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Glucose deprivation using 2-deoxyglucose and acarbose induce metabolic oxidative stress and apoptosis in female mice bearing breast cancer

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ABSTRACT

A characteristic of cancer cells is increased glucose uptake and glycolysis for energy production and hydroperoxide detoxification due to mitochondrial dysfunction. Thus, inhibition of glucose uptake and glycolysis represent smart novel therapy. We used 2-deoxyglucose (2DG) as a glycolysis inhibitor and acarbose (ACA), a specific alpha-glucosidase inhibitor, to decrease glucose uptake. Mice bearing mammary adenocarcinoma tumors were treated by 2DG and/or ACA. Relative tumor volume, tumor growth inhibition rate, relative body weight, glucose concentration, hexokinase-1 protein level by ELISA, pyruvate, and ATP (glycolysis products), reactive oxygen species (ROS), total glutathione T-GSH, apoptosis, and histopathology were measured in treated and untreated groups. Our results showed that combination therapy inhibited tumor volume and increased tumor growth inhibition rate, body weight reduction, decreasing glucose level, HK-1 level, and inhibition of glycolysis products. In addition, combination therapy induced oxidative stress, increase ROS, and decrease T-GSH. Furthermore, immunohistochemistry examination showed the broader area of apoptosis in breast cancer treated by combination agents. In conclusion, our result revealed that the novel combination inhibits glycolysis and glucose uptake and induced oxidative stress and apoptosis.

1. Introduction

Cancer cells require glucose to generate energy, production macromolecule biosynthesis precursors to support cell growth and proliferation, and manage the oxidative impacts of their metabolic metabolism by decreasing reactive oxygen species (ROS) [1]. As a result of hypoxia and mitochondrial malfunction, cancer cells mainly rely on aerobic glycolysis. This is known as the Warburg effect, and it occurs when cancer cells require a large amount of glucose to support metabolic demands. Cancer cells generate energy primarily by increasing the rate of glycolysis by 200 times that of normal cells of origin; this increase is followed by lactate fermentation in the cell's cytoplasm, regardless of the plentiful oxygen supply [2]. Glucose uptake in normal cells is lower than in malignant cells [3]. This difference can be used as a target for cancer therapy by glucose deprivation (G.D.).

Cell death scenarios related with glucose metabolic limitation include ATP depletion and oxidative stress, both of which are intimately tight to mitochondrial function. The death cascade is triggered by G.D. because it depletes ATP. Due to the fact that ATP is necessary for numerous processes in apoptosis, severe ATP depletion usually results in necrosis. G.D. also causes oxidative stress, and changes in a cell's redox status trigger stress-activated or other signal transduction pathways, ultimately leading to cell death [4]. In transformed cells, G.D. primarily causes cytotoxicity via oxidative stress mechanisms and becomes cells more susceptible to starved death than normal cells [5].

Acarbose (ACA) is a glucoregulatory agent; it induced glucose deprivation by competitive alpha-amylase and alpha-glucosidase inhibitors. It inhibits complex dietary carbohydrates digestion in the small intestine, reducing the rapid rise in blood glucose after a

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meal (postprandial) [6]. It has role as an FDA-approved therapy for hyperglycemia and type II diabetes, in addition, ACA has been researched as a candidate calorie restriction mimetic (CRM) in longevity/healthy aging studies [7]. CRMs are agents that mimic the effects of calorie restriction (i.e., lifespan and a delay in the onset of age-related disease) without restricting calorie consumption. ACA extends the lifespan of mice, which supports this theory [8]. Reduced colorectal cancer is related with dose-dependent incidence of Type II diabetes [9]. Recently, it has been reported that CRMs enhanced responses to chemotherapy [10] and combination of chemotherapy and immune checkpoint inhibitors for treatment nonmetastatic subcutaneous murine tumor models [11].

Another agent, 2-Deoxy-D-Glucose that mimics glucose deprivation because it competes with glucose. 2-DG is synthetic glucose, an analog in which the 2-hydroxyl group is replaced by hydrogen. The non-metabolizable glucose analog 2-DG blocks the first step in glycolysis. It is phosphorylated by hexokinase to produce 2-DG-6P, which cannot be metabolized. It accumulates in cancer cells because the reverse reaction is also blocked [12]. Oxidative stress enhance by 2DG, which induced cell injury by disorderly thiol metabolism [13]. 2DG is depleted glycolysis intermediates, antioxidants and cellular ATP which are essential for survival and proliferation cell, result in inhibition of growth and cell death [14]. It has displayed a cytotoxic agent in tumor cells, particularly those with mitochondrial respiratory abnormalities or those caused by mitochondria-targeted medicines [15]. Metabolism of cancer cell targeting could be improved cancer therapy [16]. 2-DG is one of the most well-known cancer cell anti-metabolites [17]. It has been reported as improving effect of chemotherapies and radiation against different cancers [18]. 2-DG was found to be a breast cancer sensitizer to chemotherapy and irradiation, therefore enhancing the treatment and improving cytotoxicity by oxidative stress [19]. This work aimed to induce glucose deprivation by using ACA with 2DG that induces oxidative stress and apoptosis in breast cancer.

2. Materials and methods

2.1. Experimental animals

All animal experiments were performed according to the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) guidelines in the animal house facility. The Scientific Committee of ICCMGR, Mustansiriyah University, and College of Veterinary Medicine, Baghdad University approved all experimental studies.

2.2. Animal tumor model

In vivo experiments are carried out using the mouse mammary adenocarcinoma tumor model (AN3) [20]. The AN3 tumor line was obtained from an albino Swiss mouse's spontaneously developing mammary tumor. Continuous transplanting in inbred syngeneic mice keeps the AN3 tumor line alive.

2.3. Experimental design

About one million AN3 cells were injected into the right flank of each female Swiss Albino mouse (6–8-week) (ICCMGR, Animal House Unit, Mustansiriyah university, Baghdad, Iraq). When the tumor nodules reached 0.5–1 cm in diameter, mice were randomly distributed into four groups (each group contain ten): The first group were injected with i/p of 0.9% normal saline and received a normal regular diet (control); the second group were received acarbose 1,000 ppm with diets daily [8]; the third group treated with intraperitoneal injected of 2-deoxyglucose 500 mg/kg five days each week [21]. Finally, the fourth group were treated with acarbose 1,000 ppm daily plus 2-deoxyglucose 500 mg/kg five days each week. After eighteen days, using a lethal dose of chloroform for anesthetization the mice and then scarification.

2.4. Anti-tumor efficacy assessment

Every third day, the tumors were measured using a caliper. The tumor volume was calculated according to (product of length × width × width/2) [22]. The volume of the tumor was standardized in each volume at time zero, at which time therapy was began, in order to determine tumor growth. Relative tumor volume was measured every third day during the evaluation period by the following formula: RTV= (Tumor volume on measured day)/ (Tumor volume on day 0) x 100.

Tumor growth inhibition (TGI) was also calculated every third day during the evaluation period by the following formula GI% = Relative tumor volume of the untreated group − Relative tumor volume of treated group /Relative tumor volume of untreated group x 100 [23].

A tumor growth inhibition >50% was considered meaningful.

2.5. Relative body weight

Every third day the body weight of each mouse was weighted via a sensitive balance. The following equation determined the relative body weight: RBW = (Body weight on measured day)/ (Body weight on day 0) × 100.

2.6. Glucose levels

Glucometers and test strips (Contour, Japan) were used to measure blood glucose levels. The blood samples were obtained from the tail vein by tail amputation.

2.7. Hexokinase −1 enzyme quantification

Tumor tissue was carefully weighted one microgram and subjected to liquid nitrogen then homogenized in one milliliter PBS using a mortar and pestle on ice. Next, we centrifuged the sample at 5000×g for 5 min to obtain the supernatant. Hexokinase enzyme was quantified according to the manufacturer's recommendations (Elabscience, USA), Catalog No: E-EL-M0673. The plates were precoated with an antibody specific to mouse HK-1. All standards, reagents, and samples are performed at room temperature then added to each well for 100 μl each and were incubated at 37 °C for 90 min. After the incubation, the solution was decanted from each well. We added for each well 100 μl of biotinylated detection Ab working solution, then were decanted and washed each well by 350 μl of wash buffer 3 time. The wells were then added with conjugate, 100 μl for each well, and then were incubated at 37 °C for 1 hour and proceeded with washing the plates five times. Then, the plates were added with substrate solution of 90 μl and were incubated at 37 °C for 15 minutes. Stop solution was added 50 μl to each well and read the plates at 450 nm.

2.8. Glycolysis products measurement

Pyruvate and Intracellular ATP glycolysis products were measured in tumor tissue using colorimetric assay kits (Elabscience, USA), Catalog No of pyruvate Kit: E-BC-K130-S and ATP Kit: E-BC-K157-S. We take fresh tumor tissue for pyruvate measurement and washed with PBS at 2–8 °C. The tissue were put on the mortar and added liquid nitrogen to grind fully. Then, added homogenized medium (PBS) at ratio (mL): the weight of the cancer tissue (g) = 9:1. The tissue homogenates were centrifuged for

10 min at 10000 g at 4 °C, then take the supernatant for detection as stated in the manufacturer's recommendations. For ATP level measurement, Fresh cancer tissues were weighted, cut into pieces then added boiled double distilled water at ratio (mL): the weight of the cancer tissue (g) = 9:1. The tissue homogenates were incubated for 10 min, then oscillated for 1 min to mix fully, the homogenates were centrifuged for 10 min at 10000 g, then take the supernatant for detection as stated in the manufacturer's recommendations.

2.9. Reactive oxygen species detection

To measure ROS formation in tumor tissue, DCFH-DA, a fluorescent probe, was used to monitor intracellular ROS levels. Fresh breast cancer tissue (live cells) was taken into the pre-cooled buffer solution immediately and cleaned the blood and other contaminants. Then removed the massive composition, fiber, fat, and blood vessels (except for specialized cells) and take 500 mg from cancer tissue. This tissue cut into about 1 mm3 pieces with the ophthalmic scissors, then put these pieces to pre-cooled buffer solution to remove the cell debris, followed by add an appropriate amount of enzyme digestion (trypsin), then incubated in 37 °C water bath for 20–30 min and gently oscillated the mixture intermittently. Trypsin activity was stopped the digestion by add medium that contain fetal bovine serum. The mixture filtered to remove the tissue massive component with nylon mesh (300 mesh) and collected the cells, then centrifuged at 500 g for 10 min and discarded the supernatant, then washed with buffer solution for 1–2 times. Re-suspend to prepare the single cell suspension solution. The cell amount should be no less than 10^6. Fluorometric method using for determination ROS level by utilizing ROS assay kit (Elabscience, USA), Catalog No: E-BC-K138-F as stated in manufacturer's instruction. Fluorescence Microplate reader was analyzed at an excitation wavelength of 502 nm and emission wavelength of 525 nm.

2.10. Total glutathione assay

A Fresh sample was excised and weighted, then prepared by adding protein precipitator with buffer solution. Samples were homogenized mechanically on an ice bath. Followed by centrifugation for 10 min at 10000 rpm, then the supernatant was collected for detection. DTNB reacted with GSH to produce TNB yellow color and GSSG. Total glutathione was determined according to the amount of yellow TNB formation kit (Elabscience, USA), Catalog No: E-BC-K097-M.

2.11. Immunohistochemistry cleaved caspase −3

To study Cleaved caspase −3 expression in breast cancer. Charged slides with 3-μm − thick section 10% neutral buffered formalin-fixed, paraffin-embedded were first treated with 3% H_2O_2 for 10 min, then transferred into citrate buffer pH 6 for 40 minutes at 98 °C, followed by incubated with rabbit polyclonal anti-Cleaved caspase-3 antibody (1:50 dilution; Elabscience, USA) where indicated, for 30 minutes at room temperature. The section probed with

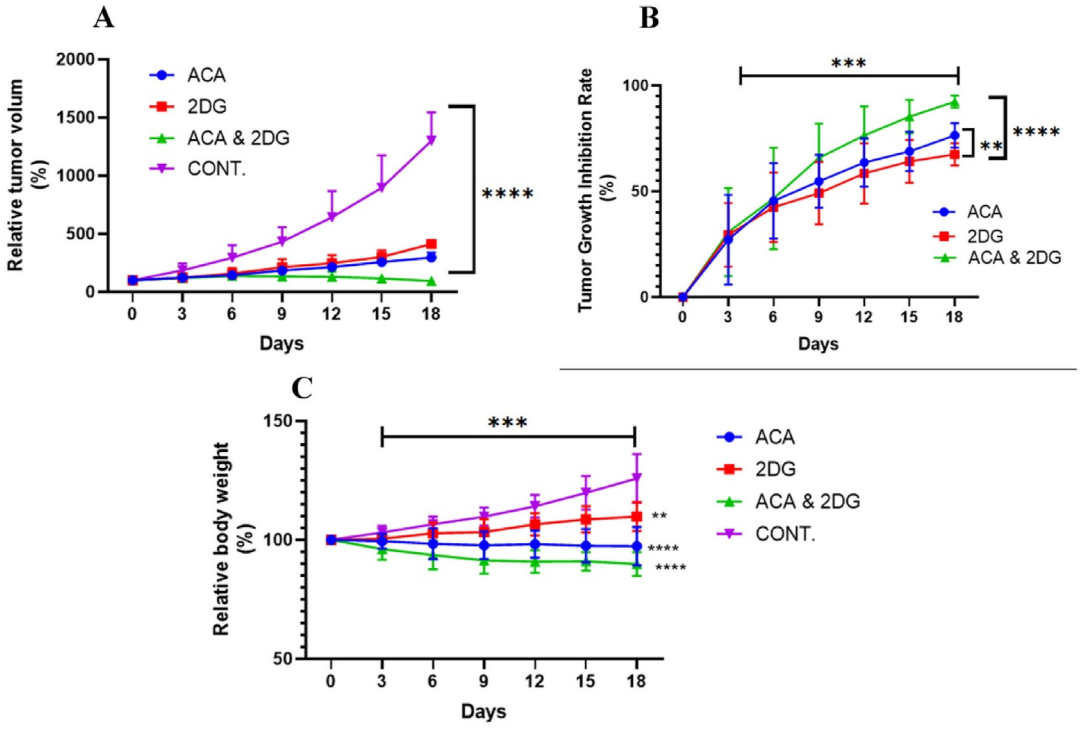

Fig. 1. ACA synergistically enhances the antitumor effect of 2DG against mouse breast cancer model (AN3). The tumor volume was measured every third day for 18-days. There were four groups. First is the untreated control group, which injected i/p with 0.9% normal saline and received a normal diet. The second group received ACA 1,000 ppm with diets daily. The third group was treated with a 500 mg/kg 2DG intraperitoneal injection for five days each week. The fourth group received combined treatment of ACA 1,000 ppm daily with diets plus 2DG 500 mg/kg five days each week. (**A**) Relative tumor volume. All treatment groups induced a significant reduction in tumor volume compared to the untreated group. Combination therapy (ACA–2DG) significantly reduced tumor size compared to 2DG and ACA mono-treatment groups. (**B**), Tumor growth inhibition percentage. The ACA combination with 2DG enhanced the highest tumor growth inhibition rate (92.39%), followed by the ACA group (76.49%). The 2DG group had the lowest growth inhibition rate (67.42%). In the control group, the tumors persist in growing during the experiment period. (**C**), Relative body weight. Bodyweight measurements were every three days for 18-day. The 2DG-ACA combined treatment induced Bodyweight reduction. ACA resulted in a marked decrease in body weight (97.40%). 2DG treated group significantly lowered body weight (109.8%) compared to the control untreated group (125.8%). 2DG-ACA induces bodyweight reduction (89.87%). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ versus CONT.

anti-Cleaved caspase-3 antibody was incubated with a labeled streptavidin-biotin reagent, following the manufacturer's protocol. Immunoreactive products were visualized with the DAB reaction. Sections were counterstained with hematoxylin for 2 minutes. The optical density (O.D.) of cleaved caspase-3 was determined using NIH Image J (Fiji, Version 1.53). The following formula was used to determine the O.D. values: OD = log (maximum intensity/mean intensity), with maximum intensity equal to 255 [24].

2.12. Statistical analyses

All data analyses were performed with Graph Pad Prism version 8.01 (GraphPad software. C.A., USA) and Excel version 10. Data were analyzed using one-way ANOVA analysis, which was used to compare between groups. All data were shown as mean ± S.D. The value of significant was set at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

3. Results

3.1. ACA enhance the antitumor effect of 2DG

To study the effect of ACA treatment on tumor growth and possible synergism anti-tumor efficacy with 2DG against breast cancer, AN3 tumor cells were injected subcutaneously in the flank of female mice to permit tumor measurement. When the tumor volume reached 0.5–1 cm in diameter, the female mice were randomly allocated into four groups (ten each group), as designated in the methods section. As exposed in Fig. 1-A, tumor volumes were plotted over 18 days. All treatment groups induced a significant reduction in tumor volume as compared to the untreated group. In addition, the combination group of ACA–2DG significantly reduced tumor size compared to ACA and 2DG treatment groups.

Moreover, the ACA- 2DG combination therapy improved the highest tumor growth inhibition rate (92.66%), followed by the ACA group (79.45%). On the other hand, the 2DG group had the lowest growth inhibition rate, 70.88%, as shown in Fig. 1- B. In the control group, the tumors persistent to grow during the periods of the experiment. In addition, the result showed significant increase in growth inhibition rate at all periods treatment in treated groups compared to zero time.

3.2. 2DG-ACA induced body weight reduction

ACA, alone or in combination with 2DG, decreased body weight in a mouse bearing mammary adenocarcinoma. ACA induces starvation because it delays the digestion of complex dietary carbohydrates and decreases glucose absorption, thus decreasing body weight. As shown in Fig. 1-C that ACA resulted in a marked decrease in body weight (97.40%). 2DG showed lower body weight (109.8%) significant compared to the untreated group (125.8%). Thus, 2DG in combination with ACA induces body weight reduction (89.87%). Furthermore, we observed that significant increase in body weight in untreated group while decrease in this parameter in ACA and

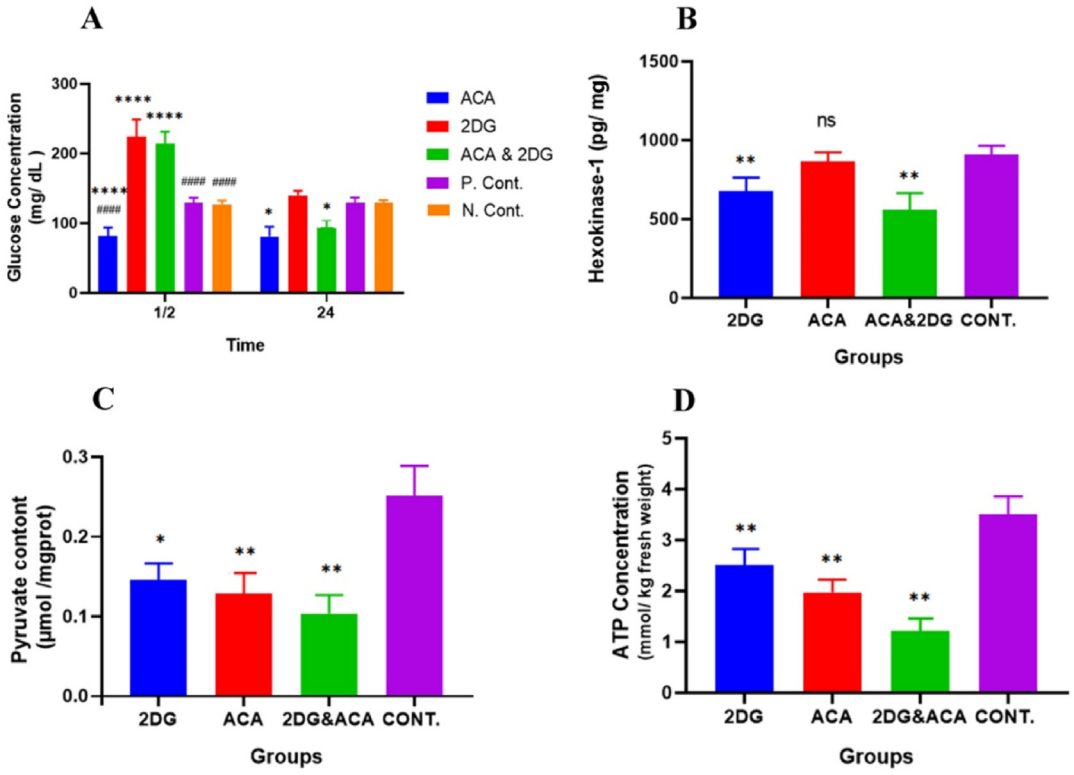

Fig. 2. Glycolysis pathway analysis. **A**, Glucose level measurements; we measured glucose concentration in blood at 1/2 h and 24 h after injection of the 2DG. We identified a significant increase in the glucose concentration after 30 minutes of 2DG injection in 2DG and ACA-2DG groups as compared with positive control)P.C((bearing breast cancer) and negative control N.C (healthy mice) groups due to 2DG presence in the blood, which the glucometer can identify as glucose. After 24 hours of 2DG injection, there was a significant reduction in glucose concentration in ACA and ACA-2DG groups compared with control groups. **B**, we quantified HK-1 protein expression in one mg of breast cancer tissue in mice confirmed by The ELISA assay. We observed a significant reduction in the HK-1 protein expressed in 2DG and ACA-2DG groups compared with the untreated groups. At the same time, there is no significant reduction in HK-1 protein expression in breast cancer tissue in the ACA group compared with the control group. **C, D,** Measurement of pyruvate and ATP concentration in tumor tissue showed a significant reduction in ACA treated group and 2DG treated group compared to untreated groups. At the same time, the ACA-2DG combination therapy had the highest inhibitory effect on the pyruvate and ATP levels in tumor tissue. *P < 0.05, **P < 0.01 ***P < 0.001 and ****P < 0.0001 versus Con; ####P < 0.0001 versus 2DG.

ACA-2DG treatment periods compared with zero time.

3.3. ACA with 2DG synergistically induces glucose deprivation

We next sought to study the effect of ACA, 2DG, and their combination on glucose level. We measured glucose concentration in blood at 1/2 h and 24 h after injection of 2DG. We identified a significant rise in the glucose concentration after 2DG injection in 2DG and ACA- 2DG groups compared with control groups. However, after 24 hours of 2DG injection, the result showed that significant reduction in glucose concentration in ACA and ACA-2DG groups compared with control groups (Fig. 2-A).

3.4. 2-DG-ACA combination therapy reduced Hexokinase-1 enzyme level

The present experiment identifies and quantifies HK-1 protein expression in one mg of breast cancer tissue in mice confirmed by The ELISA assay. We observed a significant reduction in the HK-1 enzyme in 2DG and ACA-2DG groups compared to the untreated group (Fig. 2-B). However, the results revealed that the HK-1 enzyme concentration was not significantly reduced in ACA treated group.

3.5. Glycolysis products inhibited by ACA-2DG combination

To study the effect of ACA, 2DG, and ACA- 2DG combination therapy on pyruvate and ATP glycolysis products. First, we determined pyruvate concentration in cancer tissue. We showed that ACA and 2DG significantly reduced pyruvate compared to the untreated group (Fig. 2-C). Notably, ACA- 2DG combination therapy had a significant inhibitory effect on the pyruvate level in cancer tissue. In addition, we showed a significant suppression in the ATP level in all the (2DG, ACA, and 2DG- ACA) treated groups compared with the control group (Fig. 2-D).

3.6. Oxidative stress-induced by ACA-2DG combination therapy

To explore whether ACA-2DG induced oxidative stress in breast cancer tissue. We measured total GSH and ROS in cancer tissue. We showed a significant elevation in the ROS level in ACA-treated groups compared to the untreated group (Fig. 3-A). Compared with ACA monotherapy, the ROS level was further elevated by combining ACA and 2DG. As shown in Fig. 3-B, the total GSH level significantly decreased in the ACA group compared with the control group. When the ACA and 2DG were applied together, the total GSH level was further decreased.

3.7. ACA-2DG combination therapy increases apoptosis in cancer tissue

Apoptosis analysis by immunohistochemistry for Cleaved caspase −3 showed a mild increase in mammary adenocarcinoma following ACA and 2DG monotherapy (Fig. 4). While the ACA-2DG combination group efficiently increased cas-3 compared with the untreated group. The investigation showed that ACA-2DG combined therapy efficiently induces apoptosis on breast cancer tissue. Notably, ACA-2DG increased apoptotic cells in the histological section compared with untreated groups.

4. Discussion

Metabolic pathways of cancer cell-associated energy have been altered compared with normal cells. Tumor cells rely on aerobic glycolysis, use high glucose quantities, and produce high pyruvate and lactate levels. Malignant cells have been defecting in the respiratory mechanism, which leads to the generation of excess ROS by mitochondria. This phenomenon, called the Warburg effect, was described 70 years ago [25]. Cancer cells show increased glucose metabolism, which is necessary for their survival. Glucose metabolism produces pyruvate from aerobic glycolysis and NADPH from the pentose cycle, which acts as hydroperoxide detoxification against the lethal effects of ROS production during respiration [25]. The dependence of cancer cells on glucose metabolism for ROS detoxification is a smart target for therapeutic agents to kill cancer cells without affecting normal tissues. It is hypothesized that glucose deprivation induces a switch from aerobic glycolysis to oxidative phosphorylation, and ROS production from the mitochondrial respiratory chain promotes oxidative stress and apoptosis.

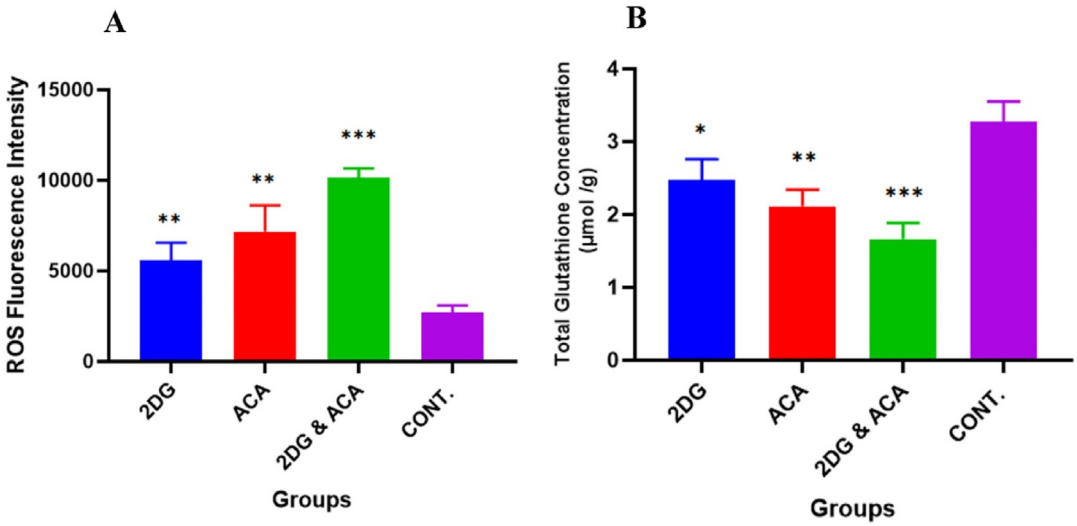

Fig. 3. Measuring oxidative stress in the treatment groups. We measured total GSH and ROS in breast cancer tissue. A, Indicates higher significant elevation in the ROS level in the ACA-2DG treated group compared to other monotherapy groups and untreated groups. B, Total GSH level is significantly decreased in the ACA-2DG treated group compared with monotherapy and control groups. ***$P < 0.01$ **$P < 0.01$, and * $P < 0.05$ versus CONT.

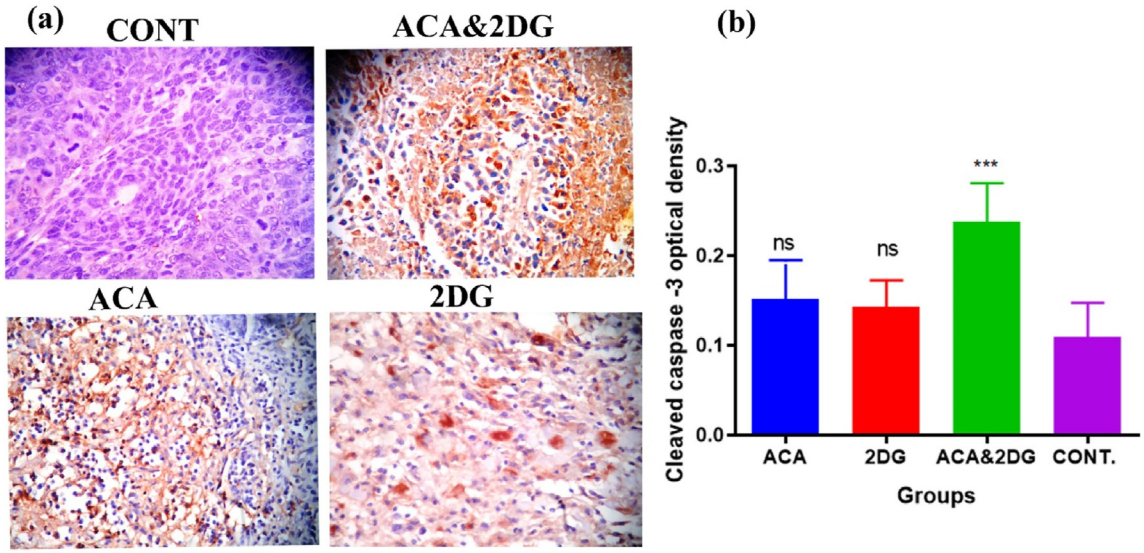

Fig. 4. Quantitative apoptosis analysis by immunohistochemistry for Cleaved caspase −3 showed a mild increase in cleaved cas-3 expression in mammary adenocarcinoma following ACA and 2DG monotherapy. While the ACA-2DG combination therapy efficiently induces cas-3 expression as compared with mono-treatment groups and the untreated group. ***$P < 0.05$ versus CONT. DAB stain.

ACA was used to induce glucose deprivation [26]. In addition, ACA, 2DG was utilized to mimic induced glucose deprivation because they are analogs of glucose that interferes with the glucose metabolism pathway [12]. The result of current work support evidence that combining ACA with glycolysis inhibitor (2DG) could synergize in vivo to induce cancer cell death and regression in tumor volume in mice model.

This work aimed to induce glucose deprivation by using ACA with 2DG that induces oxidative stress and apoptosis in breast cancer. The result of the current study indicated that ACA induces glucose deprivation by decreasing glucose concentration in groups treated with ACA compared with other groups. The combination of ACA and 2DG showed a decreased glucose level compared with control groups. Moreover, ACA, 2DG, and combination revealed decreased relative body weight compared with untreated groups. Our results are consistent with previous reports that demonstrated that 2DG alone and ACA alone reduced body weight [27,28].

To confirm the anti-cancer effect of ACA-2DG, we measured relative tumor volume and tumor growth inhibition. This study showed that the antitumor effect of ACA-2DG was better than that of alone ACA and the alone 2DG. The combination group showed more reduction than in free ACA but not significant. In previous work by our groups, we found an antitumor effect of 2DG through glycolysis inhibition and downregulation of GAPDH [29]. 2DG has been shown to reduce blood vessels number in tumor tissue and is associated with downregulation of c-Myc, PDK1, and HIF-1α [21]. Other anti-cancer mechanisms of 2DG include cellular energy depletion, increased oxidative stress, interference with N-linked glycosylation, induction of autophagy, inhibition of antioxidation, and apoptosis [14,30]. ACA showed a decrease in relative tumor and had antitumor efficacy. In addition, it induces glucose deprivation which may be one of the antitumor mechanisms. Glucose deprivation has killed cancer cells in three different ways: necrosis, caspase-8-dependent apoptosis, and mitochondrial apoptosis [31]. ACA inhibited the growth of renal tumors and delayed outgrowth in mice [32].

To understand the proposed mechanism of the 2DG, ACA, and 2DG-ACA combination, we quantified HK-1 by ELIZA assy. HK-1 is an important key enzyme in irreversible reactions in the glycolysis pathway. Also, it is considered an important enzyme in cell energy metabolism. It catalyzes the first step by catalyzing the irreversible conversion of glucose to glucose-6- phosphate [33]. Previous studies reported that glycolysis-related genes, such as HKI and HKII, overexpression in many cancer [34,35]. We have shown decreased Hexokinase-1 protein levels in groups treated with 2DG compared with the control group. 2-DG is phosphorylated by hexokinase (H.K.) to 2-DG-P, unlike G-6-P in cells because 2-DG-P cannot be metabolized by phosphohexose isomerase, leading to intracellular accumulation of 2-DG-P, inhibiting glucose-6-phosphate isomerase and the function of hexokinase [12].

In correlation with the mechanism of the combination agent, we measured the pyruvate and ATP level in treated and untreated groups. The results showed that pyruvate and ATP levels were decreased in the combination ACA-2DG group compared with the control group. 2DG alone and ACA alone also decreased pyruvate and ATP levels to lesser degrees. The concentration of pyruvate depends on glucose level; therefore, ACA decreases glucose level, leading to decreased pyruvate concentration [36]. Our work about 2DG is found to decrease pyruvate levels in breast cancer cells treated with 2DG [29]. Thus, the ATP levels of treated groups become deficient relative to that of the untreated group [37].

ROS assay finding of the current study found that combination therapy induces oxidative stress by increasing ROS levels in treated groups compared with the control group. Combination therapy induced glucose deprivation (G.D.). Therefore, G.D. is activating a positive feedback loop, including ROS generation by mitochondria and NADPH oxidase [5,38]. In addition, our results confirm that 2DG-ACA induce oxidative stress result in total GSH depletion in treated groups.

Glucose metabolism products involved pyruvate from glycolysis and NADPH from the pentose cycle, and this product participates against oxidative stress-mediated by hydroperoxide. Pyruvate acts as an antioxidant agent during reacts directly with hydrogen peroxide, leading to pyruvate decarboxylation to acetic acid and conversion of H2O2 to H2O [39]. NADPH also contribute detoxification of ROOH and H2O2 by glutathione peroxidases through provides electrons for the reduction of glutathione disulfide; therefore, increased uptake of glucose for metabolism by cancer cell is necessary to produce more glucose metabolism, which acts as a compensatory mechanism to protect the cell from intracellular ROS which generates from mitochondria [40]. Our result indicates that

G.D. decreases glucose metabolism product result in depletion in T-GSH and elevation of ROS.

Using immunohistochemistry to detect cleaved Caspase-3 expression, we observed that the ACA-2DG combination was the best inducer for apoptosis compared with monotherapy. Caspases are found inactive form in the cytosol and activated by proteolysis and/or dimerization by other caspases. 'caspase-3 is considered Executioner' caspases activated during cleavage apical' caspases or 'initiator' called caspases-8 and -9 [41]. Caspase-9 is activated by dimerization after recruitment to the apoptosome, a complex of APAF1 proteins formed in response to the release of the mitochondrial protein cytochrome c into the cytosol. This caspase activation cascade is called the mitochondrial or intrinsic pathway of apoptosis [42]. Our result supports this mechanism due to glucose deprivation-induced apoptosis by displaying hallmarks of apoptosis such as cleavage of caspase and caspase substrates [31]. Another mechanism indicates that G.D. induced stress-triggered, promoting TRAIL-RD/DR2 and receptor-mediated apoptosis [43]. Moreover, G.D. was caused by a lack of proton provition due to inhibition of glycolysis and protons consumed by mitochondrial electron transfer chain to generate energy. Lysosomal is a compensator for this deficiency through proton efflux, which leads to an increase in lysosomal pH resulting in lysosomal alkalinization triggering necrosis or apoptosis depending on alkalinization extent [44].

In conclusion, the result of the current study is a novel therapeutic strategy that provides a hypothesis for 2DG-ACA combination therapy in a treatment modality that targets the cancer cell's metabolism. This study is the first to report that ACA-induced glucose deprivation synergized with 2DG, which produced a significant anti-cancer response and safety. This therapy could be used in clinical tumor therapy.

Author contributions statement

- conception of the work: Ahmed Majeed Al-Shammari, Khalisa K. Khudair- collection of data: Qayssar A. Obaid, Ahmed Majeed Al-Shammari- analysis of data: Qayssar A. Obaidm Ahmed Majeed Al-Shammari, Khalisa K. Khudair- writing of the manuscript: Qayssar A. Obaid, Ahmed Majeed Al-Shammari- final approval of the final draft: Ahmed Majeed Al-Shammari, Khalisa K. Khudair.

Disclosure

All authors have approved the final article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2022.01.007.

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