picrate complex (Jaffe's Reaction) whose absorbance is read at 510 nm. The intensity of the color developed is proportional to the creatinine content. The concentration of creatinine in the serum is expressed as mg/dL.

2.9 Oxidative Stress Marker and Antioxidant Defense System

2.9.1 Lipid peroxidation (LPO)

LPO level in hepatic and renal tissues was estimated according to the method described by Trush et al., (1981). The oxidative deterioration of lipids leads to the formation of cycloperoxides which on cleavage form malondialdehyde (MDA). MDA reacts with thiobarbituric acid (TBA) to generate pink colored MDA-TBA chromophore whose absorbance is read at 532 nm. The concentration of MDA-TBA chromophore is indicative of LPO and is expressed as nanomoles MDA-TBA chromophore formed/mg protein using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

2.9.2 Reduced glutathione (GSH)

GSH level in hepatic and renal tissues was estimated according to the method described by Moron et al., (1979). Reduction of 5, 5´-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) by the –SH groups of GSH forms a yellow-colored chromophore, 5-thionitrobenzoic acid whose absorbance is read at 412 nm. A standard curve using GSH was run to determine the levels in the test samples and expressed as nanomoles of GSH/mg protein.

2.9.3 Glutathione Peroxidase (GPx)

GPx activity in hepatic and renal tissues was estimated according to the method described by Lawrence and Burk (1976). GPx catalyses the conversion of hydrogen peroxide to water

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in the presence of reduced GSH to form GSSG and accompanied by the oxidation of NADPH to NADP+. The oxidation of NADPH which reflects the activity of GPx was measured by the decrease in absorbance at 340nm. GPx enzyme activity is expressed as nanomoles of NADPH consumed/min/mg protein using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

2.9.4 Glutathione Reductase (GR)

GR activity in the hepatic and renal tissues was measured by the method of Williams and Arscott (1971). GR catalyzes the NADPH dependent reduction of GSSG to GSH. The enzyme activity was determined by following the decrease in absorbance at 340nm due to NADPH oxidation and expressed as nanomoles of NADPH consumed/min/mg of protein using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

2.9.5 Catalase (CAT)

Catalase activity in the hepatic and renal tissues was determined by the method described by Luck (1971). Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. The activity of catalase is reflected as the decomposition of hydrogen peroxide which is measured at 240nm. The enzyme activity was expressed as IU/mg protein using an extinction coefficient of 0.0394 $mM^{-1}cm^{-1}$.

2.9.6 Superoxide dismutase (SOD)

SOD activity in hepatic and renal tissues was determined according to the method described by Kono (1978). This enzyme activity estimation is based on the inhibitory action of SOD on the reduction of nitroblue tetrazolium by the superoxide anions which are produced by the photo-oxidation of hydroxylamine hydrochloride forming a blue color complex whose absorbance is read at 560nm. The enzyme activity was expressed as IU/mg protein.

2.10 Protein estimation

The protein content in the samples was analyzed according to the method described by Lowry et al., (1951). Proteins are reacted with copper ions in an alkaline medium and the aromatic amino acids reduce phosphomolybdate-phosphotungstic acid present in Folin's reagent to produce a blue colored complex whose absorbance is noted at 620nm. A standard curve of bovine serum albumin (BSA) was prepared to determine the protein concentration in the samples.

2.11. Histopathological studies

After excising out the hepatic and renal tissues, they were immediately immersed in buffered formalin for fixation. Post fixation, the tissues were dehydrated using ascending series of alcohol concentration, followed by clearing using benzene. Embedding of the tissues was done with paraffin wax. Using a hand driven microtome, 5µm thick paraffin sections were obtained and transferred to glass slides. These slides were then deparaffinized in xylene and stained using hematoxylin and eosin according to the procedure described by Humanson, (1961).

2.12 Statistical Analysis

Data is expressed as Mean ± S.D. SPSS software was used for the statistical evaluation. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was used for statistical analysis. $p \leq 0.05$ was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Assessment of glucose metabolism

3.1.1 Blood glucose level

A significant increase in blood glucose levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant decrease in glucose levels when compared to the animals in DEX group and increased when compared to control and LycT group. No changes were observed in the glucose levels of LycT group when compared to control group (Figure: 2a).

3.1.2 Hepatic glycogen level

A significant decrease in hepatic glycogen levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals did not

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cause any change in glycogen levels when compared to DEX group and decreased when compared to control and LycT groups. A decrease in hepatic glycogen levels was observed in LycT+DEX group when compared to control group (Figure: 2b).

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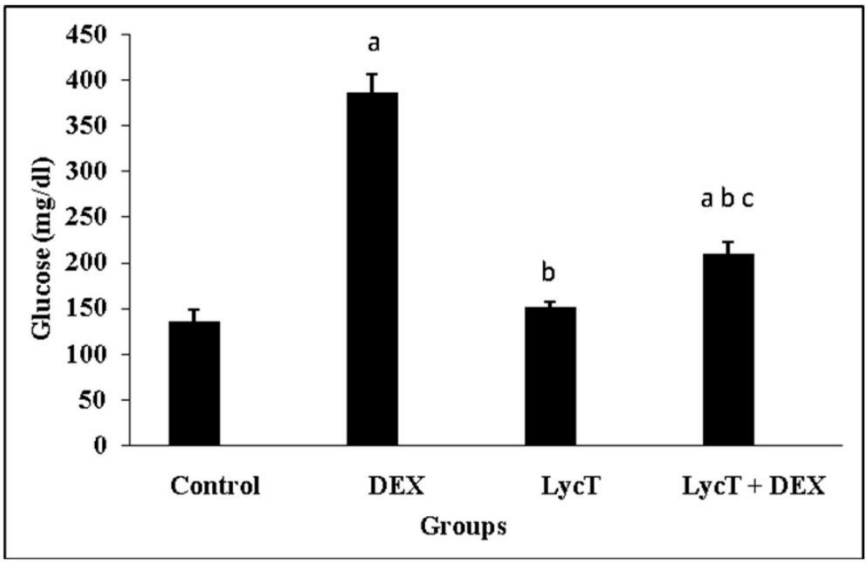
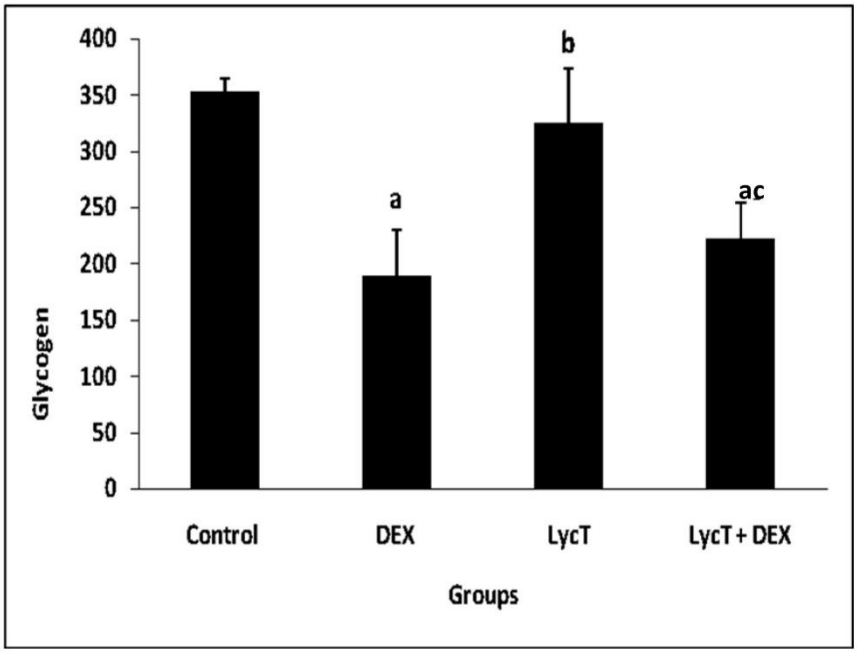


(a)



(b)

Figure 2: Modulatory effect of LycT and/ or DEX on (a) blood glucose level and (b) hepatic glycogen level

Data is represented as Mean±SD (n=5). Data is analysed by One-Way ANOVA followed by post hoc test. [a]p ≤0.05 significant with respect to control group; [b]p≤0.05 significant with respect to DEX group; [c]p ≤0.05 significant with respect to LycT group

3.1.3 Glucose metabolizing enzymes

3.1.3.1 Hexokinase

A significant decrease in hepatic and renal hexokinase levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in hepatic hexokinase levels when compared to DEX group and decreased when compared to control and LycT groups. Renal hexokinase levels remained unaltered in LycT+DEX group when compared to DEX group. No changes were observed in the hepatic and renal hexokinase levels of LycT group when compared to control group (Figure: 3a; 3c).

3.1.3.1 Phosphoglucoisomerase

A significant decrease in hepatic and renal PGI levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in PGI levels when compared to DEX group and remained unaltered when compared to control and LycT groups (Figure: 3b; 3d).

3.2. Assessment of Liver function markers

3.2.1 SGOT and SGPT

DEX administration caused a significant increase in serum levels of SGOT and SGPT when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant decrease in these levels when compared to the animals in DEX group and increased when compared to control and LycT group. No changes were observed in the SGOT and SGPT levels of LycT group when compared to control group (Figure: 4a-b).

3.2.2 Alkaline Phosphatase

A significant increase in serum ALP levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals caused no change in serum ALP levels when compared to the animals in DEX group and increased when compared to control and LycT group. No changes were observed in these levels in LycT group when compared to control group (Figure: 4c).

3.2.3 Bilirubin

A significant increase in total and direct bilirubin levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant decrease in total and direct bilirubin levels when compared to the animals in DEX group. Direct bilirubin levels increased in LycT+DEX group when compared to control and LycT groups while the total bilirubin levels remained altered between these groups. No significant difference was observed in the bilirubin levels in LycT group when compared to control group (Figure: 4e-f).

3.3 Cell damage marker

A significant increase in serum LDH levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant decrease in serum LDH levels when compared to the animals in DEX group and increased when compared to control and LycT group. No changes were observed in these levels in LycT group when compared to control group (Figure: 4d).

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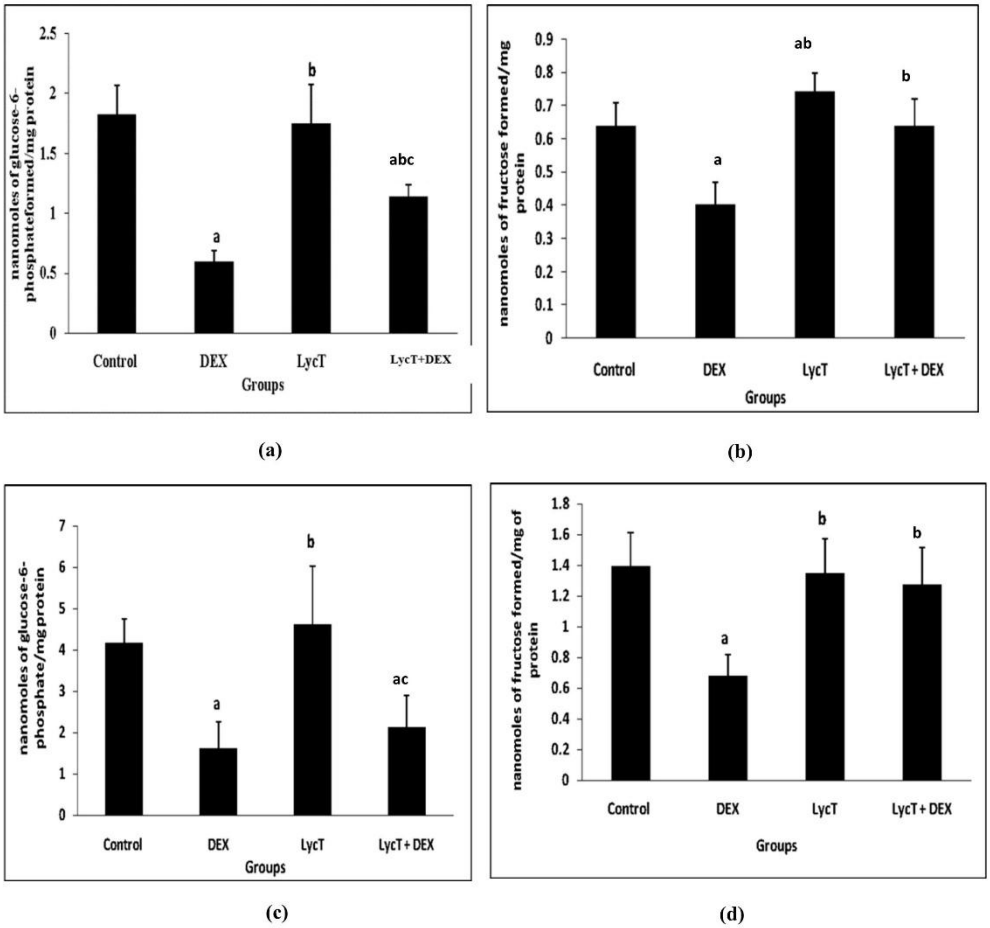


Figure 3: Modulatory effect of LycT and/ or DEX on hexokinase and phosphoglucoisomerase in (a, b) liver (c,d) kidney

Data is represented as Mean±SD (n=5). Data is analysed by One-Way ANOVA followed by post hoc test. [a]p ≤0.05 significant with respect to control group; [b]p≤0.05 significant with respect to DEX group; [c]p ≤0.05 significant with respect to LycT group

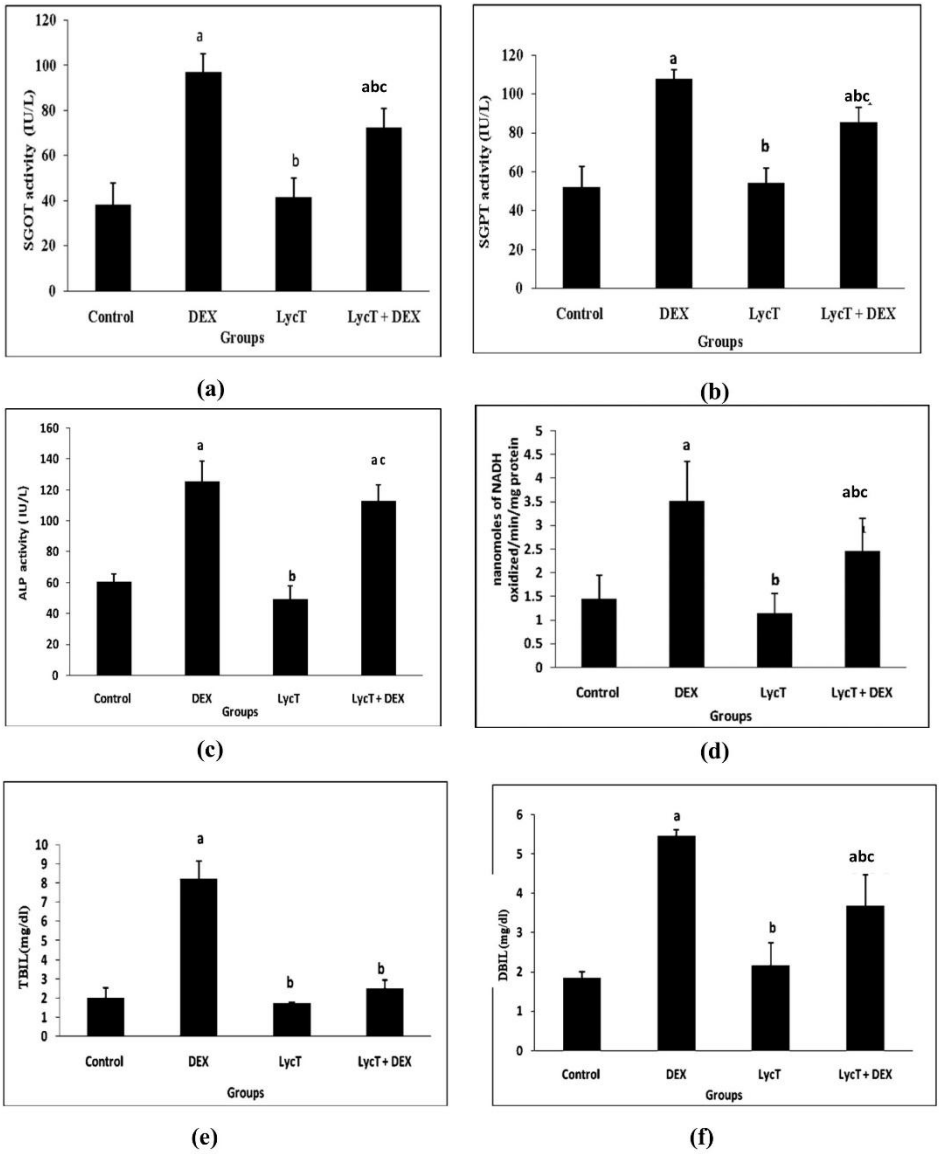
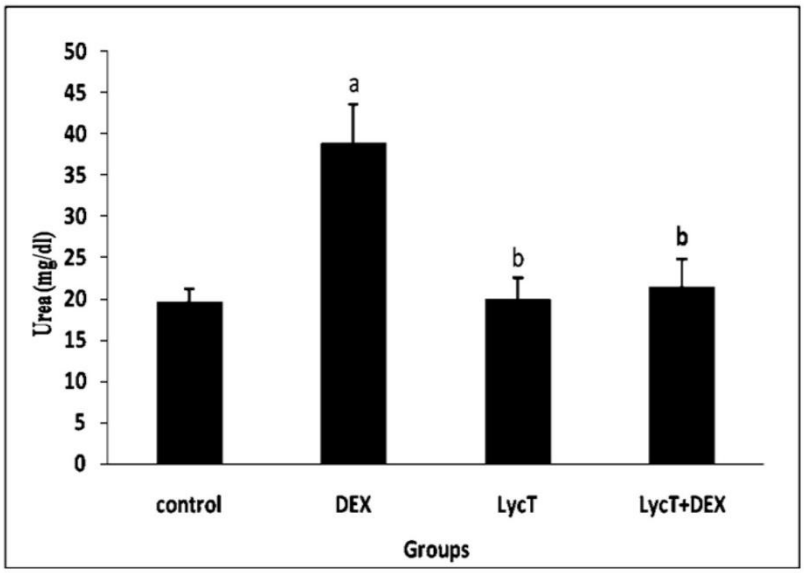


Figure 4: Modulatory effect of LycT and/ or DEX on serum (a) SGOT, (b) SGPT (c) ALP (d) LDH (e) total bilirubin (f) direct bilirubin levels

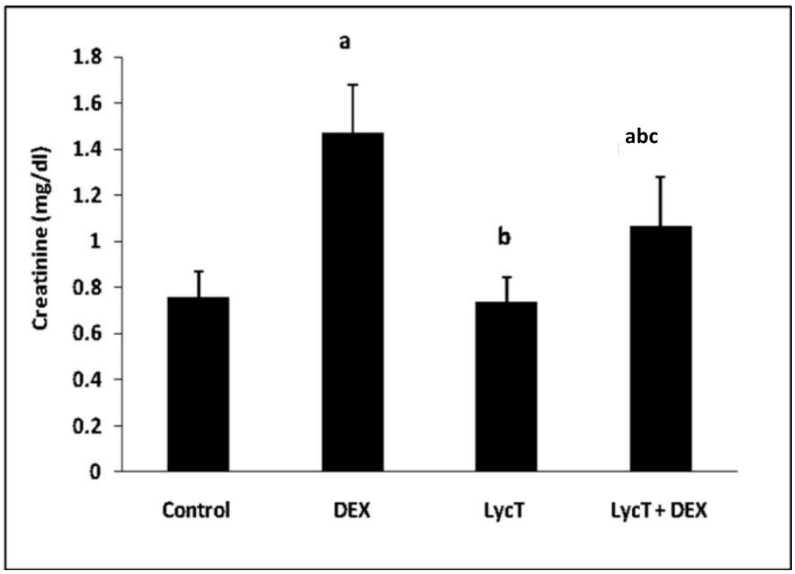
Data is represented as Mean±SD (n=5). Data is analysed by One-Way ANOVA followed by post hoc test. [a]p ≤0.05 significant with respect to control group; [b]p≤0.05 significant with respect to DEX group; [c]p ≤0.05 significant with respect to LycT group

3.4 Assessment of renal function markers

A significant increase in serum urea and creatinine levels was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant decrease in urea and creatinine levels when compared to the animals in DEX group. Urea levels remained unchanged in LycT+DEX group when compared to control and LycT groups. Creatinine levels increased in LycT+DEX group when compared to control and LycT group (Figure: 5a-b).



(a)



(b)

Figure : Modulatory effect of LycT and/ or DEX on (a) urea and (b) creatinine levels in serum

Data is represented as Mean±SD (n=5). Data is analysed by One-Way ANOVA followed by post hoc test. [a]p ≤0.05 significant with respect to control group; [b]p≤0.05 significant with respect to DEX group; [c]p ≤0.05 significant with respect to LycT group

3.5. Histopathology

Liver from control and LycT groups exhibited normal histoarchitecture. Clear cut hexagonal hepatic lobules, separated by interlobular septa and traversed by portal veins were observed. Polyhedral hepatocytes, kupffer cells and sinusoids were normal in appearance Portal triad comprised of portal vein, hepatic artery and bile duct. Liver from DEX group revealed areas with kupffer cell infiltration, abnormal lipid accumulation. The hexagonal arrangement of hepatocytes appeared disturbed. Liver from LycT+DEX group did not exhibit any significant departure from the normal histoarchitecture (Figure: 6).

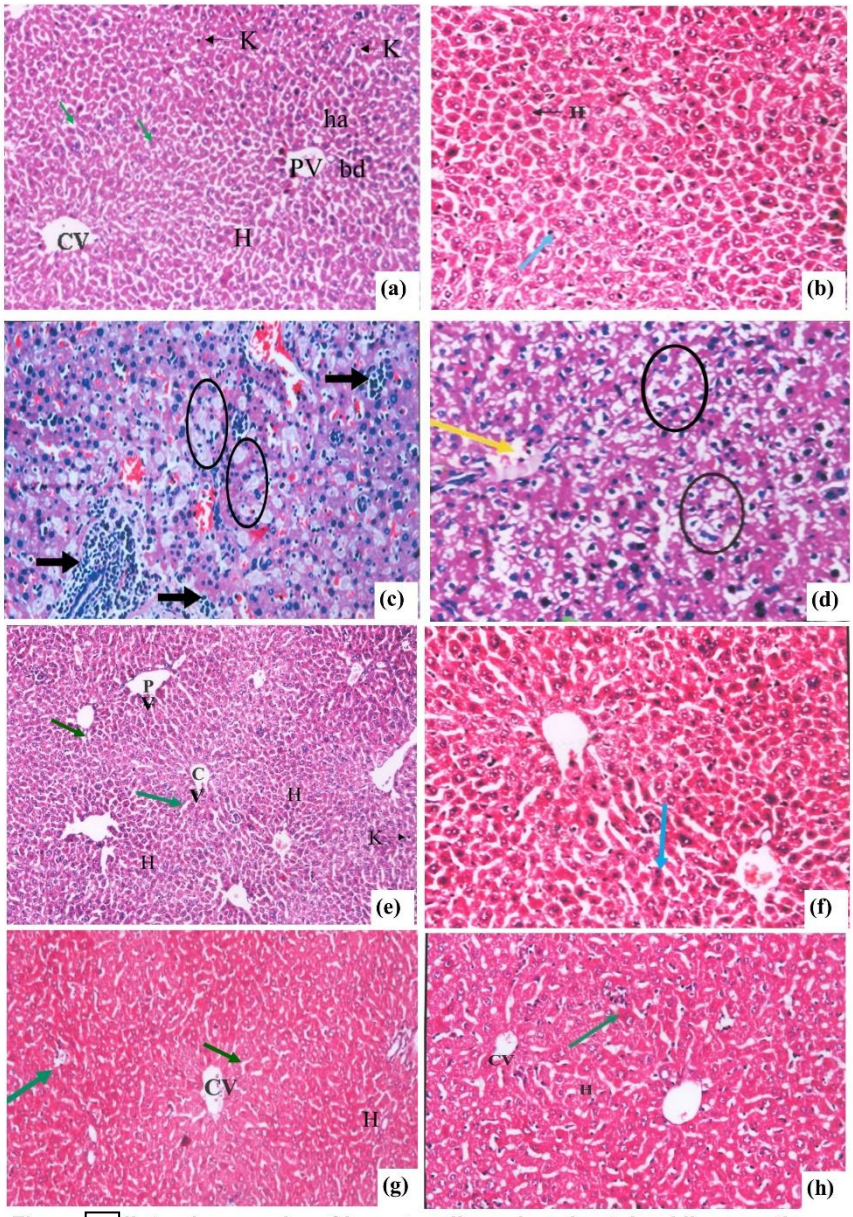


Figure Histomicrographs of hematoxylin and eosin stained liver sections
Control group (a,b); DEX group (c,d); LycT group (e,f); LycT+DEX group (g,h)
CV: central vein, PV: portal vein, H: hepatocytes, ha: hepatic artery, bd: bile duct, sinusoids (green arrow), lipid retention (encircled, yellow arrow), Kupffer cell infiltration (bold black arrow)

The kidneys from control and LycT groups exhibited normal histoarchitecture. Regions of outer cortex and deeper medulla were clearly visible with no signs of damage. Cortical labyrinth revealed proximal and distal convoluted tubules (PCT and DCT) along with renal corpuscle. The renal corpuscle consisted of bowman's capsule and tuft of capillaries known as glomerulus. The medullary region forming the renal pyramids consisted of loops of Henle and collecting ducts. Disorganization and atrophy of renal histoarchitecture was observed in DEX group. Renal lesions including marked glomerular damage and shrinkage, increased capsular space, hyaline casts, hemorrhage, tubular atrophy were observed in DEX group. In LycT+DEX group some lesions such infiltration, empty spaces (damaged histoarchitecture) were observed but the damage was less in comparison to DEX group (Figure: 7).

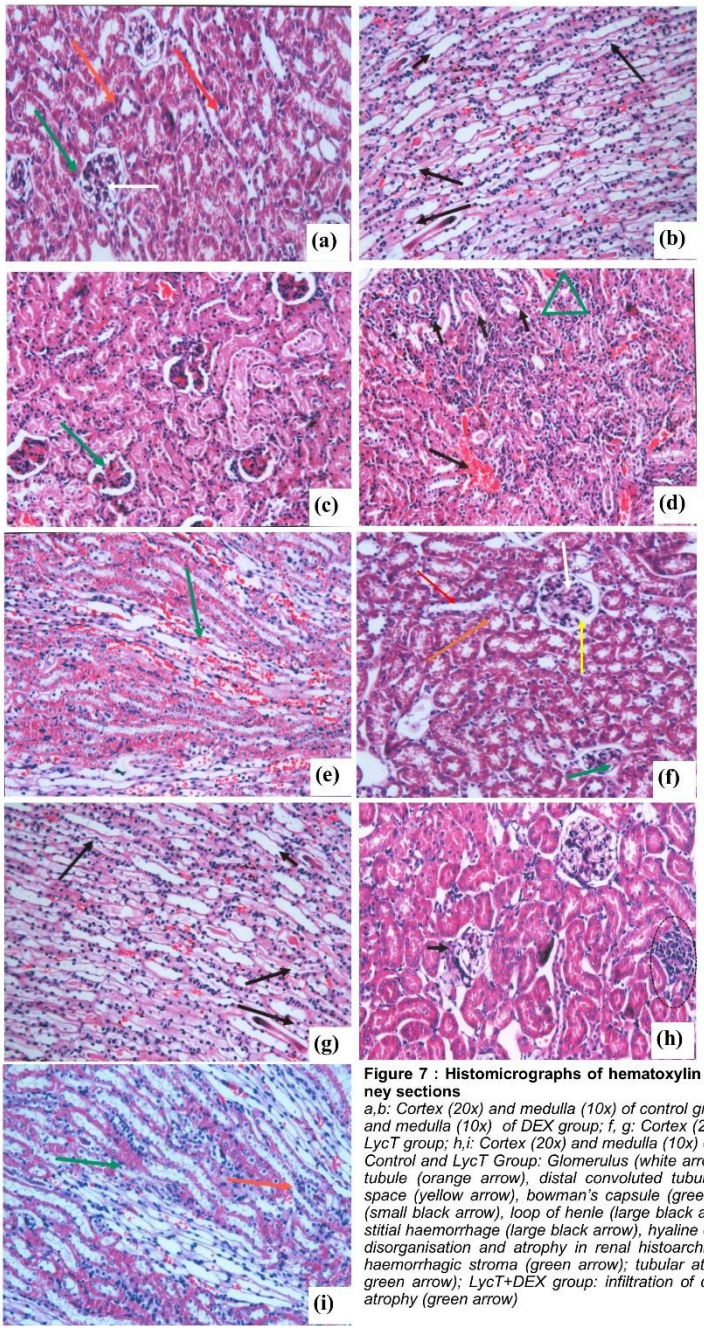


Figure 7 : Histomicrographs of hematoxylin and eosin stained kid· ney sections

a,b: Cortex (20x) and medulla (10x) of control group; c-d, e: Cortex (20x, and medulla (10x) of DEX group; f, g: Cortex (20x) and medulla (10x) o LycT group; h,i: Cortex (20x) and medulla (10x) of LycT+ DEX group

Control and LycT Group: Glomerulus (white arrow), proximal convolutec tubule (orange arrow), distal convoluted tubule (red arrow), capsula space (yellow arrow), bowman's capsule (green arrow), collecting duc (small black arrow), loop of henle (large black arrow); DEX group: Inter· stitial haemorrhage (large black arrow), hyaline casts (small black arrow disorganisation and atrophy in renal histoarchitecture (green triangle) haemorrhagic stroma (green arrow); tubular atrophy (to be marked by green arrow); LycT+DEX group: infiltration of cells (encrircled); tubula atrophy (green arrow)

3.6 Oxidative Stress marker

A significant increase in hepatic lipid peroxidation was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant decrease in lipid peroxidation levels when compared to the animals in DEX group and remained unaltered when compared to control and LycT groups. No changes were observed in LycT group when compared to control group (Table: 1).

A significant increase in renal lipid peroxidation was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant decrease in lipid peroxidation levels when compared to the animals in DEX group and remained unaltered when compared to control and LycT groups. No changes were observed in LycT group when compared to control group (Table: 2).

Table 1: Effect of LycT and/or DEX on lipid peroxidation and antioxidant defense system in hepatic tissue

Groups	Control	DEX	LycT	LycT + DEX
Lipid Peroxidation *(nanomoles of MDA-TBA chromophore formed/mg protein)*	0.650 ± 0.090	1.34 ± 0.166[a]	0.70 ± 0.05[b]	0.560 ± 0.043[b]
Reduced glutathione *(nanomoles of GSH/mg protein)*	4.84 ± 0.446	3.91 ± 0.235[a]	4.44 ± 0.647	5.08 ± 0.648[bc]
Catalase *(IU/mg protein)*	41.6 ± 4.42	31.4 ± 5.60 [a]	43.9 ± 6.23[b]	39.8 ± 6.39[b]
Superoxide Dismutase *(IU/mg protein)*	11.0 ± 1.40	8.58 ± 1.58[a]	13.2 ± 0.399[b]	11.7 ± 1.61[b]
Glutathione Peroxidase *(nanomoles of NADPH consumed/min/mg protein)*	23.6 ± 0.35	20.3 ± 2.26	22.6 ± 1.55	22.3 ± 5.15
Glutathione Reductase *(nanomoles of NADPH consumed/min/mg protein)*	31.4 ± 3.75	21.7 ± 4.90[a]	30.1 ± 4.54[b]	30.0 ± 5.30[b]

Data is represented as Mean±SD (n=5). Data is analysed by One-Way ANOVA followed by post hoc test. [a]p ≤0.05 significant with respect to control group; [b]p≤0.05 significant with respect to DEX group; [c]p ≤0.05 significant with respect to LycT group

3.7 Non-enzymatic and enzymatic antioxidant defense system

3.7.1 Reduced Glutathione

A significant decrease in hepatic reduced glutathione level was observed in DEX group when compared to control group. LycT administration to DEX treated animals led to a significant increase in reduced glutathione levels when compared to the animals in DEX and LycT group and remained unaltered when compared to control group. No changes were observed in LycT group when compared to control group (Table: 1).

A significant decrease in renal glutathione level was observed in DEX group when compared to control group. LycT administration to DEX treated animals caused a significant increase in reduced glutathione level when compared to the animals in DEX and LycT group and remained unaltered when compared to control group. No changes were observed in LycT group when compared to control group (Table: 2).

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Table 2: Effect of LycT and/or DEX on lipid peroxidation and antioxidant defense system in renal tissue

Groups	Control	DEX	LycT	LycT + DEX
Lipid Peroxidation *(nanomoles of MDA-TBA chromophore formed/mg protein)*	0.525 ± 0.112	0.88 ± 0.078 [a]	0.617 ± 0.078[b]	0.620 ± 0.14[b]
Reduced glutathione *(nanomoles of GSH/mg protein)*	4.88 ± 1.160	3.40 ± 0.668[a]	4.23 ± 0.746	5.24 ± 0.977[bc]
Catalase *(IU/mg protein)*	40.6 ± 3.68	22.7 ± 3.92[a]	37.2 ± 2.92[ab]	28.6 ± 4.01[abc]
Superoxide Dismutase *(IU/mg protein)*	8.42 ± 0.929	3.48 ± 0.300[a]	7.48 ± 1.04[b]	6.81 ± 0.545[ab]
Glutathione Peroxidase *(nanomoles of NADPH consumed/min/mg protein)*	20.8 ± 3.99	5.23 ± 1.64[a]	24.5 ± 4.62[b]	8.13 ± 1.25[ac]
Glutathione Reductase *(nanomoles of NADPH consumed/min/mg protein)*	29.5 ± 1.67	21.2 ± 1.43[a]	25.3 ± 2.74	31.4 ± 7.87[b]

Data is represented as Mean±SD (n=5). Data is analysed by One-Way ANOVA followed by post hoc test. [a]p ≤0.05 significant with respect to control group; [b]p≤0.05 significant with respect to DEX group; [c]p ≤0.05 significant with respect to LycT group

3.7.2 Catalase

A significant decrease in hepatic catalase activity was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in catalase activity when compared to the animals in DEX group and remained unaltered when compared to control group and LycT groups. No changes were observed in LycT group when compared to control group (Table: 1).

A significant decrease in renal catalase activity was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in catalase activity when compared to the animals in DEX group and decreased when compared to control group and LycT groups. No changes were observed in LycT group when compared to control group (Table: 2).

3.7.3 Superoxide Dismutase

A significant decrease in hepatic SOD activity was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in SOD activity when compared to the animals in DEX group and remained unaltered when compared to control group and LycT groups. No changes were observed in LycT group when compared to control group (Table: 1).

A significant decrease in renal SOD activity was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in SOD activity when compared to the animals in DEX group, remained unaltered when compared to LycT group and decreased when compared to control group. No changes were observed in LycT group when compared to control group (Table: 2).

3.7.4 Glutathione Peroxidase

No change in hepatic GPx activity was observed in any of the treatment groups (Table: 1).

A significant decrease in renal glutathione peroxidase activity was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in GPx activity when compared to the animals in DEX group and

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decreased when compared to control group and LycT groups. No changes were observed in LycT group when compared to control group (Table: 2).

3.7.5 Glutathione Reductase

A significant decrease in hepatic glutathione reductase activity was observed in DEX group when compared to control and LycT groups. LycT administration to DEX treated animals led to a significant increase in glutathione reductase activity when compared to the animals in DEX group and remained unaltered when compared to control group and LycT groups. No changes were observed in LycT group when compared to control group (Table: 1).

A significant decrease in renal glutathione reductase activity was observed in DEX group when compared to control group and remained unaltered in comparison to LycT group. LycT administration to DEX treated animals led to a significant increase in glutathione reductase activity when compared to the animals in DEX group and remained unaltered when compared to control group and LycT groups. No changes were observed in LycT group when compared to control group (Table: 2)

4. DISCUSSION

Despite its immense clinical benefits, dexamethasone is used with extreme caution because of the accompanying adverse effects. The need to use dexamethasone with mitigated or no side effects has provoked the search for agents that could enable its safe and effective use. Liver and kidney are exposed to high concentrations of drugs/and or their metabolites as these organs play significant roles in drug metabolism and excretion, rendering them susceptible to drug induced damage/toxicity (Irving and Elfarra, 2012; Singh et al., 2016). Generation of oxidative stress by dexamethasone has been considered as one of the underlying mechanisms responsible for its associated adverse effects including toxicity in hepatic and renal tissues (Kakali et al., 2004; Tayade et al., 2012; Hasona et al., 2017; Safaeian et al., 2018; Hasona and Morsi, 2019). Therefore, antagonizing the effects of excessive free radicals and ROS seems a rational way of counteracting dexamethasone induced ill effects. There is an ever-increasing interest in exploring the protective potential of natural antioxidants present in dietary components or other herbal preparations used for improving health and immunity. Carotenoids are a class of phytochemicals endowed with strong free radical scavenging and anti-oxidant activities. Carotenoids, importantly lycopene, are gaining considerable attention owing to their good antioxidant properties (Przybylska, 2020). The data presented here indicates the potential of LycT (carotenoid rich extract) in reducing dexamethasone induced hepatic and renal damage in mice.

In the present study, dexamethasone enhanced the blood glucose level and reduced hepatic glycogen level in mice. This indicates altered glucose metabolism by liver. Enhanced levels of glucocorticoids via endogenous production or exogenous consumption leads to increased glucose production and reduced glucose uptake by peripheral tissues (liver, muscles, adipose tissue). Dexamethasone and other glucocorticoids induce peripheral insulin resistance by inducing post-receptor defects in insulin actions, inhibition of insulin secretion by pancreatic β-cells, GLUT4 translocation, impaired insulin signaling etc (Ghasias et al., 2011). In healthy conditions, insulin secreted by β-cells of pancreas aids in glucose uptake by liver cells. However, during dexamethasone treatment, the uptake of glucose by the glucose receptors is reduced and as a consequence, dexamethasone is associated with increased blood glucose levels (Rafacho et al., 2007). With insulin resistance, pancreatic β-cell dysfunction occurs which allows further hyperglycemia to develop leading to diabetes during chronic glucocorticoid therapy (Gosmanova et al., 2012; Yates et al., 2012). In addition to the effect on peripheral tissues, steroids also regulate the synthesis and release of hormones associated with the development of hyperglycemic disorders (Kuo et al., 2015). The reduction in glycogen levels in response to dexamethasone has been attributed to decreased supply of glucose to liver due to insulin resistance (Yi et al., 2012).

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The animals belonging to LycT+DEX group exhibited decreased blood glucose level and unaltered glycogen level in comparison to DEX group. Similar results were reported earlier demonstrating the normalization of blood glucose levels in diabetic rats by lycopene treatment (Muhsin and Sefa, 2012). Rats administered with lycopene rich tomato homogenate exhibited improved glucose tolerance (Hashimoto et al., 2019). Zeng et al (2017) have demonstrated that lycopene improved insulin sensitivity in mice that were fed a high fat diet. Lycopene is known to raise insulin levels and concomitantly decrease body glucose levels (Cuevas-Ramos et al., 2013; Martin-Pozuelo et al., 2016). Tomatoes have been considered beneficial in hyperglycemic/diabetic conditions because they mitigate diabetes induced tissue damage, atherosclerosis, inflammation, oxidative stress etc (Banihani, 2018). The various constituents present in tomatoes such as ascorbic acid, lycopene, β-carotene, flavonoids, several small bioactive molecules, minerals (such as magnesium and potassium) etc are favorable in diabetic conditions (Banihani, 2018).

Glucose metabolizing enzymes are severely impaired during glucocorticoid therapy. This results in impaired oxidation of glucose resulting in hyperglycemia (Kipnis, 1959; Morgan et al., 1961; Randle and Morgan, 1962; van Raalte et al., 2011). Assessment of hexokinase and phosphoglucoisomerase activities revealed their decrease in DEX group which may be due to insulin insensitivity/resistance in response to dexamethasone treatment. Hexokinase is the first enzyme in the glycolytic pathway which converts glucose to glucose-6-phosphate. Phosphoglucoisomerase is the second enzyme of glycolysis pathway which interconverts glucose-6-phosphate to fructose-6-phosphate. Insulin increases glycolysis by increasing the activity of hexokinase and phosphoglucoisomerase (Dimitriadis et al., 2011). It has been previously reported that administration of glucocorticoids to rats and rabbits provoked inhibition of hexokinase and phosphoglucoisomerase (Ilyin, 1964). Inhibited activities of glucose utilizing enzymes were observed in intestinal mucosal scrapings of dexamethasone treated rats (Salleh et al., 1988). Cortisol treatment inhibited the phosphorylation of glucose and fructose-6-phosphate possibly due to reduced enzyme activities of hexokinase and 6-phosphofructokinase (Kipnis, 1959; Randle and Morgan, 1962). LycT administration improved the activity of glycolytic enzymes in dexamethasone treated animals. Decreased blood glucose levels and enhanced activities of glucose metabolizing enzymes in LycT+DEX group suggests that LycT was able to mitigate hyperglycemic conditions, possibly improving glucose metabolism in response to dexamethasone treatment.

The levels of liver function markers such as dehydrogenases, phosphatases, transaminases, bilirubin etc in serum indicate the status of liver. Excessive ROS production during metabolism of xenobiotics including carcinogens and drugs causes damage to several tissues of the body including liver and kidney (Gupta et al., 2013b; Meo et al., 2016). Enhanced levels of liver function markers were observed in serum of animals belonging to DEX group. These elevations indicate damage to the structural integrity of liver and has been observed in hepatic toxicity (Hamza and Al-Harbi, 2015; Uchida et al., 2017). It has been previously reported by several authors that elevated serum levels could be explained by the damaged and leaky hepatic cell membrane (Wahid et al., 2016). Hepatic damage as revealed by altered histoarchitecture and raised SGOT and SGPT levels has been reported after dexamethasone administration to rats (Al-Fartosi et a., 2017). DEX associated hepatic necrosis in rats was also associated with increased serum levels of SGOT and SGPT (Jackson et al., 2008). Hasona and Morsi (2019) and Hasona et al (2017) have reported elevations in liver function markers in dexamethasone administered rats. Abou-Seif (2019) have also demonstrated that dexamethasone induced liver injury was reflected by raised SGOT, SGPT and LDH levels along with disturbed histoarchitecture. LycT supplementation to dexamethasone treated mice decreased the enhanced levels of these markers probably

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by mitigating hepatic damage and protecting membrane integrity. We have previously demonstrated the effect of LycT in protecting membrane integrity and decreasing levels of liver function markers in serum during NDEA induced hepatocellular carcinoma in mice (Gupta et al, 2013c). Lycopene supplementation was effective in improving hepatic function which was impaired during hepatitis caused by lipopolysaccharide (Sheriff and Devaki, 2013). The derangement in hepatic function markers in NDEA and phenobarbital treated rats was improved upon lycopene supplementation (Das et al., 2016). We have also reported that lycopene was effective in mitigating DMBA induced hepatotoxicity (Koul et al., 2010).

Serum creatinine level indicate the excretion capability of kidneys and are generally elevated due to renal damage. Although, liver is the primary site responsible for the synthesis of urea from ammonia however, elevated levels of urea in blood also serve as an indicator of renal malfunctioning (Salazar, 2014). Both these levels serve as important diagnostic marker for kidney pathology (Guyton and Hall, 2006; Salazar, 2014). In the current study, dexamethasone treatment significantly increased the serum creatinine and urea levels. Previous reports demonstrate that dexamethasone administration to rats increased their serum uric acid and creatinine levels indicating renal damage (Hasona et al., 2017). Significant reduction in the levels of creatinine and urea were observed when LycT treated mice were challenged with dexamethasone. These results are in harmony with those previously reported. Lycopene has been effective in mitigating several drug induced nephrotoxicity in animals (Yilmaz et al., 2006; Karahan et al., 2005; Moawad, 2007; Gerbed, 2012; Dai et al., 2015). We have also previously reported that LycT conferred protection against doxorubicin induced renal toxicity in mice (Koul et al., 2013). Lycopene was able to attenuate colistin induced nephrotoxicity in mice as evident from improved renal function markers and histoarchitecture (Dai et al., 2015).

Light microscopy studies of hepatic and renal tissues indicated marked damage in these tissues in DEX group. Such deviations in histoarchitecture signifying damage have been reported previously (Welt et al., 2007; Koul et al., 2013; Bala et al., 2018; El-Kordy et al., 2019). The tissue sections from LycT+DEX group revealed less damage as compared to the DEX group. Improved levels of organ function markers and mitigated damage in histoarchitecture suggested that LycT conferred protection against dexamethasone induced deleterious effects.

High levels of circulatory glucocorticoids accelerate the metabolic rates, inhibit the defense capacity of cells leading to elevated levels of free radicals in various organs (Beytut et al., 2018). NADPH oxidases are involved in dexamethasone mediated increase in ROS and apoptosis in various tissues (Bai et al., 2019; Macedo et al., 2020). JNK-P38 MAPK signaling pathway has been implicated in dexamethasone induced oxidative damage in mice (Zhu et al., 2018). Free radical scavenging enzymes are the first line of cellular defense against oxidative injury. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage (Rodrigo et al., 2013). In the present study, increased MDA levels in hepatic and renal tissues of DEX group indicates enhanced lipid peroxidation which is suggestive of oxidative stress in the tissues. The activities of various antioxidant enzymes and reduced glutathione level were significantly decreased in the DEX group when compared to its control counterparts. The declined activities/levels of antioxidant defense system components partially explain the deleterious effects caused by dexamethasone. The toxic effects of dexamethasone have been linked to increased MDA levels along with a decrease in GSH content (Kakali et al., 2004). Dexamethasone administration decreased antioxidant enzyme activities, increased ROS production, lipid peroxidation, mitochondrial dysfunction, leading to apoptosis in thymocytes (Yi et al., 2016). Recent reports demonstrated that dexamethasone administration leads to elevated MDA

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levels and suppressed the activities of antioxidant defense enzymes (Hasona et al., 2017; Hasona and Morsi, 2019).

GSH is a ubiquitous thiol containing tri-peptide that serves as an essential role in maintaining cell integrity because of its reducing properties. It is an important antioxidant molecule involved in the protection against LPO reactions. Osama and Abo-Salem (2012) have demonstrated decreased levels of GSH during gentamicin induced nephrotoxicity. SOD is involved in catalyzing the dismuation of $O^{2.-}$ to H_2O_2. CAT and GPx enzymes catalyze the decomposition of H_2O_2 to water and oxygen and thus protect the cell from oxidative damage. GR is the enzyme directly involved in reduction of GSSG to GSH. Low activity of GPx is responsible for disturbance of the prooxidant/antioxidant balance (Benabdeslam et al., 1999). Hepatic and renal tissues of mice belonging to DEX group exhibited decrease in GSH and various antioxidant enzymes. These observations are in concordance with other reported studies (Eid et al., 2007; Yi et al., 2016; Hasona et al., 2017; Hasona and Morsi, 2019).

Lycopene is a forty-carbon acyclic hydrocarbon carotenoid containing eleven trans conjugated double bonds. The extended conjugated polyene chain of lycopene is an electron-rich system, susceptible to attack by electrophilic agents. This makes lycopene unstable and highly reactive towards oxygen and free radicals (Peters et al., 2007). It has an exceptionally high singlet oxygen quenching ability (Stahl and Sies, 2003). The other carotenoids present in tomatoes and tomato-based products also exhibit antioxidant activities (George et al., 2004; Gama et al., 2006). Reducing xenobiotic induced increased LPO by intervention of lycopene has been reported previously (Koul et al., 2010). Lycopene efficiently scavenges peroxyl radicals and thus, contributes to the defense against LPO induce by dexamethasone (Bose et al, 2006). We have previously reported that LycT was effective in mitigating doxorubicin induced nephrotoxicity in mice by boosting the enzymatic and non-enzymatic antioxidant defense system (Koul et al., 2013). Kujawska et al (2014) have reported antioxidative effects of lycopene enriched tomato paste against nitrosamine induced oxidative stress in rats. In the present study, LycT intervention to the dexamethasone administered mice boosted the enzymatic and non-enzymatic antioxidant defense system which subsequently gets reflected in the fall in the MDA levels in the conjunctive treated group when compared to the group treated with DEX only.

5. CONCLUSION

The mitigation in damage to histoarchitecture, along with improvement in specific organ function markers and glucose metabolism suggest the protective potential of LycT against dexamethasone induced deleterious effects. The strengthening of antioxidant defense response as observed from decreased lipid peroxidation levels and upregulation of enzymatic and non-enzymatic defense system suggest a possible mechanism for its protective effects. Although these observations indicate alleviation of dexamethasone induced adverse effects by LycT, however further studies are warranted.

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ETHICAL APPROVAL

All the experimental protocols were initially approved by the Institutional Ethics Committee of Panjab University, Chandigarh (India) and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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