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Tumor Growth, Glycolytic and Cell Cycle Inhibitory Effects of Ketogenic Diet in Ehrlich Solid Tumor-Bearing Female Mice

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Abstract

Background: The ketogenic diet (KD) is a high-fat, low-carbohydrate diet, its role in cancer is based on the premise that cancer cells exhibit a heightened dependence on glucose metabolism, termed the Warburg effect. **Aim:** the study aimed to investigate the impact of the ketogenic diet on cancer cell growth and proliferation in female mice with Ehrlich Solid Tumor (EST). **Materials and Methods**: Swiss female mice were divided into three groups (n=15): (G1) Healthy control mice fed with a balanced diet, (G2) EST mice fed with a balanced diet and (G3) EST mice fed with KD. Through the experiment, some biological parameters and tumor growth were monitored. Biochemical measures and aerobic glycolysis enzyme activities were measured. Also, cell cycle analysis by flow cytometry and histopathological examination were done. **Results:** The treatment of EST-bearing mice with KD demonstrated an inhibitory effect on tumor growth rate. This was evident through the modulation of glycemia, glycolytic enzymes activities and induction of cell cycle arrest. **Conclusion:** The results suggest a potential therapeutic strategy of KD to target the metabolic and proliferative vulnerabilities of EST, providing a novel avenue for enhancing the efficacy of cancer treatment.

Keywords: Ehrlich solid tumor, ketogenic diet, tumor growth rate, aerobic glycolysis, cell cycle.

1. Introduction

Cancer is a complex condition marked by the body's abnormal cells multiplying and uncontrollably spreading. Any part of the body could be impacted, and through a process called metastasis, it could spread to different parts of the body [1]. Cancer cells, rapidly proliferate and divide without ever stopping to fix any harm[2]. Cancer cells preferentially use glucose for energy through glycolysis, even in the presence of oxygen, instead of oxidative phosphorylation, which occurs in the mitochondria a phenomenon known as the Warburg effect [3]. Glycolysis enables cancer cells to produce ATP quickly, which is necessary for their accelerated growth and multiplication, it can also provide intermediates that are employed in other metabolic processes and promote proliferation of cancer cells[4]. Multiple effects result from cancer cells' increased dependence on glycolysis. First, it may result in the buildup of metabolic waste products like lactate, which results in an acidic microenvironment that favors the survival and growth of cancer cells. Second, it leads to a reduction in the amount of glucose and other nutrients that are available to nearby cells, which affect their functionality and encourage the growth of tumors [5]. Despite changes in the types of human cancer animal models, Ehrlich ascites tumor (EAT) remains a crucial technique for unraveling pathogenesis and identifying novel therapeutic agents against cancer, Ehrlich ascites tumor-bearing mice-induced DNA damage, apoptotic P53 and proliferating cell nuclear antigen (PCNA) alterations. The Ehrlich ascites tumor model has been utilized to investigate the anti-tumor activity of a

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variety of natural and synthetic chemical compounds [6].

ketogenic diet (KD) is a very low carbohydrate eating plan that provides moderate amounts of dietary protein of the highest quality and high amounts of dietary fat [7]. The ketogenic diet forces the body to metabolize fat instead of the necessary carbohydrates it needs to operate correctly. The KD causes a switch from glycolysis to respiration in normal cells by lowering blood sugar levels and increasing ketone body utilization, whereas cancer cells cannot use ketone bodies because of altered oxidative phosphorylation. The tumor microenvironment including factors such as oxygenation levels, nutrient availability and immune cell infiltration can influence tumor growth and response to treatment. The ketogenic diet may interact with the tumor microenvironment in complex ways, impacting its efficacy [8].

This study investigates the biological and biochemical effects of the ketogenic diet on Ehrlich solid tumors as well as their effects on cell cycle and tumor growth in an EST-bearing female mice model.

2. Experimental

2.1. Ehrlich Cell Line

The parent line was obtained from female Swiss albino mice that had Ehrlich ascites cells intraperitoneally (IP). The Ehrlich ascites tumor cell line was maintained by intraperitoneal (IP) injection of 2.5 million cells into female Swiss albino mice, after dilution with sterile saline solution and quantification using a bright line hemocytometer. Ehrlich solid tumor was induced by inoculation of 0.2 mL of EAT cells (containing 2.5×10^6 cells/mouse) subcutaneously into the left thigh of the lower limb of each female mouse [9].

2.2. Preparation of Ketogenic Diet

A ketogenic diet was prepared at a ratio of 3:1 (fats: proteins and carbohydrates) with some modification in its fat composition [10]. In a nutritional composition, the ingredients per kilogram (g/kg) include 200g casein, 435g soybean oil, 145g flaxseed oil, 110g butter, 30g starch, 45g fiber, 30g mineral mixture and 10g vitamin mixture. The vitamin and mineral mixtures used were prepared according to the American Institute of Nutrition (AIN-93G) [11].

2.3. Experimental Design

All animal procedures were performed following the guidelines for the care and use of experimental animals established by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the protocol of the National Institute of Health, and ethical approval was obtained from the Faculty of Women for Arts, Science and Education, Ain Shams University (Study Code #ASU/W/Sci-6M/23-2-42). The experimental animals used throughout the present work were 45 female Swiss albino mice weighing about 24-28g obtained from the Research Institute of Ophthalmology, Giza. The animals were allowed to acclimate for 7 days before the experiment and fed on a balanced diet prepared according to AIN-93G [11], they were maintained under standard laboratory conditions (25°C, 70% relative humidity and a 12-hour light/dark cycle). After the adaptation period, the mice were divided into three groups (n=15); (G1) (Healthy control mice) healthy mice were inoculated with 0.2 ml of physiological sterile saline in the left thigh and fed on the balanced diet, (G2) (EST mice) mice were induced with EST in left thigh and fed on the balanced diet and (G3) (EST+ KD) mice were induced with EST and fed on KD starting after one week from induction.

Continuous weekly monitoring of blood glucose and β-hydroxybutyric acid was done to detect the states of ketosis; blood glucose level was determined weekly by glucometer fine test (Auto-Coding Premium). At the end of the experimental period (5 weeks), all mice were fasted for 12 hours with water ad libitum. The animals were sacrificed under sodium barbiturate anesthesia and blood samples were obtained via heart puncture, blood was left to coagulate at room temperature and serum was obtained after centrifugation at 4000 r.p.m for 20 min, for the assessment of carbohydrate metabolism and ketosis marker (Blood glucose, insulin and β-Hydroxybutyric acid). The tumor was removed using sterile scissors and forceps washed with 0.9% sterile sodium chloride solution then weighed. Tumor tissue samples were stored at -80°C for estimation of biochemical parameters and cell cycle analysis by flow cytometer.

2.4. Biological Measurements:

2.4.1. Body Weight Change (g):

Mice were weighed weekly to monitor body weight changes that were calculated by subtracting initial body weight from final body weight.

2.4.2. Feed Intake (g):

Feed intake was measured daily by subtracting the residual and refusal diet from the supplied diet [12].

Feed Intake (g) = diet supply - (residual diet + refusal diet)

2.4.3. Feed Efficiency Ratio (FER):

The feed efficiency ratio was calculated for each group of mice by the following equation [12].

FER= (body weight change (g))/(feed intake (g))

2.5. Tumor Volume (mm3) and Tumor Weight (g) Measurements

After 11 days from EST induction, tumor volume was measured twice a week using a Vernier caliper and determined by applying the following equation [13].

Tumor volume (mm3) = 1/2(length × width^2)

Where length is the tumor's higher diameter and width is the lower diameter.

Tumor weight was determined by weighing the solid tumor tissue during sample collection after animal sacrifice at the end of the study.

2.6. Inhibition Rate of Tumor Growth (IRT) Measurement

Growth inhibition (GI) is a medical term pertaining to cancer therapy and the specific reduction in the growth of tumors and oncogene cells by the specific therapeutic agent [14]. The relative tumor volume (RTV) was calculated using the following formula [15]:

RTV = (tumor volume on measured day)/ (tumor volume on day 0)

The tumor growth inhibition ratio (TGI %) was calculated according to [14] using the following formula:

TGI (%) = [1 − (RTV of the treated group)/ (RTV of the control group)] × 100.

2.7. Biochemical Measurements:

Blood glucose level was measured utilizing the colorimetric kit (Catalog No. GAGO-20), following the manufacturer`s protocol. Insulin and β-hydroxybutyric acid concentrations were determined employing enzyme-linked immunosorbent assay (ELISA) kits from My BioSource, specifically Cat. No. MBS2881867 for insulin and MBS722965 for β-hydroxybutyric acid, following the manufacturer's instructions. Additionally, glycolysis markers, namely pyruvate kinase M2 (PKM2), pyruvate dehydrogenase kinase 4 (PDK4) and pyruvate dehydrogenase (PDH) activities were assessed using ELISA kits from My BioSource with Cat. No. MBS260089, MBS1602984 and MBS730537, respectively, according to the manufacturer's recommended procedures.

2.8. Cell Cycle Analysis by Flow Cytometry

Tumor tissue samples, frozen in liquid nitrogen, were thawed in cold PBS/ EDTA [PBS (pH 7.4), containing 0.1% (w/v) EDTA] for 3 min. 3mg of tissues were dispersed using nylon cell strainers about 100 μm and 35 μm (BD Falcon #352360 and #352235). Tissue specimens were pressed through a 100 μm mesh using the plunger of a syringe (5 ml). The mesh was placed in a tissue culture dish containing PBS/EDTA, such that the mesh was in contact with the surface of the buffer, causing cells to be released into the liquid. The mesh was then rinsed with a cold buffer (4°C). The obtained cell suspension was then filtered using 35 μm cell strainers followed by an adjustment of the total volume with PBS/EDTA to 15 ml/sample. It is then kept on ice for 20 minutes. The cell pellet was suspended in a cold buffer (0.5 ml 4°C), and the supernatant was removed after the centrifugation step (310 g; 4°C; 6 min). 5 cc of cold 80% ethanol (-20°C) was added dropwise while being gently and continuously rotated. Before being stained, samples were first incubated for 30 minutes on ice and then for an additional night at -20°C [16]. Cell suspensions were taken out of -20°C storage and given around ten minutes to acclimatize to room temperature (RT) in preparation for cell cycle analysis. Pellets were resuspended in 5 ml PBS/EDTA at RT, centrifuged (310 g; RT; 6 min), and then incubated for 30 min at RT before being pelleted once more (310 g, RT; 6 min). The staining solution (PBS without EDTA, 30 mg/ml propidium iodide (PI) and 0.3 mg/ml DNase-free RNase) was added to the cell sediments in a volume (2 ml) that was sufficient to saturate all the DNA with PI. After being cooled to room temperature and incubated for 30 minutes in a dark 37°C water bath, the samples were processed using flow cytometry [17].

2.9. Histological Examination of Muscle and Tumor Tissue

Samples of muscle and tumor tissues were collected, preserved in a 10% formalin solution, sectioned at a thickness of 5 µm and stained with hematoxylin and eosin (H&E) for a later histological analysis [18]. An Olympus BX43 light microscope was used to analyze the tissue slides and an Olympus DP27 camera connected to the Cellsens dimensions program was used to take pictures of the slides.

2.10. Statistical Analysis

The statistical software for social science (SPSS) version 16.0 was used to analyze the data statistically. The presentative variable was described using the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the groups statistically; the significance was at the level of ($P \leq 0.05$) [19].

3. Results

3.1. Effects of the Ketogenic Diet on Tumor Volume, Weight and Inhibition Rate

Results in Table (1) showed that there was a significant decrease ($P \leq 0.05$) in tumor volume (0.44 ± 0.04 mm^3) and tumor weight (0.97 ± 0.06 g) in EST mice treated with the ketogenic diet as compared to the non-treated EST bearing mice. Accordingly, the ketogenic diet showed a marked inhibition of tumor growth which recorded 41.55 ± 5.80 as IRT.

3.2. Effects of Ketogenic Diet on Biological Measurements

The results indicated that induction of EST in female mice significantly ($P \leq 0.05$) reduced the animals' feed intake, FER, and body weight compared to the healthy control mice. However, treating the EST-bearing mice with a ketogenic diet markedly improved the feed intake and FER as compared to the control mice bearing EST, although body weight was not changed after treatment with the ketogenic diet for 4 weeks (table 2).

3.3. Ketogenic Diet Reduced Blood Glycemia and Enhanced Ketosis in EST-Bearing Mice

From the results in Figures 1 & 2, there was a significant reduction ($P \leq 0.05$) in blood glucose (80.01 ± 3.27 mg/dl), blood insulin (8.55± 0.90 µIU/ml) and β-hydroxybutyric acid (0.68 ± 0.14 µmol/l) levels in EST mice as compared to healthy control mice. Furthermore, treatment of EST-bearing mice with a ketogenic diet further reduced the blood glycemia and insulin levels significantly compared to the EST control mice. Alongside it significantly enhanced the ketosis status by increasing the blood level of β-hydroxybutyric acid (4.77 ± 0.25 µmol/l) level in EST mice treated with a ketogenic diet as compared to EST mice.

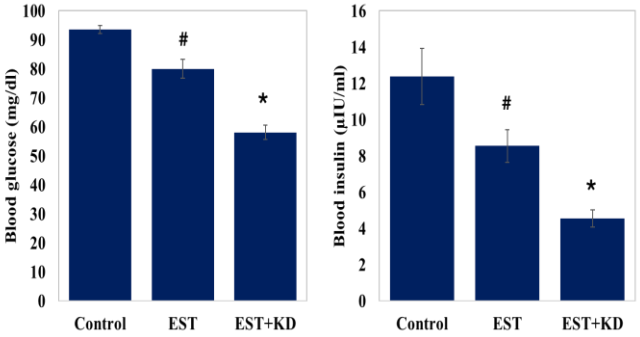

Fig. 1: Blood glucose and insulin levels in experimental groups. Values are presented as means ± SD. #represents a significant difference compared to healthy control group. *represents a significant ($P \leq 0.05$) difference compared to control EST group.

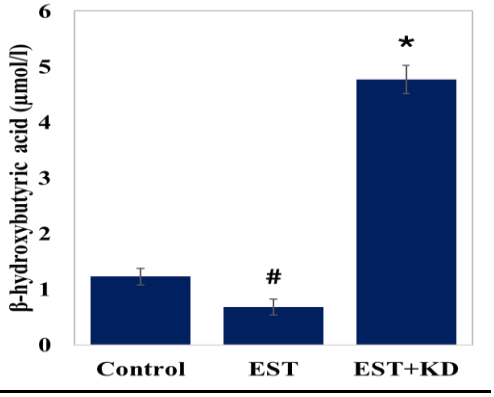

Fig. 2: Serum B-hydroxybutyric acid levels in different study groups. Values are presented as means ± SD. #represents a significant difference compared to healthy control group. *represents a significant ($P \leq 0.05$) difference compared to control EST group.

3.4. Modulation of Glycolytic Enzymes by Ketogenic Diet in EST-Bearing Mice

The activities of glycolytic enzymes were significantly altered by the induction of EST in female mice to significantly enhance the aerobic glycolytic pathways (figures 3). The activity of pyruvate kinase M2 and pyruvate dehydrogenase kinase 4 were significantly increased while pyruvate

dehydrogenase was decreased in control EST-bearing mice as compared to healthy control mice. However, treatment of EST-bearing mice with the ketogenic diet significantly (P≤ 0.05) reduced the activity of the glycolytic enzymes pyruvate kinase M2 and pyruvate dehydrogenase Kinase 4, and increased pyruvate dehydrogenase as compared to EST mice.

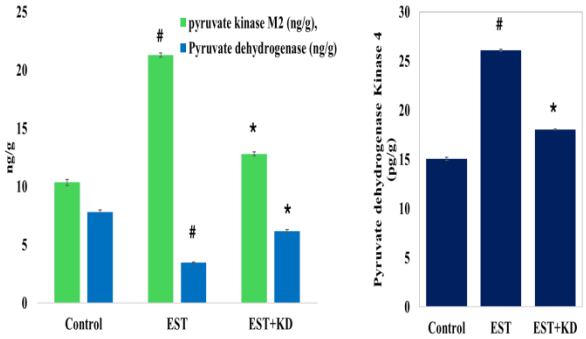

Fig. 3: Pyruvate kinase M2, pyruvate dehydrogenase and pyruvate dehydrogenase kinase 4 levels in experimental groups. Values are presented as means ± SD. #represents a significant difference compared to healthy control group. *represents a significant (P ≤ 0.05) difference compared to control EST group.

Table 1: Effect of ketogenic diet on tumour volume, tumor weight and IRT in experimental groups

Parameters / Groups	Tumor volume (mm^3)	Tumor weight (g)	Inhibition rate of tumor (IRT)
EST (n=10)	0.73 ± 0.05	1.44 ± 0.34	-
EST+KD (n=11)	0.44 ± 0.04*	0.97± 0.06*	41.55± 5.80

Values are presented as means ± SD. * represents a significant (P ≤ 0.05) difference compared to control EST group.

Table 2: Change in body weight, feed intake, and feed efficiency ratio in experimental groups

Parameters / Groups	Change body weight (g)	Feed intake (g)	Feed efficiency ratio (FER)
Healthy Control (n=14)	1.00 ± 1.30	805.71 ± 299.53	0.001 ± 0.002
EST (n=10)	-7.00 ± 1.63#	380 ± 53.33#	-0.019 ± 0.006#
EST+KD (n=11)	-7.00 ± 2.93	106.36 ± 50.65*	-0.071 ± 0.054*

Values are presented as means ± SD. #represents a significant difference compared to healthy control group *represents a significant (P ≤ 0.05) difference compared to control EST group.

3.5. Ketogenic Diet-Induced Cell Cycle Arrest in EST-Bearing Mice

Cell cycle distribution of cell fractions in the different phases was determined through flow cytometry using propidium iodide staining (Table 3 & Figure 4). The induction of EST in mice enhanced the cell cycle progression as 43.4% of the cell population was in the S phase and 16.2% were in the G2/M phase accompanied by lower populations found in the sub-G1 and G0/1 phases of the cell cycle. The values were significantly compared to the healthy cells in which a higher population was found at the G0/1 phase. On the other hand, treatment of EST-bearing mice with a ketogenic diet significantly induced cell cycle arrest, where a higher cell population was found at the G0/1 phase (69.1%), and a lower cell population was found at the G2/M phase (1.7%).

Table 3: Cell cycle analysis in experimental groups

Groups	ub-G1	G0/1	S phase	2/M
Healthy control	2.60±0.30	54.4±6.0	42.6±3.0	1.0±0.20
EST	1.10±0.20#	40.1±2.0#	43.4±4.0	16.20±0.8#
EST+KD	4.1±0.50*	69.1±6.0*	21.80±1.51*	1.7±0.40*

Values are presented as means ± SD. #represents a significant difference compared to healthy control group. *represents a significant (P ≤ 0.05) difference compared to control EST group.

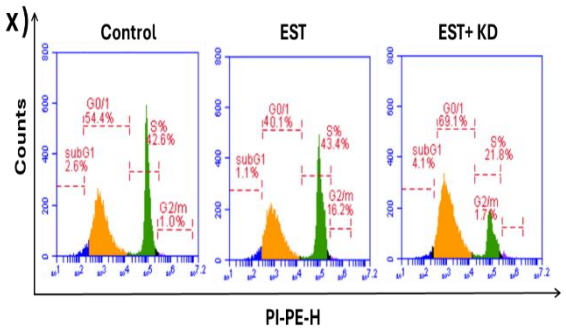

Fig. 4: Cell cycle analysis and cell population % in each phase were quantified of experimental groups.

3.6. Microscopic Examination of Tumor Tissue

The histological examination revealed the presence of normal muscle bundles and intact striated muscle fibers without any abnormalities in the healthy control mice fed on a balanced diet (figure 5). Contrastingly, in the neoplastic cells of the EST mice, the tumor cells exhibited characteristics indicative of malignancy, such as anisocytosis, nuclear pleomorphism and hyperchromatic nuclei. Notably, frequent atypical mitotic figures were consistently observed, often accompanied by the presence of numerous multinucleated giant cells (figure 6). Moderate improvement was observed in EST mice treated with KD characterized by extensive necrotic areas in neoplastic sheets and reduced mitotic activity (figure 7).

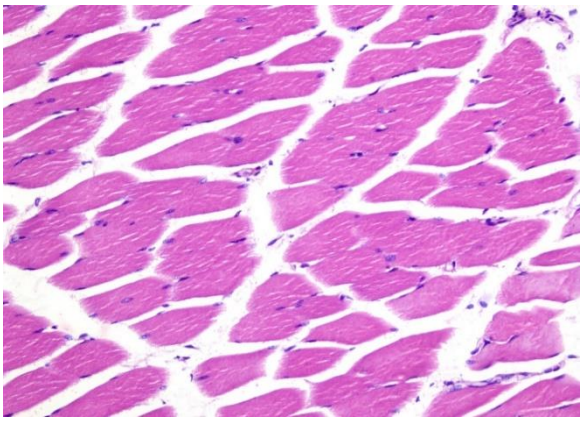

Figure (5): Left thigh muscle tissue section of the healthy control mice, showing normal muscle bundles.

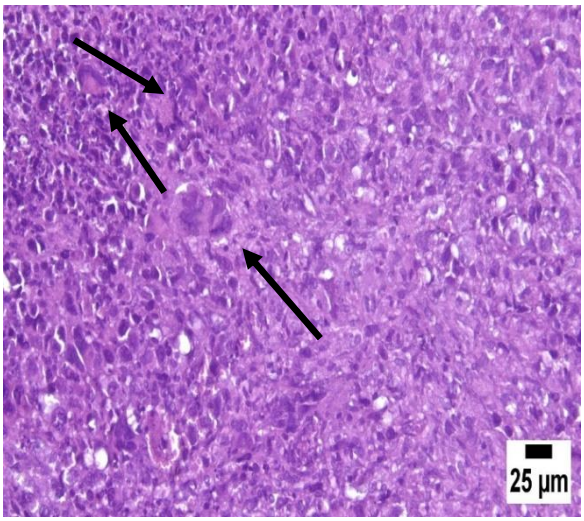

Fig. 6: Photomicrograph of Ehrlich solid tumor from EST mice, showing dense cellular neoplasm invade the muscle bundles with severe necrosis of muscle bundles.

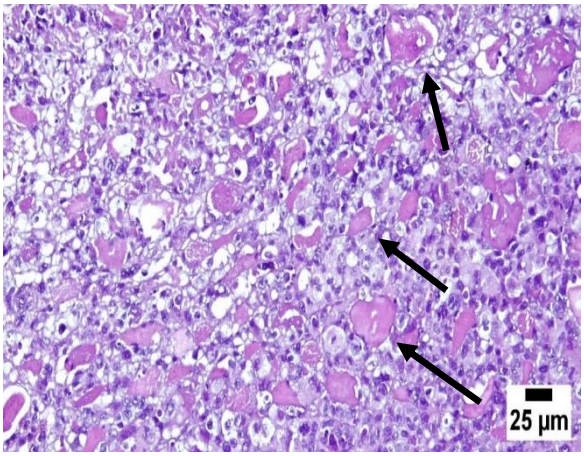

Fig. 7: Photomicrograph of Ehrlich solid tumor from EST mice treated with KD, showing lower apoptotic spaces

4. Discussion

The present study aimed to elucidate the potential effects of the ketogenic diet in inhibition of tumor growth and glycolytic activity as well as their effects on cell cycle progression in EST-bearing female Swiss albino mice.

Ehrlich solid tumor is known for its aggressive behavior including invasive characteristics, it may infiltrate surrounding tissues and structures contributing to both the increase in tumor volume and weight [20]. The factors contributing to the increase in tumor volume and weight include angiogenesis to support the growing tumor and potential infiltration into surrounding tissues. These factors collectively contribute to the aggressive behavior of EST and its impact on tumor size [21]. The result of this study was in parallel with the finding reported that the Ehrlich cells

subcutaneously inside the thigh of the mice' left leg, caused an increase in the volume of the portion over the week of the experiment because of tumor growth that was confirmed by weighing the tumor after its separation from the leg [22]. On the other side, feeding EST-bearing mice with a ketogenic diet could inhibit the tumor growth rate which can be explained by the fact that during a ketogenic diet, the body produces ketone bodies as an alternative energy source and cancer cells have a reduced ability to utilize ketone bodies compared to normal cells. This metabolic shift creates an unfavorable environment for tumor growth that leads to decreased tumor weight and tumor volume [23]. A ketogenic diet increases the sensitivity of cancer cells to oxidative stress. Cancer cells, already under stress due to the altered metabolic environment may become more susceptible to additional stressors, potentially leading to decreased tumor growth [24]. Furthermore, the ketogenic diet may impact signaling pathways involved in cell growth and survival, this could include modulation of the mammalian target of rapamycin (mTOR) pathway, which is often dysregulated in cancer. The alteration of such pathways may contribute to the inhibition of tumor growth in EST mice [25].

The effect of EST on an animal's body weight, and feed intake establishes a direct correlation between the tumor's high metabolic demand and its impact on essential physiological processes. This interconnection elucidates how EST, by actively engaging in nutrient competition with normal tissues, precipitates a decline in key biological parameters. The diversion of crucial nutrients away from regular cellular activities not only underscores the aggressive nature of the Ehrlich solid tumor but also provides a mechanistic link to the observed reduction in overall biological measurements [26]. The results were in hand with Aguilar-Cazares et al [27]who stated that the presence of a tumor triggers an inflammatory response, inflammatory cytokines released during this response had direct effects on appetite-regulating centers in the brain, led to reduced feed intake, and it can contribute to a condition known as cancer-associated cachexia, that involves significant weight loss, muscle wasting and weakness. Tumors can influence the endocrine system, leading to alterations in hormone levels. Hormones involved in appetite regulation, such as leptin and ghrelin, may be affected, contributing to changes in feeding behavior [28]. However, feeding EST-bearing mice with a ketogenic diet led to the maintenance of body weight and reduced muscle mass wasting compared to control EST-bearing mice, which can be confirmed by Khan et al [29] who explained that tumor cells may have a reduced ability to utilize ketone bodies compared to normal cells, potentially leading to a preferential supply of energy to normal tissues. Ketogenic diets are known to improve mitochondrial function and energy efficiency. This could be particularly relevant in the context of cancer, where mitochondrial dysfunction is often observed. EST cells are more dependent on glucose, the ketogenic diet's restriction of carbohydrates might limit the glucose supply to the tumor, affecting its growth and potentially contributing to reduced muscle mass wasting. The ketogenic diet may influence the metabolic and inflammatory factors contributing to cachexia, potentially slowing down muscle wasting in the context of EST [30].

Induction of solid tumors can lead to a reduction in blood glucose and ketosis because cancer cells exhibit a high rate of glucose consumption. Even in the presence of oxygen, cancer cells prefer glycolysis (the Warburg effect) to generate energy. This increased glucose uptake by cancer cells can result in a relative decrease in glucose availability in the bloodstream, contributing to lower blood glucose levels [31]. Cancer is often associated with chronic inflammation, inflammatory processes can affect insulin sensitivity and glucose metabolism, potentially contributing to changes in blood glucose levels. The results were confirmed by El-Saied et al [32] who stated that the decrease in serum glucose in mice bearing solid tumors may be due to the tumors exhibiting a high rate of glycolysis. This is a way of stimulating the body into a constant state of gluconeogenesis. Cachexia condition in cancer can resulted in a decrease in overall energy stores and contribute to lower blood glucose levels. Conversely, based on the current data, the ketogenic diet significantly decreased blood glucose and blood insulin while increasing β-hydroxybutyric acid in EST-bearing mice because going aligns with the expected metabolic response to a ketogenic diet, by restricting carbohydrates, the mice likely entered a state of ketosis, relying on ketone bodies, such as β-hydroxybutyric acid, for energy instead of glucose [33]. These results go in hand with Dilliraj et al [34] who stated that in a ketogenic diet, carbohydrate intake is severely restricted and when carbohydrates are scarce, the body's glycogen stores in the liver and muscles are depleted, and its reduction leads to decreased glucose release into the bloodstream, the liver shifts its metabolic pathways to break down fats into fatty acids through lipolysis process. Fatty acids are then transported to the liver, where they undergo oxidation. This process generates energy-rich molecules in the form of ATP, during fatty acids oxidation, excess acetyl-CoA is produced, which cannot be effectively processed through the citric acid cycle (Krebs cycle) due to limited oxaloacetate availability, instead, the liver initiates ketogenesis, a process that converts acetyl-CoA into ketone bodies. Beta-hydroxybutyrate is one of these ketone bodies,

along with acetoacetate and acetone, once produced, β-hydroxybutyrate is released into the bloodstream. It serves as an alternative fuel source for various tissues, including the brain, which can utilize ketone bodies when glucose availability is low [35]. Insulin is released by the pancreas in response to rising blood glucose levels. Since a ketogenic diet leads to lower and more stable blood glucose levels, the pancreas doesn't need to produce as much insulin. This reduction in insulin secretion contributes to lower overall insulin levels [36]. Ketogenic diets have been associated with reduced inflammation. Chronic inflammation is linked to insulin resistance and impaired glucose regulation thus by potentially lowering inflammation, a ketogenic diet contributes to better insulin sensitivity and glucose control [37].

The results of the present study indicated a marked disruption in the glycolytic enzyme activity in mice after EST growth. This might be potentially a result of the metabolic reprogramming of cancer cells to support their rapid proliferation. This reprogramming includes increased reliance on glycolysis, even in the presence of oxygen, as part of the tumor's adaptation to its high-energy demands [38]. The results of this study were confirmed by Schiliro and Firestein [39] who stated that the cancer cells create conditions that favor their growth, increased glycolysis and reduced reliance on oxidative phosphorylation could provide advantages to cancer cells by supporting their energy and biosynthetic demands. Cancer cells preferentially utilize glycolysis for energy production even in the presence of oxygen, so the level of hexokinase was increased. The reliance of cancer cells on glycolysis for energy production and biomass synthesis leads to elevated levels of PDK and PDK4 which lead to increased phosphorylation and inhibition of pyruvate dehydrogenase and diverted pyruvate away from entering the citric acid cycle [40]. The inhibitory phosphorylation by PDK leads to a decrease in PDH levels preventing the conversion of pyruvate to acetyl-CoA and thus reducing the entry of carbon into the citric acid cycle. This contributes to the redirection of metabolites towards glycolysis [41]. A ketogenic diet led to a reduction of aerobic glycolysis enzyme activity and an increase in PDH activity in EST-bearing mice, the results of this study were confirmed by Schiliro and Firestein [39] who showed that a ketogenic diet restricts carbohydrate intake, this can shift the metabolism of the mice towards utilizing fatty acids for energy through beta-oxidation, this shift in metabolic preference could lead to downregulation of enzymes involved in glycolysis, such as hexokinase and pyruvate kinase M2. With lower glucose availability and reduced reliance on glycolysis, there is a decreased demand for PDK and PDK4 to inhibit PDH activity [42]. The activity of PKM2 is influenced by the NADH/NAD+ ratio, a ketogenic diet can alter this ratio due to changes in the utilization of NADH during fatty acids oxidation and ketogenesis, changes in the NADH/NAD+ ratio could impact the activity and expression of PKM2. On a ketogenic diet, the production of acetyl-CoA from fatty acids oxidation and ketone bodies metabolism is elevated, the increased availability of acetyl-CoA could directly inhibit hexokinase and pyruvate kinase M2 activity and contribute to its downregulation while promoting the activity of PDH [43]. Ketone bodies can directly inhibit the activity of PDK, reducing its inhibitory effect on PDH [44]. Nutrient-sensing pathways, such as AMP-activated protein kinase (AMPK) and mTOR, play crucial roles in regulating cellular metabolism. A ketogenic diet can activate AMPK, which, in turn, may inhibit glycolysis and enhance fatty acids oxidation. The modulation of these signaling pathways could contribute to the observed metabolic changes [45].

The observed changes in cell cycle distribution in EST mice suggested a complex interplay of molecular events, indicating that cancer cells' resistance to apoptosis leads to a lower accumulation of cells in the sub-G1 phase. Cancer cells can acquire mutations or dysregulations that allow them to evade normal cellular checkpoints and apoptosis [46]. Ehrlich solid tumors tend to have more rapid and uncontrolled cell division, which can result in a higher accumulation in the S phase and G2/M phase [47]. Cancer cells often carry mutations or alterations in genes that control the cell cycle checkpoints, such as p53 and RB (retinoblastoma protein). These mutations can lead to the loss of normal regulatory mechanisms, allowing cancer cells to bypass G1 phase checkpoints and progress through the cell cycle more rapidly. Dysregulation of signaling pathways involved in cell cycle control, such as the PI3K/Akt/mTOR pathway, can affect the progression of cancer cells through the cell cycle phases [48]. The results of this study were in line with Fatehi et al [49] who showed that in cancer, the cell cycle undergoes abnormal regulation characterized by accelerated progression through G1, S and G2 phases. This results from compromised checkpoint controls, leading to heightened DNA synthesis, uncontrolled mitotic cell division and evasion of programmed cell death. These disruptions collectively contribute to the unrestrained proliferation observed in cancer cells, ultimately culminating in tumor formation. On the other hand, the metabolic shift towards ketone bodies production caused by the ketogenic diet in EST-bearing mice might cause some cells to undergo stress due to the altered energy balance. This cellular stress could potentially trigger apoptosis, leading to a higher accumulation of cells in the sub-G1 phase. This

metabolic shift may impact the activity of key signaling pathways involved in cell cycle regulation. For example, changes in AMP-activated protein kinase or mammalian target of rapamycin pathways could influence cell cycle progression. The ketogenic diet activates the tumor suppressor protein p53, which plays a critical role in regulating the cell cycle and apoptosis [50]. This could be a protective mechanism to eliminate damaged or cancerous cells. The ketogenic diet is known to lower insulin and insulin-like growth factor 1 levels which can influence the activity of the phosphatidylinositol 3-kinase pathway, which is crucial for cell cycle progression, downregulation of this pathway may contribute to cell cycle arrest [51]. In a ketogenic diet, cancer cells require more time to prepare DNA replication due to changes in energy substrate utilization [52]. Metabolic changes induced by a ketogenic diet can create cellular stress, which impacts DNA repair mechanisms. Delayed DNA replication and cell division could be a response to increased genomic stress in cancer cells [53]

The histological findings in the EST mice revealed several features indicative of malignancy within the neoplastic cells, these observations provide valuable insights into the aggressive nature of Ehrlich solid tumor [54]. This pattern signifies uncontrolled proliferation and a lack of the organized tissue structure seen in normal tissues. Anisocytosis refers to significant variation in cell size within the tumor [55]. This is a characteristic feature of malignancy, reflecting the loss of normal cell size regulation. Nuclear pleomorphism refers to the irregularity in the size, shape and staining intensity of cell nuclei. This is another hallmark of malignancy, indicating genetic instability and aberrant nuclear structure [56]. Genetic alterations within the neoplastic cells may include mutations in key genes responsible for regulating cell growth and division, leading to uncontrolled proliferation and the observed anisocytosis. Activation of oncogenic signaling pathways may drive the malignant phenotype, promoting uncontrolled cell growth and the development of hyperchromatic nuclei [57]. Chronic inflammation, induced by the presence of neoplastic cells, can contribute to the malignant phenotype, and inflammatory signals may promote cell survival, proliferation and invasiveness. Activation of the PI3K/Akt/mTOR pathway can promote cell survival and growth. Dysregulation of this pathway is commonly associated with malignancy, including increased cell size and hyperchromatic nuclei [54]. The decreased mitotic activity observed in the EST mice treated with KD indicated that cancer cells in this group are dividing less actively compared to the EST mice. The ketogenic diet has impacted their ability to proliferate. Ketogenic diets promote the use of ketone bodies as an alternative energy source, this metabolic shift could have influenced cell cycle regulation, leading to reduced mitotic activity [58]. The ketogenic diet may induce cell cycle arrest, preventing cells from progressing through the cell cycle and entering mitosis, this can be a consequence of altered signaling pathways or nutrient availability. Ketogenic diets have been associated with enhanced DNA repair mechanisms, improved DNA stability may lead to a reduction in the need for cell division, resulting in decreased mitotic activity [59].

5. Conclusion

In an effort to identify the anti-tumor effects of the ketogenic diet in EST bearing female mice, the tumor inhibition rate, the blood glycemia, ketosis, and glycolytic enzyme activity, as well as cell cycle analysis, were determined. Obviously, treatment of EST-bearing mice with the ketogenic diet markedly reduced tumor weight and volume leading to increased tumor inhibition rate. This might be due to their effects on depriving cancer cells of glucose causing significant reduction in blood glucose and elevated ketosis causing reduced glycolytic enzyme activity, energy depletion, and cellular stress in cancer cells. This was reflected in affecting cell cycle progression in cancer cells towards cellular arrest at the G0/G1 phase. Overall, this indicates the potential antitumor effects of the ketogenic diet in EST-bearing mice.

6. Conflicts of interest:

There are no conflicts to declare.

7. References:

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