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Antiproliferative effect of ketogenic diet on hormone independent mammary gland carcinoma *via* harnessing glucose metabolism: *In-vitro* and *In-vivo* investigations

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ABSTRACT

The ketogenic diet (KD) has been emphasized as a complementary strategy for management of several clinical conditions including cancer. Therefore, in this study we explored the effect of KD in mammary gland carcinoma through *in-vitro* and *in-vivo* studies. *In-vitro* studies were performed on MCF-7 and MDA-MB-231 cells with different experimental conditions such as high glucose (HG), low glucose (LG) and no glucose(NG) in conjugation with β-hydroxy butyrate(BHB) treatment. The MTT assay revealed that glucose deprivation alongwith BHB(10 mM) treatment significantly reduces the viability of MDA-MB-231 cells as compared to MCF-7 cells. Moreover, apoptotic and antiproliferative potential (via AO/EtBr, JC-1, cell migration assay) were analyzed on MDA-MB-231 cells which indicate that NG with BHB treatment induce cell death.Furthermore, we investigated the *in-vivo* anticancer efficacy against DMBA-induced mammary gland carcinoma in female Wistar rats. KD treatment effectively restored autonomic dysfunction, altered mammary gland morphology and histology; as evident through decrease in lobules, alveolar bud, restoration of the surface architecture and loss of tumor micro-vessels. The altered levels of antioxidants such as TBARs(0.85 ± 0.19 nM of MDA/μg of protein), SOD(2.26 ± 0.05 U/μg of protein), PC(41.36 ± 2.94 μM/μg of protein), GSH(10.58 ± 3.08 μM/μg of protein) were also restored after KD treatment. Overall findings suggested, that deprived glucose concentration alongwith BHB can impart anti-proliferative and apoptotic effect as observed through MDA-MB-231cells. Moreover, KD also diminished the carcinogenic effects of DMBA in albino wistar rats. In view of above, the KD was utilised as adjuvant therapy in the management of mammary gland carcinoma,possibly by providing unfavourable microenvironment for highly proliferating tumour cells due deficiency of quickly available glucose.

1. Introduction

Mammary gland carcinoma, a multifaceted disease significantly reducing life expectancy, quality of life with increased morbidity among women worldwide [1]. Although therapies like chemotherapy and surgery are critical components of breast cancer treatment, their efficacy is often limited due to contraindications, side effects and other considerations [2]. New and novel technologies/products based on nanotechnology (Doxil,

Abbreviations: **AcAc**, Acetoacetate; **ANOVA**, Analysis of variance; **AO/EtBr**, Acridine orange/Ethidium Bromide; **ATP**, Adenosine tri phosphate; **BHB**, Beta hydroxyl butyrate; **DAPI**, 4′,6-Diamidino-2-Phenylindole; **DMEM**, Dulbecco's modified eagle medium; **ETC**, Electron transport chain; **FBS**, Fetal Bovine serum; **HG**, High Glucose; **HGKD**, High glucose with ketogenic diet; **IGF1**, Insulin growth factor; **JC-1**, 5′,6,6′Tetrachloro,1′3,3′tetraethylbenzimidazolylcarbocyanine; **KD**, Ketogenic diet; **LG**, Low Glucose; **LGKD**, Low glucose with ketogenic diet; **MMP**, Mitochondrial membrane potential; **NG**, No Glucose; **NGKD**, No glucose with ketogenic diet; **OXPHOS**, Oxidative phosphorylation; **ROS**, Reactive oxygen species.

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Genexol-PM etc); natural compounds (Polyphenols, alkaloids etc) and drug delivery systems (polymeric conjugates, nano or microparticles, hydrogels, microneedles etc) have provided some edge in the clinical management but the limitations like post treatment complications, poor life quality after treatment are the major restrictions [3–5].

As a result, there is considerable interest in developing adjuvant therapies to selectively target the cancer metabolism to overcome tumor progression, metastasis, angiogenesis, drug resistance, and recurrence. The hypothesis of Otto Warburg opened new avenues for treating cancer and consequently the studies on cancer metabolism and nutrition has emerged as a prominent area of focus in pre-clinical and clinical research [6,7].

Cancer cells undergo enhanced glycolytic metabolism, even in the presence of oxygen (Warburg effect). This metabolism stabilizes cancer cells to adapt intermittent hypoxia, enhances the proliferation rates, and resist oxidative damage resulting from oxidative phosphorylation. Furthermore, cancer cells show reliance on fatty acid synthesis and amino acid metabolism to support their cellular proliferation and metastasis [6,8]. Recently, the metabolism of ketone bodies (KBs) has become a significant focus in cancer research. KBs, which consist of acetoacetate (AcAc), acetone, and β-hydroxybutyrate (BHB), functions as a crucial alternative energy source to glucose during nutrient scarcity [9,10]. Consequently, when glucose is limited, cells may be compelled to seek alternative energy sources through fat and protein. A diet low in carbohydrates and high in fat, and protein can lead to the production of ketone bodies and fatty acids by the liver; which can be utilised by the normal cells but not by the cancer cells as a source of energy. Such diet is considered as ketogenic diet (KD) [11,12]. The KD limits 1gm of protein per kg of body weight alongwith 10–15 gm of carbohydrate, whereas the rest of the caloric requirements are to be completed by fat [13].

Preliminary research has shown that KD is a promising approach for cancer treatment. Preclinical studies on mouse models, including endometrial, bladder, pancreatic cancer, alongwith acute myeloid leukemia has shown that KD enhances the efficacy of targeted therapies [14]. The studies has also argued that a low carbohydrate KD diet might be a fundamental element of a complete treatment strategy for augmenting the effectiveness of conventional cancer therapies and can also improve the post treatment recovery and quality of life [15,16].

It would be appropriate to mention that findings from the previous studies have variable reports in terms of effect of KD on mammary gland carcinoma particularly in terms of its efficacy towards hormone-independent/ER- and hormone-dependent/ER + carcinoma [17,18]. Henceforth, it was hypothethized that low carbohydrate KD may provide an environment to which would be unfavourable for highly proliferating cells due to deficiency of quickly available glucose. In other words low carbohydrate KD will not provide the readily avaibale glucose which is highly requisite for the fastly proliferating cancer cells and therefore, low carbohydrate KD may act as an adjuvant to the chemotherapy. In view of the above the present study was designed to study the effect of low carbohydrate KD on MCF-7 (ER +) and MDA-MB-231 (ER-) cancer cells. The mammary gland carcinoma has been clasified into hormone dependent and independent, consequently we preferred to use ER- (MDA-MB-231) and ER+(MCF-7) types of cells in our study.

In addition, we studied the effect of KD against DMBA induced mammary gland carcinoma in female albino wistar rats. In view of above, the current study has been undertaken to study the effect of KD/low glucose environment on cellular proliferation using *in-vitro* and *in-vivo* models.

2. Materials and methods

2.1. Chemicals, kits, and reagents

Dulbecco's modified eagle medium (DMEM, Thermo Fischer 11966–065); β-hydroxy butyrate (BHB, Sigma Aldrich, 150–83-4); DMEM (Thermo Fischer 11966–025); Dextrose (Finar, CASR-50–99-7); Fetal Bovine Serum (Gibco USA – 10–27-0); 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, 28718–90-3); Hank's balanced salt solution (HBSS- Himedia, TL1190); 7, 12-Dimethylbenz (a) anthracene (DMBA) (Sigma Aldrich, 57–97-6); Collagenase type 4 (Himedia, TC-214); Hyaluronidase (Himedia, TC331); RIPA lysis buffer (Amresco, N653); Hematoxylin (Himedia, S058); Sodium Cacodylate (Sigma Aldrich, C0250); Eosin (Himedia, S007); Bovine serum albumin (BSA) (Genetix, PG-2330); Bradford solution (Amresco, M173); MTT (Duchefa biochemic, Netherland, 298–93-1); Acridine orange (AO, Himedia, MB116, 10127–02-3); Ethidium bromide (EtBr, Himedia, MB071, 1239–45-8); JC-1 assay kit (Cayman chemical Michigan, 100009172). All other chemicals of molecular biology grade were procured from Genetix Biotech Asia Pvt. Ltd., New Delhi.

2.2. In-vitro study

2.2.1. Cell procurement

MCF-7 and MDA-MB-231 cells were obtained from the National centre for cell sciences (NCCS), Pune, India, with authentication no. 997/2021–22. The cell lines obtained from NCCS, Pune were certified for the lack of mycoplasma contamination and were tested for short tandem repeat (STR) analysis on sixteen STR loci. The cells were grown in DMEM medium with 10 % FBS and 1 % antibiotic–antimycotic solution at 37 °C with 5 % CO_2 in a humidified condition.

2.2.2. In-vitro experimental conditions

In this study, both cell lines were subjected to six different experimental conditions: Group I (HG): DMEM with high glucose, 25 mM; Group II (LG): DMEM with low glucose, 1 mM; Group III (NG): DMEM with no glucose; Group IV (HGKD): DMEM with high glucose (25 mM) + BHB (10 mM); Group V (LGKD): DMEM with low glucose (1 mM) + BHB (10 mM); GroupVI (NGKD): DMEM with no glucose + BHB (10 mM).

2.2.3. Cell viability assay

MCF-7 and MDA-MB-231 cells (1×10^4 cells) were seeded in 96 well plates and incubated for 24hr and 48hr with different experimental conditions (HG, LG, NG, HGKD, LGKD, and NGKD)in triplicate. After incubations, 20 µl of MTT (5 mg/ml) was added in each well and incubated for 4 hr. Thereafter, 100 µl DMSO was added to each well for dissolving the fromazon crystals and plates were analyzed at 570 nm wavelength on an ELISA plate reader (Spectra Max, ABS Plus Microplate Reader). Cells kept with HG were utilized as control [19,20].

2.2.4. AO/EtBr staining

MDA-MB-231 cells were seeded into 12 well plate (1×10^6 cells/ml/well) and exposed to different experimental conditions (HG, LG, NG, HGKD, LGKD, and NGKD), followed by incubation for 48 hr. Cells were rinsed with PBS and stained with AO (100 µg/mL) and EtBr (100 µg/mL) (1:1) for 20 min Thereafter, cells were visualized for the changes under the fluorescence microscope (IX53, Olympus, Japan) at 20x to see the apoptotic changes [21].

2.2.5. JC-1 staining

JC-1 staining performed to assess the mitochondrial membrane potential by measuring the change in the red and green intensity ratio.Cells were seeded into 12 well plate (1×10^6 cells/ml/well) and exposed to different experimental conditions (HG, LG, NG, HGKD, LGKD, and NGKD, followed by incubation for 48 hr. Cells were stained with solution of 5 µg/ml JC-1 prepared in 1 % DMSO for 30 min and excess dye was removed by washing with PBS (three times). Further morphological changes were visualized under fluorescence microscope (IX53, Olympus, Japan) at green and red channel at 20x magnification [22].

2.2.6. Cell scratch assay/wound healing assay

MDA-MB-231 cells were seeded into 12 well plate (1×10^6 cells/ml/

well) and exposed to the experimental condition of HG, LG, NG, HGKD, LGKD, and NGKD, followed by incubation for 48 hr. 12 wells plates were scratched by keeping the 10 μL tip in the perpendicular position to the bottom of well. Images were taken at 0 hr, 24 hr, and 48 hr to analyses the migration ability of cells in different conditions [23].

2.3. In-vivo study

2.3.1. KD pellets diet preparation

Locally available food materials including dalda, butter, sesame oil, salt, protein, fiber, multi-vitamin, and dextrose were used to prepared the ketogenic diets (Supplementary Table 1). The prepared KD pellets were stored at −80 °C for long-term preservation and nutritional integrity [24].

2.3.2. Ethical statement and experimental protocol

Female albino Wistar rats (4–6 weeks old with 110–120 gm weight) were obtained from the animal house facility at the United College of Pharmacy,Prayagraj, Uttar Pradesh, India with approval no (UIP/IAEC/June-2023/06). The animal experimental protocol was conducted in accordance with the guidelines set forth by the Committee for Control and Supervision of Experiments on Animals (CCSEA), Department of Animal Welfare, Government of India.

Following a two-week adaptation period with unrestricted access to food and water *ad libitum*, female albino Wistar rats were divided into four groups (n = 6) Group-I: normal control received normal saline (3 ml/kg, p.o.); Group-II: carcinogen control (CC) received DMBA (8 mg/kg, i.v. single dose); Group-III: Keto control (KC) received KD diet; Group-IV: Keto carcinogen control (KCC) received DMBA 8 mg/kg i.v. + KD diet. Initially, the animals were provided a standard diet (SD) for 90 days. afterthat, the diet of groups III and IV was switched to a manually prepared KD diet for 30 days. On 121st day of the study, blood samples were collected from tail veins, and animals were euthanized via cervical dislocation under anesthesia comprising of combination of ketamine hydrochloride (100 mg/kg, i.m) and diazepam (5 mg/kg, i.m). Mammary gland tissues were collected, rinsed with normal saline and stored at −20 °C for further morphological and biochemical estimations.

2.3.3. ECG and HRV analysis

A combination of ketamine hydrochloride (100 mg/kg, i.m.) and diazepam (5 mg/kg, i.m.) was administered to anesthetize the animals on 120th day. After the anesthesia, the animals were securely placed on a wax tray. ECG was recorded using platinum hook electrodes on the dorsal and ventral thorax. Electrodes were coupled to a Bio-amplifier (ML-136) and channel Power Lab (ML-826) system to convert analog to digital (AD Instruments, Australia). Lab chart Pro-8 (AD Instruments, Australia) was used to analyze ECG data stored on a hard drive offline. HRV analysis was performed using continuous ECG segment using Lab chart Pro-8 [25].

2.3.4. Weight variation

Initial body weight was measured on the day of the commencement of the experiment and final weight was measured on 121st day [26]. Initial and final weights were used to calculate percentage weight variation by using formula I.

$$\%\textbf{\textit{Weight change}} = [(\textbf{\textit{Final body weight of the animals}} - \textbf{\textit{Initial body weight of the animals}})/\textbf{\textit{Final body weight of the animals}}] \times \mathbf{100}$$

2.3.5. Carmine staining

Morphological changes in mammary gland tissues were assessed by performing carmine staining. The mammary gland was extracted from animals and spread on frosted glass slide. Forsted slide was immerged in fixative solution of ethonal:cholorfom: acetic acid (60:30:10). Further, tissues were stained with carmine for the 48 hr. Subsequently, tissues were washed with 90 %, 70 %, 35 % and 15 % alchol at the interval of 2 hr, followed by three washing with distilled water for 5 min. Dehydration of samples was performed by using ascending concentration of alcohol, followed by immersion in xylene for 48 hr. Each group sample slides were observed under a light microscope and images were taken at 4x resolution to observe the presence/absence of alveolar buds (AB), terminal end buds (TEB) and lobules (LOB) [27].

2.3.6. Scanning electron microscopy (SEM)

The SEM was performed using the method previously reported. In brief, tissue was prepared using a sodium cacodylate and HBSS buffer-based HCl collagenase hyaluronic acid enzymatic digestion technique. The sample was dried employing acetone of increasing concentration (70, 80, 90, and 100 %). The acetone specimens were dried out and replaced by the critical point method soak/flash liquid CO_2 fluid exchange with ethanol or acetone. The platinum coating was used to further treat the samples, and results were analysed using a SEM (JEOL JSM-6490LV, X1000) [27].

2.3.7. Histopathological evaluation

Histopathological evaluation was performed using hematoxyline and eosin staining (H&E) staining using the protocol previously established at our laboratory. Tissues were fixed in formalin (10 %) and dehydrated gradually. The dehydrated tissue samples were embedded in melted paraffin wax and cut into 5 μm fine sections. The sections were stained using H&E and visualised (40X) under the biological microscope (CX 43, Olympus Medical Systems India Private Limited, New Delhi) [28,29].

2.3.8. Biochemical estimation

Antioxidant parameters including superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), protein carbonyl (PC), glutathione (GSH), and catalase were analysed by using 10 % (w/v) mammary gland tissues homogenate in 0.15 M KCl. The tissue homogenate was centrifuged for 15 min at 10,000 rpm to collect the supernatant and the resulting supernatant was used to perform an antioxidant profile of the sample using established methods at our laboratory [25,27].

2.3.9. Statistical analysis

All statistical analysis was performed using GraphPad Prism (8.01) and the results were presented as means ± SD. One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test were used to determine the statistical significance. Results with P-values of $p < 0.05$*, $p < 0.1$**, $p < 0.001$***, and $p < 0.0001$**** were considered statistically significant. All groups were compared to Group-II.

3. Results

3.1. Cell viability assay

Preliminary screening of cell viability was performed using MTT assay and findings revealed that MCF-7 and MDA-MB-231 exhibited a high proliferation rate in HG, whereas decreased proliferation was recorded in LG supplymented group.Glucose deprivation and BHB (KD, the ketone body) lowered the viability of both cells. The growth rate of MDA-MB-231 was more affected by BHB as compared to MCF-7 cells

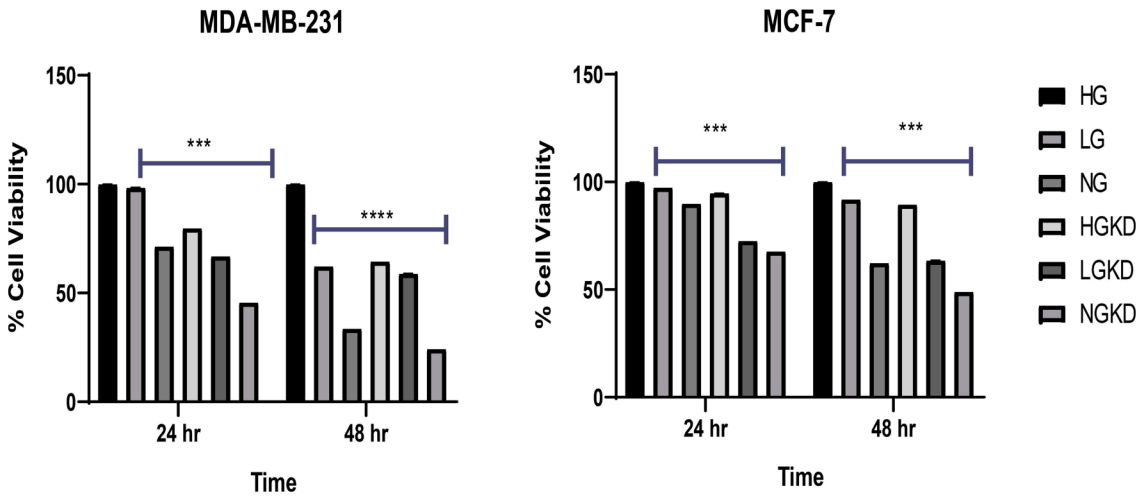

Fig. 1. Effect of KD on cell viability. The above bar diagram represents the effect of different experimental conditions alongwith treatment with BHB (10 mM) on MDA-MB-231 and MCF-7 cells. After 24 and 48 hr incubation cell viability was determined using MTT assay. BHB reduced cell viability of both cells in duration dependent manner with more significant effect on MDA-MB-231 in NG. The data was presented as mean ± SD (n = 3) and statistical analysis was done by one-way ANOVA followed by Bonferroni multiple comparison test using GraphPad Prism-8 software. All the groups were compared to control. Values with ($P < 0.05$*), ($P < 0.01$**), ($P < 0.001$***), ($P < 0.0001$****) were considered as statistically significant.

(Fig. 1). On the basis of cell viability findings further *in-vitro* experiment were performed on MDA-MB-231 cells.

3.2. AO/EtBr staining

AO/EB staining was performed to analyzed morphological changes in different experimental conditions alongwith BHB treatment. Fluorescence microscopy of HG showed viable cells with increased green fluorescence and large nucleus of cells. Whereas, LG showed the induction of apoptosis as evident by increased yellow fluorescence. Supplementation with BHB in LG showed increased number of early apoptotic cells. Whereas both NG and NGKD showed increased number of early and late apoptotic cells (Fig. 2).

3.3. JC-1 staining

The depolarization of mitochondria is a key factor of apoptosis, which may be identified by observing a shift in the intensity ratio of JC-1 fluorescence from red to green. In the current experiment we found that HG and HG alongwith BHB treated MDA-MB-231 cells displayed

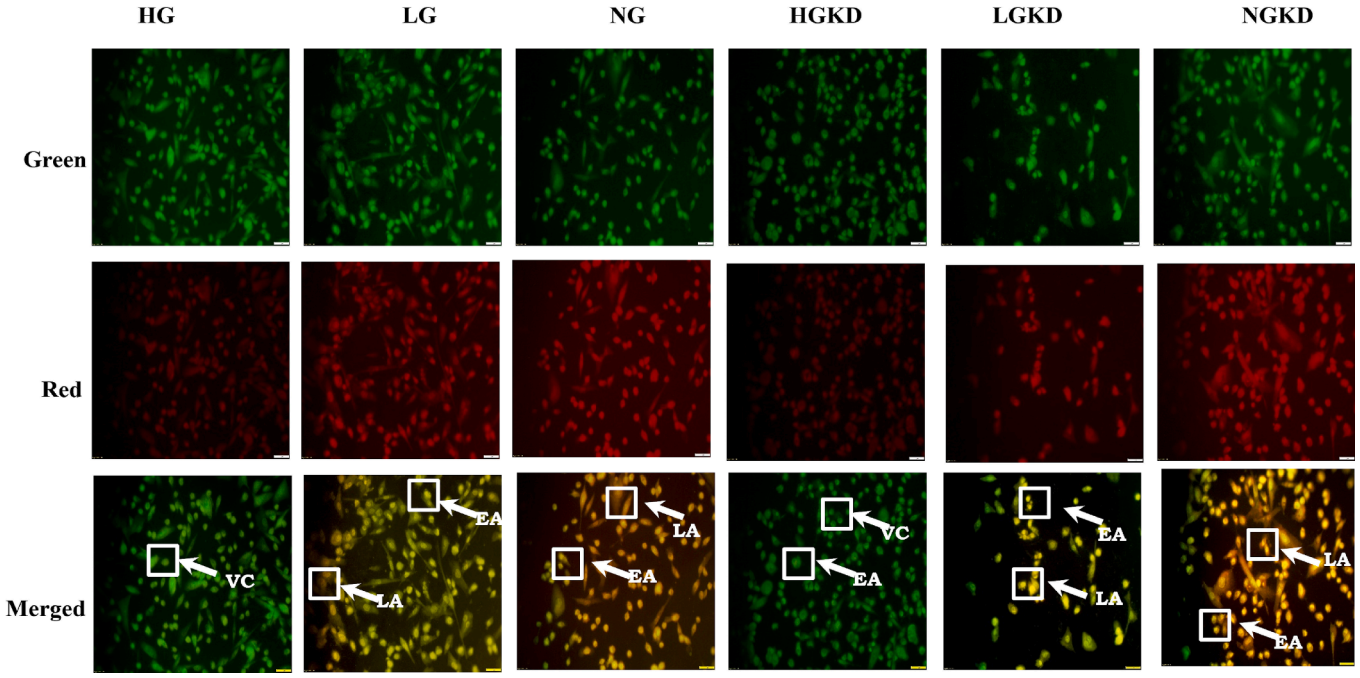

Fig. 2. Determination of apoptotic morphological changes using AO/EtBr staining. MDA-MB-231 cells were stained with AO/EtBr cocktail solution with different experimental conditions alongwith with BHB treatment. Fluorescence microscopy of untreated control (HG) cells showed viable cells(VC) with a bright green nucleus as compared to LG and NG alongwith BHB (10 mM) treatment, depicted more early apoptotic (EA) cells with yellow areas and late apoptotic(LA) cells with a bright orange nucleus. The images were captured with a fluorescence microscope at 20x magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

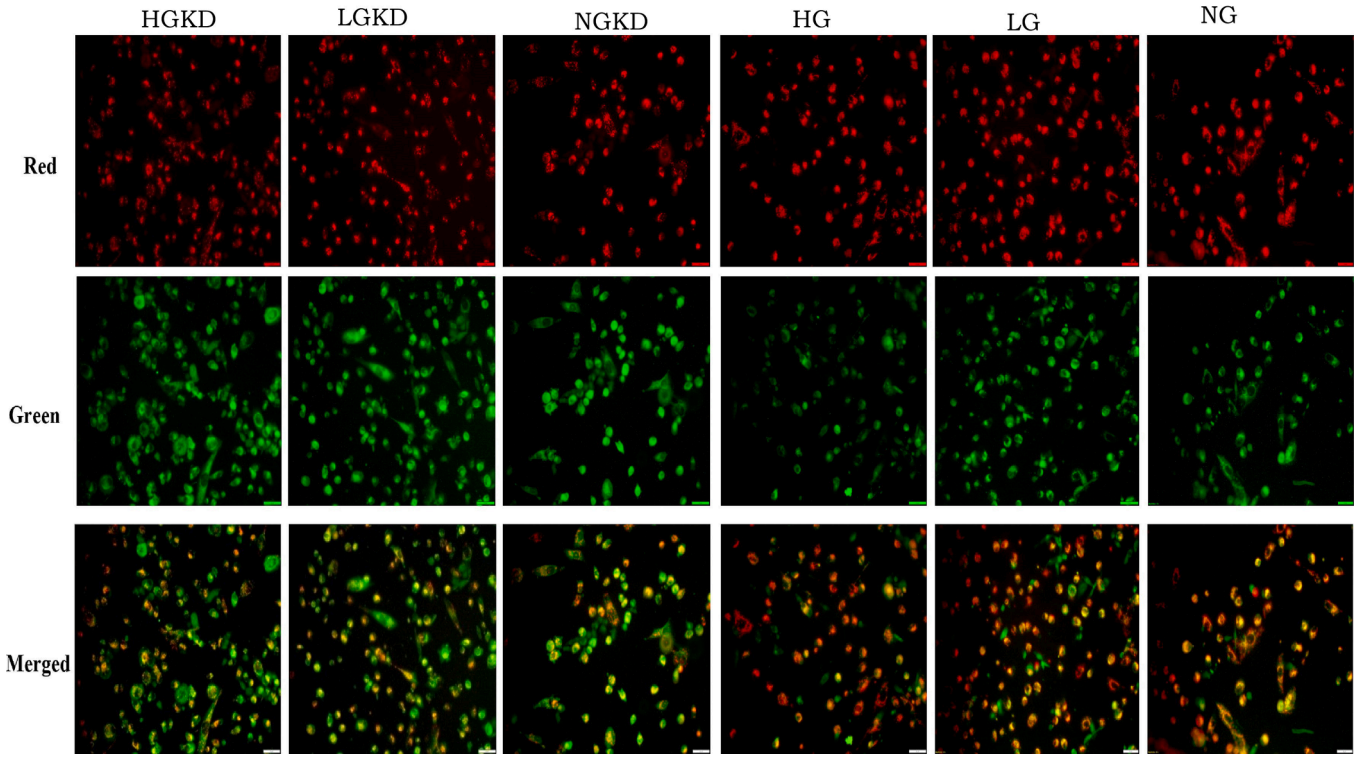

Fig. 3. Determination of MMP (mitrochondrial membrane potential) using JC-1 dye. This figure shows the effect of different experimental conditions alongwith BHB treatment on MDA-MB-231cells. Untreated control (HG) MDA-MB-231 cells showed increased MMP which is reflected by increased red flouresence. While LG and NG alongwith BHB treatment showed increased green flouresence due to decreased MMP. The images were captured with a fluorescence microscope at 20x magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased red fluorescence, indicating intact mitochondrial transmembrane potential. However, LG and NG along with BHB exhibited decrease in the intensity ratios of red to green, indicating loss of mitochondrial membrane potential (Fig. 3).

3.4. Cell scratch assay/ wound healing assay

The wound was completely closed within 48hr when cells were supplemented with HG. As the concentrations of glucose decreased wound healing decreased indicating that glucose starvation affects the

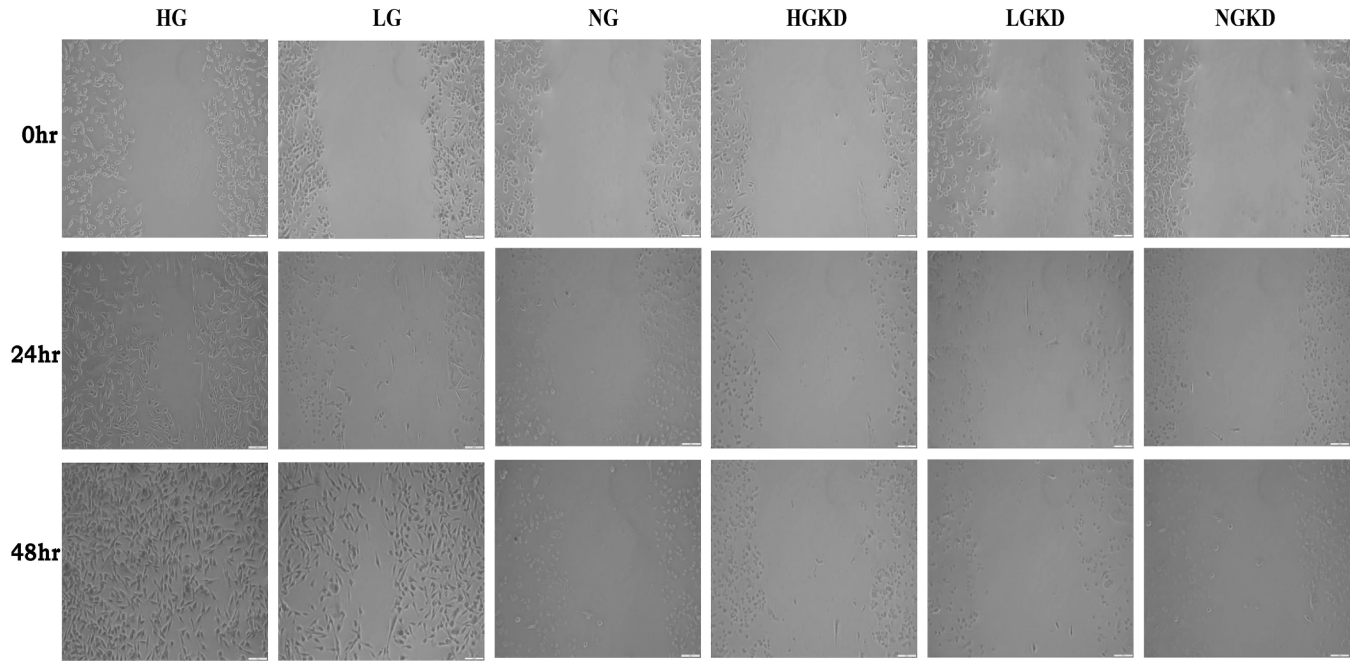

Fig. 4. Determination of cell migration using cell scratch assay. MDA-MB-231 cells migration was measured at 0, 24 and 48 hr after plated into 6- well plate with different glucose concentration alongwith BHB treatment. The figure depicted that BHB supplemented cells shows decreased cell migration as compared to untreated control (HG).

migration properties of cancer cells. However in BHB supplemented groups cell migration was slower than the glucose supply alone. These results were similar and consistent to those observed in the MTT assay and indicate that BHB decreases the proliferation and migration properties of the MDA-MB-231 cells (Fig. 4).

3.5. ECG and HRV

The findings from the ECG analysis revealed a significant protective effect by KD to restore the heart rate (HR) in KC (328.46 ± 24.20) and KCC (341.5 ± 19.56) which was increased in CC (374.88 ± 13.71) compared to NC. KD also minimizes the alteration in QRS interval in KC (0.02 ± 0.001) and KCC (0.02 ± 0.002). P amplitude (atrial depolarization, 0.01 ± 0.03), Q amplitude (0.03 ± 0.02), R amplitude (ventricular depolarization, 1.53 ± 0.22), S amplitude (−0.22 ± 0.11) and T amplitude (ventricular repolarization, 0.35 ± 0.10) were found significant in KCC when compared to CC group (Table 1). Significant decrease in the average RR (163.61 ± 11.4) and median RR (163.5 ± 11.43) in KCC was observed (Table 2).

3.6. Weight variation

Significant weight variation was observed in KC (−13.32 ± 16.48) and noticeable weight loss in KCC (−8.53 ± 10.67) animals compared to NC (9.47 ± 8.67) and CC (8.67 ± 8.62) at the end of the study (Fig. 5).

3.7. Morphological studies of mammary gland tissue

Carmine staining was used to assess the cellular proliferation and angiogenesis.The microscopy of the CC-treated animal showed

Table 1
Effect of ketogenic Diet on ECG changes in different groups.

ECG Parameters	Normal Control (NC)	Carcinogen Control (CC)	Keto Control (KC)	Keto Carcinogen Control (KCC)
RR Interval (s)	0.19 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.17 ± 0.01
Heart Rate (BPM)	314.93 ± 21.9***	374.88 ± 13.71	328.4 ± 24.20**	341.5 ± 19.5*
PR Interval (s)	0.05 ± 0.002	0.04 ± 0.004	0.04 ± 0.004	0.05 ± 0.003
P Duration (s)	0.02 ± 0.004****	0.01 ± 0.003	0.01 ± 0.001	0.02 ± 0.002****
QRS Interval (s)	0.02 ± 0.004****	0.01 ± 0.002	0.02 ± 0.001****	0.02 ± 0.002****
QT Interval (s)	0.04 ± 0.01	0.06 ± 0.02	0.06 ± 0.02	0.1 ± 0.01***
QTc (s)	0.09 ± 0.04	0.15 ± 0.05	0.15 ± 0.04	0.2 ± 0.02
JT Interval (s)	0.02 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.008
T Peak Interval (s)	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.006
P Amplitude (mV)	0.024 ± 0.02***	−0.02 ± 0.02	0.04 ± 0.02****	0.01 ± 0.03*
Q Amplitude (mV)	−0.02 ± 0.07	−0.03 ± 0.02	0.02 ± 0.007	0.03 ± 0.02*
R Amplitude (mV)	1.12 ± 0.47	0.91 ± 0.18	1.33 ± 0.419	1.53 ± 0.22*
S Amplitude (mV)	−0.11 ± 0.06	−0.002 ± 0.03	−0.002 ± 0.03	−0.22 ± 0.11**
ST Height (mV)	−0.01 ± 0.05	0.03 ± 0.02	0.04 ± 0.03	0.11 ± 0.03**
T Amplitude	−0.001 ± 0.1	0.07 ± 0.08	0.31 ± 0.04***	0.35 ± 0.10****

The experiment data represented as mean ± SD. Statistical significance was calculated using one- way ANOVA followed by Bonferroni multiple comparison test. All the groups were compared to CC group (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Table 2
Effect of ketogenic diet on HRV changes.

HRV Parameters	Normal Control (NC)	Carcinogen Control (CC)	Keto Control (KC)	Keto Carcinogen Control (KCC)
Average RR	172.11 ± 12.63	204 ± 46.2	170.21 ± 21.84	163.61 ± 11.4*
Median RR	172 ± 12.97	204.16 ± 46	170.83 ± 22.9	163.5 ± 11.43*
SDRR	3.69 ± 3.20	4.39 ± 1.73	3.70 ± 4.00	1.59 ± 1.29
SDARR	4.10 ± 4.09	3.87 ± 2.22	2.10 ± 1.63	1.44 ± 1.50
CVRR	0.022 ± 0.02	0.022 ± 0.01	0.020 ± 0.01	0.01 ± 0.008
Average Rate	350.51 ± 26.6	306 ± 62.50	357.33 ± 43.01	368.16 ± 24.56
SD Rate	8.24 ± 7.80	6.94 ± 3.76	6.98 ± 5.30	3.68 ± 3.16
SD	1.07 ± 0.13*	3.24 ± 2.27	1.45 ± 0.54*	0.95 ± 0.11**
RMSSD	1.07 ± 0.13*	3.24 ± 2.27	1.45 ± 0.54*	0.95 ± 0.11**

The experiment data represented as mean ± SD. Statistical significance was calculated using one- way ANOVA followed by Bonferroni multiple comparison test. All the groups were compared to CC group (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

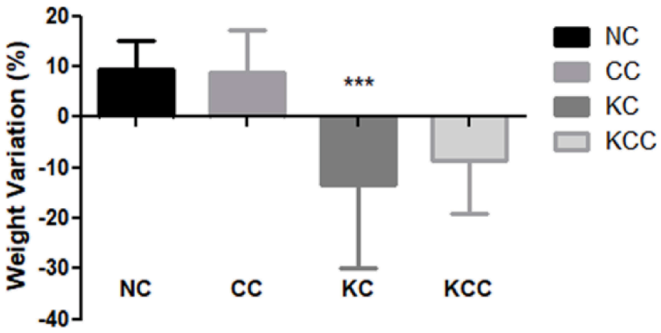

Fig. 5. Effect of ketogenic diet on weight variation. In this figure date was presented as Mean ± SD. Each group contains six animals and comparisons were made using one-way ANOVA followed by Bonferroni multiple comparison test. All groups were compared to the CC group (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).Groups are as follows: (I) NC (3 ml/kg normal saline, p.o.); (II) CC (DMBA 8 mg/kg, i.v.); (III) KC (KD diet); (IV) KCC (DMBA 8 mg/kg i.v. + KD diet).

increased number of AB, and LOB, indicating enhanced cellular proliferation. Administration of KD inhibited proliferation and angiogenesis, as exhibited by a decrease in the number of TEB, AB, and LOB as compared to DMBA treated animals (Fig. 6,I).

Surface architecture in the mammary glands of the experimental animals was studied through SEM. The NC and KC groups revealed collagenous covering (cc), duct (d), and lobules (L); whereas the CC group was evident of the formation of tumor micro-vessels (tmv), loss of cc, large blood vessels (bv) and formation of ducts. KCC group was recorded with restored CC with reduced L and tmv (Fig. 6,II).

The histological section of the NC group showed characteristic architecture of mammary ducts surrounded by basement membrane, with a dense layer of connective tissue. Adipose tissue (AT), and mammary duct (MD) stromal tissue (ST) were equally distributed. Subsequently, CC treated animals depicted degraded ducts, intermittent ST and AT layers signifying *in-situ* ductal carcinoma. Administration of KD restored the histological changes (Fig. 6,III).

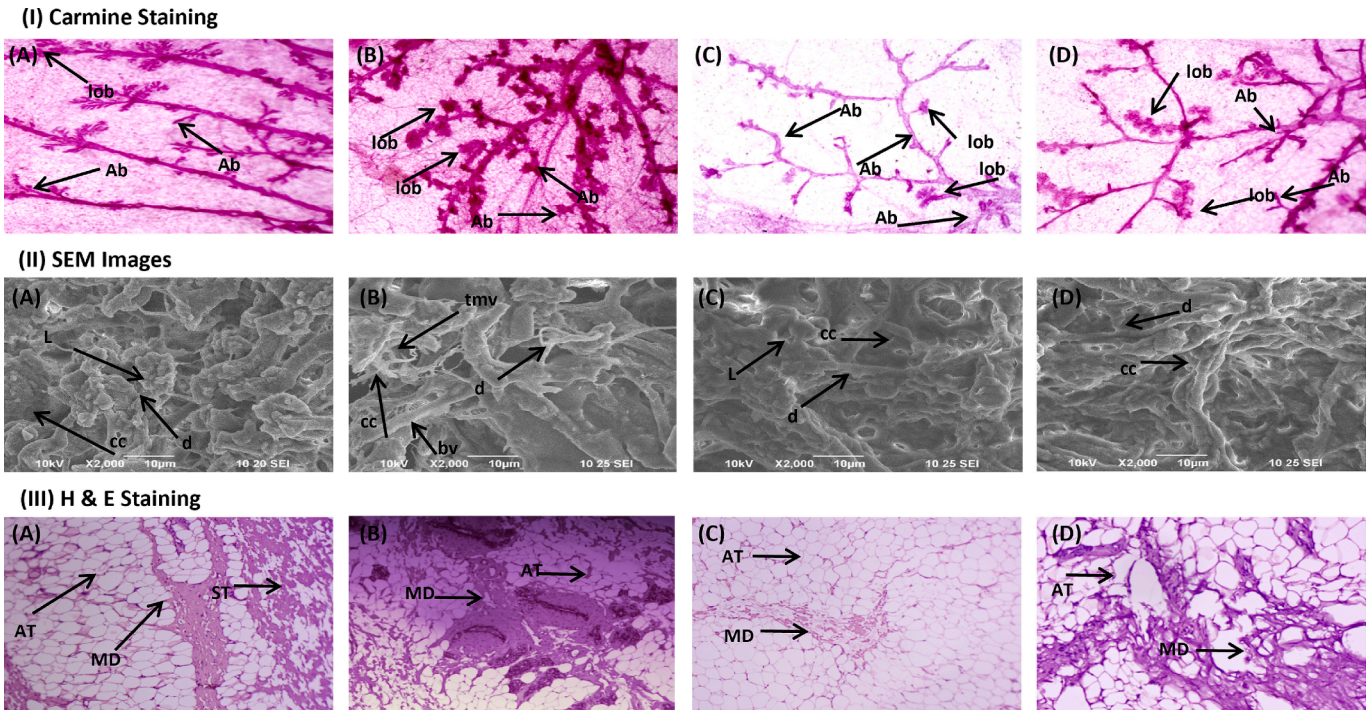

Fig. 6. Effect of ketogenic diet on morphology of mammary gland tissue. Angiogenesis and cellular proliferation are two key parameters used to evaluate the progression of cancer. **(I) Carmine Staining** of mammary gland tissue reveals the presence of alveolar buds (Ab), lobules (lob). [**A.** Normal Control (NC); **B.** Carcinogen Control (CC); **C.** Keto control (KC); **D.** Keto Carcinogen Control (KCC)]. The extent of ab and lobules formation was excessive in the CC which reduced after KD treatment. **(II) SEM Analysis** of NC and KC demonstrated the presence of Collagenous covering (cc), duct (d), lobules (L), and CC showed formation of tumor micro-vessels (tmv), loss of cc, large blood vessels (bv) formation of ducts; where in treatment group, KD causes no loss of cc and maintained the structure of mammary ducts. **(III) Histological analysis** of mammary glands tissue at 40X. NC shows adipose tissue (AT), mammary duct (MD), and stromal tissue (ST). In CC it shows loosely bound AT, poorly organized MD epithelial cell (5) whereas in KC, AT structure is well-organized. KCC group shows deformation of mammary gland structure but well-organized adipose tissue.

Table 3
Effect of ketogenic diet on oxidative stress markers.

Groups	TBARS (nM of MDA/ µg of protein)	PC (µM/µg of protein)	SOD (U/µg of protein)	GSH (µM/µg of protein)
Normal control (NC)	0.53 ± 0.38*	41.02 ± 2.91*	2.18 ± 0.06	14.94 ± 1.49**
Carcinogen control (CC)	0.87 ± 0.02	45.45 ± 1.90	2.07 ± 0.18	9.04 ± 1.69
Keto control (KC)	0.71 ± 0.22	40.68 ± 2.19*	2.28 ± 0.14*	14.45 ± 4.12**
Keto carcinogen control (KCC)	0.85 ± 0.19	41.36 ± 2.94*	2.26 ± 0.05*	10.58 ± 3.08

The experiment data represented as mean ± SD. Statistical significance was calculated using one- way ANOVA followed by Bonferroni multiple comparison test. All the groups were compared to CC group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

3.8. Determination of oxidative stress parameters

In TBARs estimation, we found increased TBARs formation in CC (0.87 ± 0.02) in comparison to NC (Table 3). In PC estimation KCC (41.36 ± 2.94) showed no variation with NC (41.02 ± 2.91) and PC levels were higher in CC (45.45 ± 1.90) in comparison to other groups. There was a noticeable decrease level of SOD in the CC (2.07 ± 0.18) group and an increase level in KC (2.28 ± 0.14) and KCC (2.26 ± 0.05). Moreover, we observed a significant decrease level of GSH in CC (9.04 ± 1.69) with concomitant increase in NC (14.96 ± 1.49) and KC (14.45 ± 4.12) (Table 3).

4. Discussion

KD inhibits the tumour cell to utilized ketone bodies by imitating a starving metabolic state, promoting a biological shift toward fat metabolism (24). However, numerous reports about inconsistent efficacies of this treatment broke the illusion of the possibility to cure cancer with KD therapy [2].Therefor, in our study, we explored the effect of KD/low glucose environment on cellular proliferation using *in-vitro* and *in-vivo* models. As per *in-vitro* cell viability assay, BHB with LG and NG efficiently reduce the proliferation of ER- MDA-MB-231 cells as compared to ER + MCF-7 cells. The ER- MDA-MB-231 cells is an aggressive cell and require more energy for the proliferation, in comparison with ER + MCF-7 cells. The more efficacy of low glucose environment on ER- cells (MDA-MB-231) cells could be attributed to the fact that ER- cells (MDA-MB-231) need quick glucose being aggressive in nature and therefore deprivation imparted an immediate effect on the proliferation. On the contrary, since MCF-7 cells are less aggressive so the effect of glucose deprivation was not that much significant. However, authors are of the opinion that effect of long-term glucose deprivation on both cell types would be equal and is a subject for further studies. Our finding is in alignment with previous reports, revealing that short term glucose deprivation (24 hr) in various breast cancer cell lines induces cell death, which was higher in MDA-MB-231 than MCF-7 cells [30].

Mitochondria play crucial role in the apoptosis and goes through several morphological and physiological changes during the process of programmed death [31]. Loss of mitochondria membrane potential is an early and irreversible stage of cell death [32]. JC-1 staining demonstrated a significant shift from red to green fluorescence, indicating disrupted MMP in BHB supplemented group (HGKD, LGKD, and NGKD). This disruption showed less pronounced effect in HG and LG experimental condition. Further cell death was confirmed through AO/EtBr dual

staining which used to differentiate between live, apoptotic and necrotic cells [33]. AO/EtBr staining revealed notable absence of apoptosis in HG, whereas in LG conditions exhibited an increased number of early apoptotic cells. BHB treatment in LG conditions demonstrated both late and early apoptosis. Hyperglycaemia play crucial role in breast cancer metastasis, proliferation, and chemotherapy resistance cancer [34]. Scratch assay data demonstrated that HG treated cells having increased cell growth, indicating enhanced cell motility and proliferation compared to NGKD and LGKD groups.

Based on *in-vitro* findings, we expanded our work to validate the efficacy of KD against DMBA –induced mammary gland carcinoma. DMBA is a chemical carcinogen that is capable of causing malignant lesions that resemble human ductal carcinoma *in situ* [35].

ECG and HRV parameters are associated with advanced cancer and serve as marker of autonomic dysfunctions and same was evident in our study after DMBA administration [36]. Increase RR interval and R amplitude signify the autonomic dysfunction in CC group. Increases heart rate, and decrease QRS interval in CC group also support the autonomic dysfunction during ECG and HRV analysis. KD treated animals significantly recovered the autonomic dysfunction.

KD function through a dual mechanism, involving the reduction of muscle mass and appetite control. The decrease in body weight is accomplished by suppressing appetite-stimulating hormones, such as insulin. Additionally, the formation of ketone bodies initiates a metabolic shift from glucose to fat burning for energy production [37]. We also observed substantial weight variations in KD treated animals, in contrast to the NC and CC groups, highlight the potential efficacy of KD in influencing body weight.

Cellular proliferation is the well-established hallmark of cancer progression[38]. Carmine staining is considered as a reliable technique used to visualized alveolar structures, ductal elongation and branching. KD treated animals showed decreased number of LOB,TEB and AB; indicating anti-proliferative potential, in comparison to CC group. Furthermore, SEM analysis was performed to explore the loss in collagen covering due to loss of collagen fibres and capillary networks. CC group showes formation of tumor micro-vessels (tmv) and loss of ducts formation which restored with KD. In histological analysis CC showed loosely bound AT, poorly organized MD epithelial cell whereas KD treated groups showed deformation of mammary gland structure but well-organized adipose tissue with KD.The overall, *in-vivo* morphological finding in accordance with our previously reported findings [39].

Over the past decade, extensive research has indicated that ROS play a critical role in cancer cell proliferation and survival by causing damage to lipids and proteins [40]. Previous study reported that generation of ROS and dysregulation of antioxidants in DMBA induced carcinogenesis. In cancer SOD and GSH works to fight against the free radicals and showed the decreased levels in CC group. Earlier it has been reported that keton bodies reduce the ROS production and increases the antioxidant level, which was evident in our study after KD administration [41,42].

Previous evidence indicates potential benefits of KD for breast cancer treatment with direct correlation with hormone-independent/ER- and hormone-dependent/ER + mammary gland carcinoma [43] Our findings indicates that KD demonstrates more pronounced effect against hormone-independent/ER- carcinoma compared to hormone-dependent/ER + carcinoma and the mechanism beneath the same needs to explored further.

5. Conclusion

The findings from our study points towards a promising role of KD in solid tumour. KD demonstrated significant potential in inhibiting breast cancer progression by reducing the viability of MDA-MB-231 (ER-) cells and induce cell death when treated with BHB in glucose deprived condition. Moreover, KD also diminished the carcinogenic effects as evident through enhanced antioxidant activity, restored morphological architecture in DMBA treated animals. In view of the above,authors would like to conclude that KD can be utilised as adjuvant therapy in the management of mammary gland carcinoma, possibly by providing unfavourable microenvironment for highly proliferating tumour cells due to deficiency of quickly available glucose. However, further research is needed to explore molecular mechanisms through transcriptomic and metabolomic analyses.

6. Ethical approval and consent to participate

The research protocol was carried out in accordance with CCSEA (Committee for Control and Supervision of Experiments on Animals), Government of India. Prior to experiment the animal study was approved by the Institutional Animal Ethical Committee (IAEC) with approval no. (UIP/IAEC/June-2023/06)).

7. Consent for publication

Not applicable.

CRediT authorship contribution statement

Sneha Yadav: Investigation, Formal analysis, Data curation. **Neha:** Data curation, Investigation. **Mohammad Arman:** Investigation, Formal analysis, Data curation. **Anurag Kumar:** Software, Methodology, Investigation. **Archana Bharti Sonkar:** Methodology, Project administration. **Neeraj Kumar Shrivastava:** Validation, Methodology. **Jyoti Singh:** Visualization, Validation, Methodology. **Mohd Nazam Ansari:** Resources, Conceptualization. **Sara A. Aldossary:** Software, Resources, Conceptualization. **Abdulaziz S Saeedan:** Resources, Conceptualization. **Gaurav Kaithwas:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

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

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2025.100480.

Data availability

Data will be made available on request.

The data that support the findings of this study will be made available upon reasonable request.

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