GLA supplementation regulates PHD2 mediated hypoxia and mitochondrial apoptosis in DMBA induced mammary gland carcinoma

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A R T I C L E   I N F O

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- Gamma linolenic acid
- 7, 12-Dimethylbenz (a) anthracene
- Hypoxia
- Mitochondria mediated death apoptosis
- Breast cancer
- alpha-7-nachr

A B S T R A C T

The aim of the present study is to evaluate the effect of gamma linolenic acid (GLA) on mitochondrial mediated death apoptosis, hypoxic microenvironment and cholinergic anti-inflammatory pathway against 7, 12-dimethylbenz (a) anthracene (DMBA) induced mammary gland carcinoma. The effects of GLA were evaluated morphologically and biochemically against DMBA induced mammary gland carcinoma. The metabolic study was done for evaluation of biomarkers using 1H NMR. The present study was also verified through immunoblotting and qRT-PCR studies for the evaluation of various pathways. GLA treatment has a delineate implementation upon morphology of the tissues when evaluated through carmine staining, hematoxyline and eosin staining and scanning electron microscopy. GLA also demarked a commendatory proclamation of the fifteen key serum metabolites analogous with amino acid metabolism and fatty acid metabolism when recognized through 1H NMR studies. The immunoblotting and qRT-PCR studies accomplished that GLA mediated mitochondrial death apoptosis, curtail hypoxic microenvironment along with hindrance of de novo fatty acid synthesis and also mediate the cholinergic anti-inflammatory pathway to proclaim its anticancer effects.

1. Introduction

Polyunsaturated fatty acids (PUFAs) play a predominant role in the cell membrane formation and are also important for the functioning of membrane proteins and membrane fluidity. PUFAs synchronize several cellular processes, functions and gene expression (Kaur et al., 2014). GLA is a member of the ω-6 family of PUFAs and is transfigured into arachidonic acid (AA) by series of desaturation and elongation reactions. AA is further metabolized by cyclooxygenase enzyme into 2-series prostaglandins or through the 5-lipoxygenase enzymes into leukotrienes and 5-hydroxy-eicosatetraenoic acid, which are the major determinants for cellular inflammation (Ricciotti and FitzGerald, 2011). GLA is found in animals and plants, oils like sunflower, soy bean and grape seed and is very much found in daily diet (Bederska-Lojewska et al., 2013). Considering the fact that GLA is metabolized to AA and majority of the product of AA metabolism are pro-inflammatory, the GLA has been also considered to be pro-inflammatory in nature. Consistent intake of GLA is expected to promulgate inflammatory cascade...
and harmonize the risk of associated clinical conditions like cancer.

Several corroborations including in vitro and in vivo studies recommend the anticancer activity of GLA. It was previously reported that GLA hinders cell growth of several human neuroblastoma and several rat carcinosarcoma cell lines (Das and Madhavi, 2011; Xu and Qian, 2014). GLA also diminished the tumor growth in the implanted WBC256 rat model (Colquhoun, 2002). It was previously reported that supplementation with GLA rich diets suppressed the mammary gland carcinogenesis and transplanted tumor growth. GLA also inhibits the growth of various cultured human cancer cell lines like ZR-75-1, A549 and PC-3 cells (Xu and Qian, 2014). Thus, GLA seems to be an auspicious cancer therapeutic agent with prudent characteristics although the reason for its high selectivity in GLA induced antitumor effect is still unexplored. The mechanism for the same is elucidated herewith questioning several aspects of cancer progression.

2. Materials and methods

2.1. Chemicals

GLA (GLA-120) was acquired from Dr. Reddy’s Laboratory (India). 7, 12-Dimethylbenz (a) anthracene (DMBA) (Sigma Aldrich, 57-97-6); eagle balanced salt solution (EBSS) (Gibco, 2018-11); RNase (SRL, 58895); ponceau S (Himedia, ML045); sodium cacodylate (Sigma Aldrich, C0250); collagenase type 4 (Himedia, TC-214); hyaluronidase (Himedia, TC331); hematoxylin (Himedia, 5058); eosin (Himedia, 5007); RIPA lysis buffer (Amresco, N653); protein assay kit (Amresco, M173); bovine serum albumin (BSA) (Genetix, PG-2330); transfer buffer (Genetix, GX-9411AR), trizol reagent (Sigma-T9424), cDNA synthesis kit (Genetix-K1612). Caspase 3 (SC-4263) and caspase 8 (SC-4267) assay kits were procured from Santacruz Biotechnology Inc., California, Delaware. All others chemicals were of molecular biology grade and purchased from Genetix Biotech Asia Pvt. Ltd., New Delhi.

2.2. Experimental protocol

The female albino wistar rats were housed in polypropylene cages and randomized into four groups. Each group contained eight animals per group and maintained with controlled condition of light and temperature (23 °C ± 2 °C, 12 h light: dark cycle). The animals were fed with a standard laboratory diet and water ad libitum. The animals were acclimatized for two weeks prior to the experimentation. The study was approved by the Animal Ethical Committee in accordance with approved guidelines for the treatment of laboratory animals (BBBDNIT/IAEC/021/2014). Animals randomized to group I received normal saline (0.9 ml/kg, p.o.); group II served as toxic control and received DMBA 8 mg/kg, i.v. through single tail vein injection; group III and IV served as treatment groups and received GLA (0.25 ml/kg, p.o) and GLA (0.5 ml/kg, p.o.) along with DMBA 8 mg/kg, i.v. respectively. Toxicity was induced by single tail vein injection of DMBA on day 1st. The GLA was administered from 7th to 110th day at the dose specified above. The study was terminated on 112th day and the animals were sacrificed by cervical dislocation under light ether anesthesia.

2.3. Hemodynamic changes

The changes in electrocardiogram (ECG) and heart rate variability (HRV) were recorded according to the method established in our laboratory (Mishra et al., 2016).

2.4. Morphological evaluation

2.4.1. Carmine staining

A detailed methodology for the carmine staining has been elaborated previously (Manral et al., 2016). The mammary gland whole mounts were examined under light microscope and evaluated for the presence of alveolar buds (ABs) (De Assis et al., 2010; Manral et al., 2016).

2.4.2. Histopathology of mammary gland tissue

The mammary gland tissues were promptly fixed in 10% formalin, embedded in paraffin wax. The sections were cut into 5 μm size by using microtome and then stained with hematoxylin and eosin (H&E). The histopathological sections were visualized and photographed using digital biological microscope (N120, BR-Biochem Life Sciences, New Delhi, India) (Feng et al., 2015; Liska et al., 2016).

2.4.3. Surface texture analysis of mammary gland tissue using scanning electron microscopy (SEM)

The mammary gland tissue was digested using collagenase (type 4) and fixed using glutaraldehyde. The tissue was processed further using the protocol elaborated by us previously and examined under SEM (JEOLJSM-6490LV) (Roy et al., 2017; Yasugi et al., 1989).

2.5. Antioxidant parameters

The mammary gland tissues (10% w/v) were homogenized in 0.15 M KCl and centrifuged at 10,000 rpm. The supernatants were evaluated for the presence of antioxidant parameters including thio-barbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase, glutathione (GSH) and protein carbonyl (PC) using the methods established in our laboratory (Raj et al., 2014).

2.6. 1H NMR spectroscopic analysis of serum samples

2.6.1. Serum sample preparation

The stored serum samples were thawed, vortexed and centrifuged at 10,000 rpm for 5 min at 40 °C to remove precipitates before the execution of NMR. 500 μl of the serum sample was taken from each group for NMR recording. The serum samples were prepared by mixing 250 μl of serum with 250 μl of saline sodium phosphate buffer (20 mM, pH 7.4) with 0.9% saline in D2O (Kumar et al., 2016). The prepared serum samples were transferred into a 5 mm NMR tube, with a sealed coaxial insert containing the known concentration of 0.1 mM TSP (sodium salt of 3-trimethylsilyl-(2,2,3,3-d4)-propionic acid) prepared in D2O, to provide lock for NMR experiments and as an external standard reference to aid chemical shift referencing for metabolite quantification and assignment.

2.6.2. 1H NMR measurements

The 1H NMR spectra was recorded at 300 K on a Bruker NMR spectrometer (Avance-III) operating at a 1H frequency of 800.21 MHz and equipped with a Cryoprobe using a method described elsewhere (Roy et al., 2017).

2.6.3. Spectral assignment

The 1D 1H Carr-Purcell-Meiboom-Gill (CPMG) NMR spectra metabolite resonances were assigned using the Chenomx NMR Suite (Chenomx Inc., Edmonton, AB, Canada). The left over peaks in the 1H NMR spectra was recorded at 300 K on a Bruker NMR spectrometer (Avance-III) operating at a 1H frequency of 800.21 MHz and equipped with a Cryoprobe using a method described elsewhere (Roy et al., 2017).

2.6.4. Multivariate statistical analysis

The NMR spectra was manually phased, baseline corrected and referenced internally to the methyl resonance of lactate at δ 1.3102 before the multivariate analysis. The CPMG δ (0.7–9.5) spectra were
automatically integrated using AMIX package (Version 3.9.15, Bruker). The region δ (4.64–5.11) contorted due to water suppression were omitted from the CPMG data set to avoid the effects of imperfect water suppression. Finally, the selected regions were diminished to spectral bins of δ 0.02 and each spectral bin was further normalized using the total spectral intensity. For statistical data analysis and modeling, the binned data from CPMG experiments were submitted to the MetaboAnalyst, an open access web-based metabolomics data processing tool (Xia et al., 2009, 2015). The imported data in MetaboAnalyst was Pareto scaled before performing the multivariate and univariate analysis. Orthogonal projection to latent structure with discriminant analysis (OPLS-DA) and partial component analysis (PCA) were used for combined and pairwise analysis of spectral data. PCA was used for an initial overview of the grouping trend and outlier detection in the data set. Next, the data was modeled with the supervised method, OPLS-DA to reveal class separations between the groups and S-plot to identify the significant metabolites responsible for class separation. The OPLS-DA model needs to be stringently validated to see whether the separation is statistically significant or is due to random noise because OPLS-DA inclines to over-fit the data. The quality of the model was described by the cross-validation parameters R2Y and Q2, representing a good fitness and predictability of the constructed OPLS-DA model respectively. The permutation statistic was further used with 100 permutations to validate the OPLS-DA models. The boxplot representation was evaluated through ANOVA for visualizing the variation in the levels of significantly altered metabolites identified in the multivariate analysis. A probability level of 0.05 (p value ≤ 0.05) was used as the standard for statistical significance.

2.7. Assay for caspase 3 and caspase 8

Caspase 3 and caspase 8 assays were carried out in amber colored 96-well plates. The serum samples from all the groups including control, toxic and treatment were taken in equal volumes and diluted with reaction buffer and dithiothreitol (DTT) to a final concentration of 10 mM. 5 μl of DEVD-AFC for caspase 3 and IETD-AFC for caspase 8 were added to the reactant mixture and incubated for 1 h at 37 °C. The free AFC levels formed were measured in a plate reader with 400 nm excitation and 505 nm emission. The results were expressed as fluorescence units/mg of protein (Pu et al., 2017).

2.8. Western blotting

The mammary gland tissues were homogenized in RIPA lysis buffer. Total protein concentration was assayed by the method of Bradford (Kruger, 2009). The protein sample was extracted through acetone precipitation and quantified using the Bradford reagent. SDS-PAGE analysis was performed according to the principles of Laemmle with slight modifications (Yu et al., 2017). A detailed methodology for the same has been described by us previously (Roy et al., 2017). The blots were incubated overnight with primary antibody against Bcl-xl (MA-5-15142), Bcl-2 (SC-7382), BAX (SC-23959), BAD (SC-8044), VDAC (SC-390996), cytochrome c (SC-13561), Apaf-1 (SC-65891), procaspase 9 (SC-73548), FasB (MA5-1616), UCHL-1 (MA1-83428), polyh-3, oxypia inducible factor-1α (HIF-1α) (SC-13515), fatty acid synthase (FASN) (SC-55580), SREBP-1c (SC-13551), α-7nACHr (SC-5544), TNF-α (SC-13590) and HMGB-1 (SC-56698) in 4 °C. The standard reference was β-actin (MA5-15739-HRP). The membrane was washed thrice with TBST and further incubated at room temperature for 3 h with corresponding anti-rabbit (SC-2030), anti-goat (SC-2020) and anti-mouse (31430, Pierce Thermo Scientific, USA) HRP conjugated secondary antibody (1:5000 dilutions). After single TBST wash, the membranes were developed using an enhanced chemiluminescence substrate (Western Bright ECL HRP substrate, Advansta, Melanopark, California, US) in gel dock system. Protein quantification was done through densitometric digital analysis of protein bands using ImageJ software (Perumalsamy et al., 2017).

2.9. qRT-PCR

The primers were designed using primer quest tool from IDT DNA technologies website (www.idtdna.com). The criteria of primers selection was considered as: amplicon size between 100–200 base pairs, GC% above 50% and melting temperature between 58 °C–62 °C. The sequences of the forward and reverse primers used in this experiment were specified in Table S1.

Trizol reagent was used in the extraction of mammary gland tissue RNA and quantified using the method previously established at our laboratory (Roy et al., 2017). 1 μg of total mammary gland RNA was used in cDNA synthesis in a 96 well thermal cycler (BioRad, C1000) with steps including, incubation at 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min. 125 ng of cDNA was used as a template for each reaction of qRT-PCR with β-actin as a housekeeping control using light cycler 480 machine (Roche Diagnostics, Germany). The program in brief was an initial incubation of 50 °C for 2 min hold (UDG incubation) and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s (denaturation), 58 °C for 30 s (annealing) and final extension at 72 °C for 20s. Differential expression was calculated by 2-ΔΔCT method (Pujari et al., 2016).

2.10. Statistical analysis

The data were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni test for the possible significance identification between the various groups. *p < 0.05, **p < 0.01, ***p < 0.001 and a p < 0.001, b p < 0.01, c p < 0.05 were considered as statistically significant. Statistical analysis was performed using Graph Pad Prism software (5.02).

3. Results

3.1. Changes in hemodynamic parameters

After DMBA treatment, the heart rate (HR) was increased (355.2 ± 0.42 beats/min) which was restored after GLA treatment significantly (299.8 ± 0.79 beats/min). P wave duration has no significant effect after DMBA and GLA treatments (Fig. S1, Table S2). The HRV analysis revealed decrease in the low frequency (LF) (9.84 ± 0.01 ms²), high frequency (HF) (37.97 ± 0.05 ms²) and very low frequency (VLF) (41.81 ± 0.08 ms²) domains after the DMBA treatment. GLA treatment perceived dose-dependent restoration of the HRV parameters (Table 1).

3.2. Morphological studies

3.2.1. Carmine staining

DMBA treated group revealed increase in the lobules (1) and ABs (2). GLA treatment afforded a marked protection against the same (Fig. 1A–D).

3.2.2. Histopathological analysis

The histopathological section of control tissue revealed presence of duct (3); adipocytes (4); loose connective tissue (LCT) (5); dense connective tissue (DCT) (6) myoepithelial cells (MEC) (7); lymphocytes (8) and cuboidal epithelial cells (CEC) (9) (Fig. 1E). Concomitant treatment with GLA imparted dose dependent restoration of the cellular architecture close to control (Fig. 1G and H).
3.2.3. SEM analysis

Control tissue has intra-arterial cushion/collagenous covering, collagen layer (10) and duct (11) (Fig. 1I). DMBA treatment evidenced loss of intra-arterial cushion (Fig. 1J); development of small tumor micro-vessels (12) and development of nodules (13) (Fig. 1J). Subsequent GLA administration perceived decrease in tumor micro-vessel formation representing the deep impression of GLA on the branching sites along with restoration of intra-arterial cushion. (Fig. 1K and L).

Table 1
Effect of GLA on HRV changes in DMBA induced mammary gland carcinogenesis.

<table>
<thead>
<tr>
<th></th>
<th>Control (0.9% normal saline, p.o)</th>
<th>Toxic control (DMBA 8 mg/kg i.v.)</th>
<th>GLA + DMBA (0.25 ml/kg, p.o. + 8 mg/kg i.v.)</th>
<th>GLA + DMBA (0.5 ml/kg, p.o. + 8 mg/kg i.v.)</th>
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<tbody>
<tr>
<td><strong>Time Domain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average RR (ms)</td>
<td>167.2 ± 0.02***</td>
<td>174.11 ± 0.02</td>
<td>183.3 ± 0.05***</td>
<td>167.5 ± 0.01***</td>
</tr>
<tr>
<td>Median RR (ms)</td>
<td>168.6 ± 0.06***</td>
<td>175.83 ± 0.05</td>
<td>188.3 ± 0.09***</td>
<td>169.5 ± 0.09***</td>
</tr>
<tr>
<td>SDRR (ms)</td>
<td>6.35 ± 0.01***</td>
<td>3.76 ± 0.7</td>
<td>6.89 ± 0.01***</td>
<td>5.93 ± 0.01***</td>
</tr>
<tr>
<td>CVRR</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.18 ± 0.02***</td>
<td>0.21 ± 0.02***</td>
</tr>
<tr>
<td><strong>Frequency Domain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF (ms²)</td>
<td>11.72 ± 0.06***</td>
<td>9.84 ± 0.01</td>
<td>14.92 ± 0.04***</td>
<td>12.72 ± 0.06***</td>
</tr>
<tr>
<td>HF (ms²)</td>
<td>39.80 ± 0.09***</td>
<td>37.97 ± 0.05</td>
<td>46.94 ± 0.09***</td>
<td>43.80 ± 0.07***</td>
</tr>
<tr>
<td>LF/HF</td>
<td>0.44 ± 0.02</td>
<td>0.42 ± 0.04</td>
<td>0.44 ± 0.08</td>
<td>0.41 ± 0.1</td>
</tr>
<tr>
<td>VLF (ms²)</td>
<td>42.93 ± 0.07***</td>
<td>41.81 ± 0.08</td>
<td>38.67 ± 0.04***</td>
<td>42.77 ± 0.09***</td>
</tr>
</tbody>
</table>

(Values are presented as Mean ± SD). Each group contains eight animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the toxic control group. (*p < 0.05, **p < 0.01, ***p < 0.001).

3.2.3. SEM analysis

Control tissue has intra-arterial cushion/collagenous covering, collagen layer (10) and duct (11) (Fig. 1I). DMBA treatment evidenced loss of intra-arterial cushion (Fig. 1J); development of small tumor micro-vessels (12) and development of nodules (13) (Fig. 1J). Subsequent GLA administration perceived decrease in tumor micro-vessel formation representing the deep impression of GLA on the branching sites along with restoration of intra-arterial cushion. (Fig. 1K and L).

Fig. 1. Morphological evaluation of mammary gland tissue using carmine staining, H&E staining and SEM. Carmine staining of mammary gland tissue reveals the presence of lobules (1) and AB (2) (A–D). DMBA treated group (B) revealed the presence of presence of lobules and AB which was subsided after GLA treatment in dose dependent manner (C and D). H&E staining of control and GLA treated group (E, G and H) revealed the presence of duct (3), adipocytes (4), LCT (5), DCT (6), MEC (7), lymphocytes (8) and CEC (9). In DMBA treated group (F), the cell morphology was distorted and cell organelles were absent. SEM analysis of control (I), DMBA treated (J) and treatment group (K and L) revealed the difference in collagen layer (10), duct (11), small capillary network (12), large capillary network (13) and nodules (14) in respective groups.
In the serum metabolic profiling using 1D 1H NMR, the representative 1D 1H CPMG NMR spectra was shown in Fig. 2. The NMR spectra showed signals from lipids/lipoproteins (e.g., low density lipoprotein (LDL), very low density lipoprotein (VLDL), PUFAs etc.) and amino acids (e.g., alanine, valine, leucine, isoleucine, phenylalanine, histidine, tyrosine, glutamine, glutamate, proline etc.). Other identified metabolites were glucose, choline, creatine/creatinine, acetone, pyruvate, acetate, citrate, lactate, N-acetyl and O-acetyl glycoproteins (NAG, OAG). For an initial overview of the data set, unsupervised PCA score plots were constructed. Two outliers were detected, one from the toxic control and other from the normal control group. The outliers were excluded from both the data sets and the resulted data sets were then subjected to supervised OPLS-DA model to minimize the possible contribution of intergroup variability and to further improve the separation between the groups (Fig. 3). The combined PCA score plot (Fig. S2) showed a clear trend of clustering in different groups and no further outlier samples were detected. The combined OPLS-DA score plot also revealed that GLA (0.25 ml/kg) and GLA (0.5 ml/kg) treatments are mitigating the effect of DMBA induced toxicity as inferred by the shift of the GLA treatment group back towards the normal control group. Discriminatory metabolic marker, pairwise PCA (Fig. S2) and OPLS-DA (Fig. 3) analysis were performed with all the three treated groups (DMBA, GLA 0.25 ml/kg, and GLA 0.5 ml/kg) in respect to normal control to obtain a satisfactory classification and identification. The model parameters for the explained variation R2Y and the predictive capability Q2 were significantly high (displayed in their respective OPLS-DA score-plots) in each case, indicating that the pairwise OPLS-DA models constructed from CPMG spectra possessed satisfactory fit with good discriminatory power. The visual inspection of the pairwise score plots showed a clear differentiation between normal control group and DMBA treated group.

### Table 2
Effect of GLA on oxidative stress markers against DMBA induced mammary gland carcinoma.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBArs (nM of MDA/µg of protein)</th>
<th>GSH (mg%)</th>
<th>SOD (Units of SOD/mg of protein)</th>
<th>Catalase (nM of H2O2/min/mg of protein)</th>
<th>Protein Carbonyl (nM/ml unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control; 0.9% normal saline, p.o.)</td>
<td>0.17 ± 0.03</td>
<td>1.21 ± 0.06</td>
<td>0.044 ± 0.009</td>
<td>19.5 ± 0.03</td>
<td>20.43 ± 5.11</td>
</tr>
<tr>
<td>Group II (Toxic control; DMBA 8.8 mg/kg, i.v.)</td>
<td>0.35 ± 0.02</td>
<td>1.09 ± 0.09</td>
<td>0.059 ± 0.008</td>
<td>32.19 ± 0.07</td>
<td>41.57 ± 0.92</td>
</tr>
<tr>
<td>Group III (GLA 0.25 ml/kg, p.o. + DMBA 8 mg/kg, i.v.)</td>
<td>0.22 ± 0.09</td>
<td>1.19 ± 0.05</td>
<td>0.040 ± 0.007</td>
<td>17.6 ± 0.03</td>
<td>29.70 ± 2.18</td>
</tr>
<tr>
<td>Group IV (GLA 0.5 ml/kg, p.o. + DMBA 8 mg/kg, i.v.)</td>
<td>0.19 ± 0.07</td>
<td>1.24 ± 0.02</td>
<td>0.045 ± 0.008</td>
<td>24.2 ± 0.09</td>
<td>25.98 ± 1.57</td>
</tr>
</tbody>
</table>

(Values are presented as mean ± SD), each group contains eight animals. Comparisons were made on the basis of the one-way Anova followed by Bonferroni test. All groups were compared to the toxic control group.

* p < 0.05.
** p < 0.01.
*** p < 0.001.

### 3.3. Antioxidant parameters

The antioxidant defense system was restored after GLA treatment in comparison to DMBA treated group. GLA successfully curtailed down the level of TBArs (0.19 ± 0.07 nM of MDA/µg of protein) and PC (25.98 ± 1.57 nM/ml unit) in comparison to toxic control. The GSH level was significantly increased in DMBA treated group (1.09 ± 0.09 mg%). The GSH level was upregulated after GLA treatment (1.24 ± 0.02 mg%). After DMBA treatment, the levels of SOD (0.059 ± 0.008 units of SOD/mg of protein) and catalase (32.19 ± 0.07 of H2O2/min/mg of protein) were upregulated and significantly diminished after GLA treatment (i.e. 0.045 ± 0.008 units of SOD/mg of protein and 24.2 ± 0.09 nM of H2O2/mg of protein) (Table 2).

### 3.4. Serum metabolic profiling using 1D 1H NMR

The representative 1D 1H CPMG NMR spectra was shown in Fig. 2. The NMR spectra showed signals from lipids/lipoproteins (e.g., low density lipoprotein (LDL), very low density lipoprotein (VLDL), PUFAs etc.) and amino acids (e.g., alanine, valine, leucine, isoleucine, phenylalanine, histidine, tyrosine, glutamine, glutamate, proline etc.). Other identified metabolites were glucose, choline, creatine/creatinine, acetone, pyruvate, acetate, citrate, lactate, N-acetyl and O-acetyl glycoproteins (NAG, OAG). For an initial overview of the data set, unsupervised PCA score plots were constructed. Two outliers were detected, one from the toxic control and other from the normal control group. The outliers were excluded from both the data sets and the resulted data sets were then subjected to supervised OPLS-DA model to minimize the possible contribution of intergroup variability and to further improve the separation between the groups (Fig. 3). The combined PCA score plot (Fig. S2) showed a clear trend of clustering in different groups and no further outlier samples were detected. The combined OPLS-DA score plot also revealed that GLA (0.25 ml/kg) and GLA (0.5 ml/kg) treatments are mitigating the effect of DMBA induced toxicity as inferred by the shift of the GLA treatment group back towards the normal control group. Discriminatory metabolic marker, pairwise PCA (Fig. S2) and OPLS-DA (Fig. 3) analysis were performed with all the three treated groups (DMBA, GLA 0.25 ml/kg, and GLA 0.5 ml/kg) in respect to normal control to obtain a satisfactory classification and identification. The model parameters for the explained variation R2Y and the predictive capability Q2 were significantly high (displayed in their respective OPLS-DA score-plots) in each case, indicating that the pairwise OPLS-DA models constructed from CPMG spectra possessed satisfactory fit with good discriminatory power.

The visual inspection of the pairwise score plots showed a clear differentiation between normal control group and DMBA treated group.
Fig. 3. Combined and pairwise OPLS-DA analysis. The 2D OPLS-D analysis of 1D $^1$H CPMG NMR spectra (A) score plot derived from combined analysis comprising of all the groups: normal control (NC), toxic control (TC), GLA (0.25 ml/kg) and GLA (0.5 ml/kg). Pairwise analysis (B) between normal control (NC) and DMBA treated toxic control (TC) group, (C) between NC and DMBA + GLA (0.25 ml/kg), and (D) between NC and DMBA + GLA (0.5 ml/kg). Color circles indicate the 95% confidence interval for each class.

Table 3
List of metabolites responsible for variation and class separation between the NC, DMBA and DMBA + GLA treatment at two doses 0.25 ml/kg and 0.5 ml/kg.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Metabolite ppm</th>
<th>NC vs. GLA 0.25 ml/kg</th>
<th>GLA 0.50 ml/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LDL/HDL</td>
<td>0.79</td>
<td>↑↑</td>
</tr>
<tr>
<td>2</td>
<td>Isoleucine/excine</td>
<td>0.93</td>
<td>↑</td>
</tr>
<tr>
<td>3</td>
<td>VLDL/LDL</td>
<td>1.25</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>4</td>
<td>Arginine</td>
<td>1.71</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>5</td>
<td>Citrulline</td>
<td>1.85</td>
<td>↑↑↑↑</td>
</tr>
<tr>
<td>6</td>
<td>OAG</td>
<td>2.09</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>7</td>
<td>Glutamate</td>
<td>2.33</td>
<td>↓↓</td>
</tr>
<tr>
<td>8</td>
<td>Pyruvate*</td>
<td>2.35</td>
<td>↓↓</td>
</tr>
<tr>
<td>9</td>
<td>Glutamine</td>
<td>2.43</td>
<td>↓↓</td>
</tr>
<tr>
<td>10</td>
<td>Creatine</td>
<td>3.01</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>11</td>
<td>TMAO</td>
<td>3.25</td>
<td>↓↓</td>
</tr>
<tr>
<td>12</td>
<td>Glucose</td>
<td>3.87</td>
<td>↓↓</td>
</tr>
<tr>
<td>13</td>
<td>PUFA</td>
<td>5.25</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>14</td>
<td>Phenylalanine</td>
<td>7.29</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>15</td>
<td>Histidine</td>
<td>7.71</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

The metabolic biomarkers were identified from OPLS-DA, S-plot for the discrimination significance and p value < 0.05 for statistical significance. The up (↑) and down (↓) arrows represent, respectively, increased and decreased metabolite levels. A ↑↑↑↑/↓↓↓↓ or ↑↑↑↑/↓↓↓↓ score was given to the metabolites of the GLA treatment dose which showed ameliorating effects from DMBA towards control. "*" represents metabolites with most prominent effect of GLA treatment.

Note: Metabolites which do not have p value significant are represented by "*".

indicating that significant metabolic changes were induced by DMBA treatment (Fig. 3B). Next, to evaluate the biochemical effects of GLA treatment on DMBA induced toxicity, the altered metabolites were identified using OPLS-DA, S-plot (Fig. S4), and further tested for statistical significance at the level of p < 0.05. Overall, we identified fifteen metabolic markers significantly perturbed in the sera of DMBA treated rats compared to normal control rats. These markers metabolic entities along with their chemical shifts, their levels (increased or decreased) and p value are listed in Table 3. In comparison to normal control, DMBA treatment had significantly elevated levels of lipids, VLDL/LDL lipoprotein, PUFAs, OAG, creatine, and amino acids (arginine, citrulline, phenylalanine, histidine) whereas, decreased levels of glucose, pyruvate, trimethylamine (TMAO) and amino acids (glutamate, and glutamine) (Fig. 4 and Table 3). The metabolites which were increased or decreased in DMBA treated group such as lipids, LDL/VLDL, PUFAs, OAG, arginine, citrulline, phenylalanine returned to normal after GLA treatment (Fig. 4).

3.5. Caspase 3 and caspase 8 assay

GLA (0.25 ml/kg) significantly upregulated the level of caspase 3 and caspase 8 in comparison to other groups (Fig. 5).

3.6. Immunoblotting

After DMBA treatment, the expression of anti-apoptotic proteins (Bcl-2 and Bcl-xl) was increased with vice versa effect upon pro-apoptotic marker (BAX and BAD). GLA treatment helped to reinitiate the anti-apoptotic and pro-apoptotic markers favorably advocating apoptosis. DMBA treatment upregulated the expression of downstream markers of mitochondria mediated apoptosis (VADC, cytochrome c, Apaf-1 and pro-caspase 9) along with curtailment of cytochrome c expression (Fig. 6). GLA treatment afforded marked protection against the same and favoring apoptosis. DMBA treatment also afforded commendable hypoxia which was perceived through up-regulated expression of NFkBp65, UCHL-1, HIF-1α, FASN and SREBP-1c along with down-regulated expression of PHD2. GLA treatment upregulated the PHD2 expression and diminished the hypoxic environment by down-regulating the protein expressions of NFkBp65, UCHL-1, HIF-1α, FASN and SREBP-1c (Fig. 7). The expression of α7nAchR and HMGB-1 was downregulated and TNF-α expression was increased after GLA treatment when scrutinized through immunoblotting analysis (Fig. 8).

3.7. Fold change expression

The fold change expressions of the genes were validated through qRT-PCR assay (Fig. 6). The findings from the immunoblotting assay of the mitochondrial mediated apoptosis pathway were validated through fold change expression of genes and the same observations were found. The qRT-PCR studies for the hypoxic markers recorded similar pattern of fold changes as perceived through the immunoblotting assay (Fig. 7). The mRNA expression of α7nAchR and HMGB-1 was downregulated along with increased expression of TNF-α after GLA treatment in qRT-PCR studies (Fig. 8).

4. Discussion

DMBA is a is an immunosuppressor or organ specific laboratory carcinogen that produces mammary gland tumors which are very much similar to the tumors found in humans both morphologically and histopathologically (Choi and Kim, 2011). DMBA is metabolized by cytochrome P450 and forms diol epoxides which are transported to the mammary glands and results in the formation of DMBA-DNA adducts leading to carcinogenesis (Lakshmi and Subramanian, 2014). Autonomic dysfunction is very common to several types of cancer and it may be shepherded to increased sympathetic activity and decreased vagal tone (Fagundes et al., 2011). The HR, R wave amplitude and RR interval are the crucial markers of autonomic dysfunction, which were positively modulated after GLA treatment. Treatment with GLA embarked a marked positive regulation of the HRV factors, which is indicative of the positive regulation of autonomic dysfunction by GLA (Tables 1, S1 and Fig. S1).

Cancer progression is defined by two hallmarks named as cellular proliferation and angiogenesis (Hanahan and Weinberg, 2011). These two mechanisms bestow to make a substantial change in tissue architecture and the same was scrutinized through morphological evaluation using carmine staining, H&E staining and SEM. The carmine staining of the DMBA treated animals was in line with previous literature for the cellular proliferation, as represented by increase in number of AB count.
and DF score (Fig. 1A–D) (De Assis et al., 2010). DMBA treatment was also evident for the scattered pattern of CEC, LCT and DCT were hard to identify along with loss of duct and MEC as visualized by H&E staining (Fig. 1E–H). Marked proliferation after the DMBA administration was observed with increase in micro vessel formation, loss of intra-arterial cushion and vascular conglomeration, when perceived through SEM analysis (Fig. 1I–L). The finding from the carmine staining, H&E staining and SEM analysis are well in line with the previous reports (Manral et al., 2016). GLA treatment demarcated a marked impression on cellular architecture and morphology of the mammary gland tissue and decreased the growth of enlarged capillaries (the sign of rapidly growing tumors). Henceforth, GLA imparted dose-dependent curtailment of cellular proliferation and therefore warrants further validation through more stringent markers.

Oxidative stress is a well explicated phenomenon in carcinogenesis (Gorrini et al., 2013). It has been reported that increased production of H$_2$O$_2$ in breast cancer patients may be due to an increase in production of superoxide anion (O$_2^-$) and elevated activity of SOD, leading to tissue damage through OH (Liou and Storz, 2010). After DMBA treatment, the increased production of TBARs and PC represents damage of...
cellular lipids and proteins, which in line with previous report (Kaithwas et al., 2014). DMBA treatment also decreased the enzymatic defense of SOD, catalase and GSH, suggesting their increased utilization (Batcioglu et al., 2012). The said observations is a clear indicator of oxidative stress and GLA treatment restored the enzymatic antioxidant defense of SOD, catalase and GSH along with downregulated levels of TBARs and PC (Table 2).

NMR based serum metabolomics coupled with multivariate statistical analysis was used to investigate the effect of DMBA induced metabolic alterations and to study the ameliorating effect of GLA treatment on these alterations. After DMBA treatment, increase in the levels of lipoproteins (VLDL/LDL), lipids, and PUFAs were observed in comparison to normal control and this phenomenon suggests altered fatty acid metabolism in DMBA treated group. PUFA, on the other hand; is important intermediate for membrane metabolism and inflammatory mediators, so the elevated levels might be related to augmented utilization to repair the damaged cells and dampen the inflammation associated with DMBA induced injury to mammary gland cells (Al-Mubarak et al., 2011). OAG is acute phase protein and NAG have anti-inflammatory property and expressed more during inflammation and immune response (Guleria et al., 2016) and increased concentrations of OAG is likely a reflection of active inflammatory process. The increased levels of acetyl glycoproteins in are coherent with previous investigations in inflammatory disease and many cancers (Connelly et al., 2016).

The lower pyruvate level in DMBA treated group in comparison with normal control suggested altered TCA cycle. To maintain physiological homeostasis and meet the energy requirements of cancerous cells, there is an increased reliance on alternate energy substrates preferably amino acids (Keenan and Chi, 2015). Accordingly, the serum levels of several gluconeogenic amino acids (like glutamine, glutamate, etc.) were found to be decreased in DMBA treated group. Arginine is an important constituent of many important biological pathways and it is the precursor molecule for nitric oxide (NO), citrulline, urea, ornithine, and creatine (Geiger et al., 2016). Significant increases in serum branched...
chain amino acid (BCAA) (isoleucine and leucine) and aromatic amino acid (AAA) (histidine and phenylalanine) concentrations were found. The BCAA and AAA are catabolized to replenish the depleted levels of TCA cycle intermediates or as acetyl derivatives to generate energy during stress suggesting protein breakdown. The levels of glutamate and glutamine were also found to be significantly decreased in the serum of DMBA treatment. Glutamine is used for the biosynthesis of nucleotides for cell proliferation and it is also an alternate energy source to glucose in cancerous cells (Jin et al., 2016). Glutamate is a precursor of GSH and a metabolic product of glutamine (Balendiran et al., 2004). The main cause of cell apoptosis is oxidative damage and tumor cells may increase the concentration of the antioxidant metabolites like GSH, which might be responsible for the decreased levels of glutamate and glutamine. However, when DMBA treated rats were treated with GLA; the aforementioned metabolic changes were found to reset back to their normal level (Fig. 4 and Table 3) suggesting that GLA has been potential anti-oxidant as well as anti-tumor activity. It would be important to mention at the junction that GLA being a PUFA has the potential to get oxidized himself (pro-oxidant), thereby protects the cellular lipids (Das et al., 2001).

The process of apoptosis can be commenced by two major pathways: death receptor mediated extrinsic apoptotic pathway and mitochondrial mediated intrinsic pathway. The mitochondrial pathway is regulated by Bcl-2 family of proteins with the involvement of pro and anti-apoptotic member of proteins (O'Neill et al., 2016). During apoptosis, the pro-apoptotic protein (BAX and BAD) translocates to the outer membrane of mitochondria and promotes the release of cytochrome c. While on the other side, the anti-apoptotic proteins (Bcl-2 and Bcl-xl) inhibits the release of cytochrome c (Elmore, 2007). After GLA treatment, the expressions of pro-apoptotic (BAX and BAD) proteins were decreased along with increased expressions of anti-apoptotic (Bcl-2 and Bcl-xl) proteins. Similar findings were found with mRNA expression when scrutinized with qRT-PCR. Cytochrome c is released due to loss of channel integrity and the decreased expression of VDAC validates the same (Roy et al., 2017). After GLA treatment, the protein and mRNA expression of VDAC and cytochrome c confirm the same. Cytochrome c release then triggers the congregation of cytoplasmic apoptosome. The apoptosome is a complex formed of Apaf-1, cytochrome c and

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**Fig. 7.** Effect of GLA on hypoxic cancer cells metabolic pathway.

Immunoblotting of respective individual group [1-control, 2-DMBA treated, 3-GLA (0.25 ml/kg, p.o. + DMBA 8 mg/kg, i.v.) and GLA (0.5 ml/kg, p.o. + DMBA 8 mg/kg, i.v.)] was performed for PHD2, HIF-1α, UCHL-1, FASN and SREBP-1c. Excised mammary gland tissue sample was lysed in trizol for RNA extraction and analyzed for the mRNA expression of PHD2, HIF-1α, UCHL-1, FASN and SREBP-1c by qRT-PCR: fold induction is relative to tissue under hypoxic conditions after normalization to the β-actin expression. Each experiment was performed in triplicate. Values are presented as mean ± SD. Comparisons are made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups are compared to the DMBA treated group (p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001). β-actin is normalized with respect to β-actin ratio.
procaspase 9 (Yuan and Akey, 2013). Apoptosome formation decreased the cytosolic levels of Apaf-1 and procaspase 9 and the same findings were observed after GLA treatment. Procaspase 9 was cleaved with the formation of apoptosome and leads to the formation of active caspase 9 which results in the activation of caspase 3 and 8. The caspase activation leads to activation of downstream caspase cascade and leading to apoptosis (Fig. 6) (Bratton and Salvesen, 2010). GLA treatment increased cytosolic caspase 3 and 8 and thereby accredits apoptosis (Fig. 5). Henceforth, it was clearly derived that GLA treatment diminished the angiogenic and proliferative effects of DMBA by activating the mitochondrial-mediated death apoptosis pathway.

It is well known that tumor cells require energy from glycolysis due to hypoxic condition of the cells (Warburg effect) (Padaka et al., 2017). HIF-1α regulates the hypoxia and is further regulated by 2-oxoglutarate and iron dependent hydroxylases enzyme PHD2 (Singh et al., 2016). It was previously reported that the increased glycolytic activity in tumor cells is combined with increased fatty acid synthase to meet the fatty acid requirements through de novo fatty acid synthesis (Zaytseva et al., 2015). GLA treatment increased the expression of PHD2 and thereby curtailed the expression of HIF-1α when scrutinized through immunoblotting and qRT-PCR studies. The decreased expression of HIF-1α was cross validated through the decreased expression of NFκBp65 and UCHL-1. During deubiquination of HIF-1α, NFκBp65 imparts positive modulatory effect upon HIF-1α and UCHL-1 stabilizes HIF-1α. GLA treatment decreased the expression of VDAC after GLA treatment suggests stabilization of membrane potential transition pore (MPTP) and in Ca2+ influx. Subsequently, the role of Ca2+ influx was validated through α7nAchR proteins by which cholinergic anti-inflammatory...
pathway has been regulated (Tsong et al., 2011). In normal cell, Ca^{2+} trigger is requisite for maintaining cell physiology. The production of inflammatory cytokines is controlled by ACh and nicotine via α7nAChR (Zdanowski et al., 2015). α7nAChR have four transmembrane domains (TM1-4). A regulatory intracellular domain is located between TM3 and TM4 and forms a hetero or homo pentamers of α7nAChR which maintain integrity of central ion channel in transmembrane junction (Jonge and Ulloa, 2007). The entry of different cations (Na^+, K^+ and Ca^{2+}) is regulated through aNACHRs group of proteins and it is much more selective for Ca^{2+} influx. The cations influx reduces the negative charge on intracellular side causing membrane depolarization (Wu et al., 2015). After initiation of membrane depolarization, the gates on the intracellular side of plasma membrane is open for the entry of voltage gated Ca^{2+} and leads to downstream activation of various intracellular angiogenic cascade [vascular endothelial growth factor (VEGF), endothelial growth factor (EGF) and PI3Akt] (Moccia et al., 2012).

The present study elucidates biphasic regulation of cancer cells by GLA through inhibition of Ca^{2+} influx and activation of cholinergic anti-inflammatory pathway (Fig. 8). The α7nAChR mediated cholinergic signaling after GLA treatment was also pathway was also confirmed by increased expression of TNF-α. It was well established that TNF-α alone was ineffective in showing its anti-tumor activity without any internal or external stimuli (Johansson et al., 2012). It was hypothesized that increase TNF-α expression is mediated by TNFR-I along with an extracellular domain (ECD), transmembrane domain (TMD) and an intracellular domain (ICD). The other subunit TNFR-2 is only expressed in immune cells whereas TNFR1 is ubiquitously expressed in all cell types (Richter et al., 2012). α7nAChR pathway is initiated by TNFR-1 internal signaling consists of caspase 8. Caspase 8 is auto-activated by subtype TNFR1 which results in activation of caspase 3, 7 and endonucleases (Kim and Li, 2013). Low dose of GLA induced the expression of TNF-α as well as activated the downstream signaling cascade, which was evident through positive modulation of caspase-3, caspase-8 and downregulation in the HMGB-1 (promotes deolocalization of homotrimer and causing systemic cleavage on receptor interacting protein) (Lin et al., 1999). The tumor blood vessel permeability increased by the induction of TNF-α which deliberately augments the GLA concentration in mammary gland tissues was further confirmed by downstream expression of Fx6p65. Receptor interacting protein (RIP) is crucial for activation of Fx6p65 and plays an important role in acquisition of apoptosis. RIP consists of a death domain that can bind with other death domains in signaling molecules like fas associated death domain (FADD) and tumor necrosis factor associated death domain (TRADD) (Pobeznikayska and Liu, 2012). All in all, GLA decreased the expression of α7nAChR, HMGB-1 and Fx6p65 along with increase expression of TNF-α to execute cholinergic anti-inflammatory pathway (Fig. 8).

Henceforth, one can conclude that GLA by regulating mitochondria mediated death apoptosis, hypoxia induced cell signaling and cholinergic anti-inflammatory pathway can impart favorable effect against DMBA induced carcinogenesis.

Conflict of interest

The authors declare no conflicts of interest exist.

Author’s contribution

SR Carried out the bench work; MS performed the immunoblotting assay; AR performed the NMR studies; UD & SG performed the caspase 3 and caspase 8 assay; RKY performed the morphological analysis; JKR performed the hemodynamic studies; MNA & ASS: performed the statistical studies and compiled the data; DK evaluate the result of NMR; GK perceived the idea, designed and supervised the whole study, prepared and proof read the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocel.2018.01.011.

References
