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Comparative gene expression analysis of lactate dehydrogenase isoforms in rats treated with Baba

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ABSTRACT
This study was done to observe the effect of β-amino butyric acid (BABA) on the gene expression of five Lactate dehydrogenase isomers, which is considered one of the liver function proteins (LDH). That is used to convert pyruvate into lactate and vice versa, and the importance of inhibiting it in the presence of cancer cells because it enhances their activity. We also studied the effect of BABA on the enzyme Gamma-glutamyltranspeptidase (GGT), which plays a role in glutathione metabolism and the recovery of the amino acid cysteine. Sprague-Dawley rats male were used in this study (25 rats age 2 - 3 months and weights (152-208). Rats were divided into 5 groups (five rats each) according to the concentration of BABA as follows: Control group (A) was injected with normal saline; Group B animals were injected with a concentration of 100 mg/kg; Group C was injected at a concentration of 150 mg/kg; Group D was injected at a concentration of 200 mg/kg; Group E was injected at a concentration of 250 mg/kg. It was injected six times within three weeks. Later, blood was drawn and serum was obtained to conduct the necessary tests for LDH and GGT showed that there were significant differences at the level of probability (0.05), and the effects of the amino acid BABA were confirmed as activating or inhibiting the level of enzymes. For the LDH isoenzymes, higher levels were found in treatment E increased the gene expression levels of the five isoenzymes: LDA-1, LDA-2, LDH, LDB, LDC. This is undesirable because high levels of this enzyme indicate increased activity of cancer cells, while in the other treatments the level of the enzyme decreased compared to control samples On the other hand, the GGT appeared at the highest concentration in treatment D this treatment did not activate the enzyme, but rather it remained within its normal level represented by the control treatment as for the lowest level only during treatments B, C, and E. This may cause a defect in the processes of oxidation and reduction of glutathione and difficulty in recovering the amino acid cysteine

Key words: BABA, LDH, GGT, Cancer cells

INTRODUCTION

β-aminobutyric acid is a non-protein amino acid that can stimulate plant defense responses against biotic and abiotic stresses and as well as enhancing plant resistance to diseases and pathogens such as viruses, fungi, bacteria, and nematodes [1]. BABA is used in the pharmaceutical industry and is widely included in the chemical formulations of therapies in the preparation of anti-cancer drugs, AIDS therapies and antibiotics such as penicillin. [2].

A study evaluating the effect of BABA on Gram-negative intestinal bacteria and the tissues of the digestive system (Stomach, Minute intestine, and The Thick intestine) of male rats. The control animals as well as the histological composition of the stomach was normal under studied concentrations, however the histological composition of small and large intestine was affected with the increase in concentration. Moreover, Gram negative enterococci bacteria were also decreased with increasing concentration. On the other hand, another study demonstrated its effectiveness in the ability to increase the systemic resistance of male rats against Staphylococcus aureus, as well as increasing the numbers of white blood cells and lymphocytes and its positive effect on improving the immune system, and also to improve diabetic rats' health [3] He explained that BABA has a therapeutic role in curing type 2 diabetes By lowering the level of glucose in the blood[4]

Lactate dehydrogenase is an enzyme participate in carbohydrate metabolism, catalyzing the mutual-conversion of pyruvate and lactate with the NAD + / NADH coenzyme system. Tissue cells are a main source of lactate dehydrogenase isoforms that are normally distributed in the blood serum / plasma of humans and animals. LDH

is widely distributed in the body, and high activities are found in the liver, heart, skeletal muscle, kidney, and erythrocytes, while lower amounts are found in the lung, smooth muscle and brain [5]

Pyruvate + NADH + H+ -→ Lactate + NAD+

LDH is composed of two different subunits: lactate dehydrogenase (LDHA) and lactate dehydrogenase (LDHB). LDHA and LDHB can be grouped into groups: LDH1 contains four LDHB subunits; LDH2 contains three LDHB subunits and one LDHA; LDH3 contains Two LDHB/LDHA subunits; LDH4 has one LDHB subunit and three LDHA subunits while LDH5 has four LDHA subunits [6]. LDH can be used as a marker for various tissue injuries due to its enzymatic form and widespread distribution. When tissues are damaged, cells release it into the bloodstream. Depending on the type of tissue infection. High serum LDH occurs as a result of organ damage to extensive cell death leading to loss of cytoplasm. The causes of tissue damage can be diseases such as hepatitis, acute myocardial infarction, anemia, pulmonary embolism, acute renal failure, etc. [7].

Gamma-glutamyltranspeptidase is an enzyme fall on the outer membrane of cells where it regulates the metabolism of glutathione (GSH), the most abundant antioxidant thiol within cells. GGT plays a key role in controlling redox homeostasis, by hydrolyzing extracellular GSH and providing the cell with cysteine recovery, which is essential for intracellular GSH synthesis and protein biosynthesis. Therefore, to organize of GGT gives the cell greater resistance to oxidative stress and the property of rapid growth. [8]

GGT is a sensitive marker for biliary liver damage, liver tumors, viral hepatitis, and fatty liver disease. However, it lacks specificity and can also be elevated by alcohol consumption, many drugs including barbiturates, phenytoin, NSAIDs, and other non-hepatic conditions including pancreatitis, diabetes, and obesity. It can be used to help determine the origin of elevated alkaline phosphatase (ALP) activity, and in the management of patients with alcohol use disorders [9].

Materials and working methods

Laboratory animals

The study was carried out on 25 Sprague-Dawley male rats, aged 2-3 months, and weights (152 - 208) as averages for each cage respectively according to the sequence of concentrations.

Rats were divided into 5 groups (five rats each) according to the concentration of BABA as follows: group (A) Control was injected with normal saline only; Group B animals were injected with a concentration of 100 mg/kg; Group C was injected at a concentration of 150 mg/kg; Group D was injected at a concentration of 200 mg/kg; Group E was injected at a concentration of 250 mg/kg.

Cages placed in the animal house and provided with appropriate conditions for their living, including rich in proteins nutrition, constant abundance of water, and an appropriate atmosphere. Rats left 10 days before the injection to allow the appropriate period for adaptation to the new place.

Ethical declaration

All animal procedures adhered to the National Institutes Guide for the Care and Use of Laboratory Animals and were approved by the University of Anbar, College of Education for Women, Department of Biology, No. 20 on 2023/9/15.

Preparation of concentrations

BABA was prepared in the form of an injectable liquid by adding normal saline, depending on the required concentration and body weight according to the following equation: Concentration (%) = body weight (kg) * 1000 / weight of the substance (mg).

Later, they were injected in the Peritoneum area (IP) at a rate of 6 doses for each of them across 3 weeks.

Sample collection

Two days after completing the necessary investigations, blood was drawn with three random samples from each cage, and each sample was transferred to two types of test tubes: Gel tube for the purpose of analyzing Gamma glutamyltransferase and Trizole tube for studying the gene expression of the enzyme Lactate dehydrogenase according to the required study.

Gene Expression

RNA extraction:

The Wizard Genomic Extraction Kit (Promega) was used and the method was described by [10] as follows:

The samples were collected, and then were placed in 1 ml of Trizol solution for each (1-5X 10) of blood size. Using a micropipette, blood was drawn and well homogenized until the DNA analysis stage was finally completed.

1- Chloroform Added (200 microliters) to every 1 ml of Trizol and shake well, then centrifuged at 10,000 rpm for 10 minutes at 4 C. After that, it will separated into three layers, the upper (aqua phase), the middle layer (enter phase) and the lower layer (lower phase). The aqueous phase (top layer) was withdrawn, then 500

microliters of isopropanol was added, then left on ice for 10 minutes. Centrifuge then carried out at 10,000 rpm for 10 minutes at a temperature of 4°C, After performing the centrifugation, all the isopropanol withdrawn and 500 microliters of 70% ethanol was added to it, then it was centrifuged at 11,000 rpm for 5 minutes at temperature volatilized.
Add 90% Nuclease free water and place the tubes in a water bath at a temperature of 65°C for 15 minutes. Later, The RNA concentrations were measured using a QuantnsFluorometer. 1 microliter of the previously extracted RNA solution was added to 99 ml of diluted fluorescent dye and mixed well, then left for 5 minutes at room temperature, and the RNA concentration was measured in nanograms/liter. Electrophoresis was performed on an agarose gel for the purpose of confirming the presence of genetic material
RT-PCR reaction to convert RNA into cDNA
The genetic expressions analysis performed using AccuPowerRocktScript RT PreMIX kit (Bioneer, Korea).
According to the recommended concentrations, the primers were replaced with oligonucleotides by adding 175 Nuclease Free Water to make the concentration 100, then 50 of the primer was taken and 50 ml of Nuclease Free Water for dilution purpose and was used in the transformation, then mixed 18 of RNA with 2 ml of primer to make the final volume 20 and added it to the kit for the transformation, then the contents were mixed in PCR tubes to homogenize using vortex and placed in the RT-PCR device according to the special program. As shown in Table (52), the DNA samples were later stored at a temperature of (-20) C to preserve them for a longer period

Primers Solution
The primers were designed using the Primer3 Plus program, based on the sequence of genes found within the NCBI. Then, the primer solutions were prepared according to the instructions (Macrogen Company, Korea) as shown in (Table 1), using sterile non-ionic distilled water, with a concentration of 100 picomoles being obtained

Table 1: Represent Primersused in the molecular study of the enzyme lactate dehydrogenase

Gene	Primer	Product size
Lactate dehydrogenase A1	Forward GAGCTGTGGTTGGTCCAGTT Reverse GCAGTTGGCAGTGTGTCTTG	14
Lactate dehydrogenase A2	Forward TGTGGTTGGTCCAGTTGTGT Reverse ATGCAGTTGGCAGTGTGTCT	139
Lactate dehydrogenase B	Forward TCATCCATCCGCTGCGAAA Reverse TTCCAACTTGTCCAACGCCT	186
lactate dehydrogenase C	Forward GGAGGCCAGAGGAAATCAGG Reverse AAGGCTGCCATGCAGAAGAT	236
lactate dehydrogenase A-like 6B (Ldhal6b)	Forward AGCGTGGAAATTGAGTGGGT Reverse TACACCACTCCACACAGGGA	175

Gene expression technology
Quantitative qRT-PCR Reverse Transcription (Real Time PCR) High-performance real-time polymerase chain reaction uses DNA as a template, and the β actin gene has been used as a house keeping gene. Results then calculated depending on Livak formula [11].
The Two-step Quantitative Serial Reaction RT-PCR program
After mixing the contents of the tubes well using a mixer, they were placed in the GRT-PCR device, and replication steps were performed to investigate the amount of gene expression for the studied genes. The RT-PCR device was programmed as follows and as shown in Table 2.

Table 2: Represent Components for binding the genetic material of cDNA with primers

The Components	The Size
Master mix	10µl
Forward primer	1 µl
Revers primer	1 µl
Templete CDNA	2.5 µl
Free Nuclease water	5.5 µl
Final volume	20 µl

Quantitative Serial Reaction the Two-step RT-PCR program

Tubes mixed well, they were placed in the GRT-PCR device, and replication steps were performed to investigate the amount of gene expression. The RT-PCR device was programmed as follows and as shown in Table 3.

Table 3: Show Programming the qRT-PCR device

Step	Temperature	Time	Cycle
Initial Denaturation	94 C	30 sec	
Denaturation	94 C	30 sec	
Annealing	68-45 C	15-60 sec	30
Extension	68 C°	1 min	
Final	68 C	5 min	
Hold	4-10 C		

Then, the Cycle of threshold(Ct) values for each gene appeared separately according to the following figures

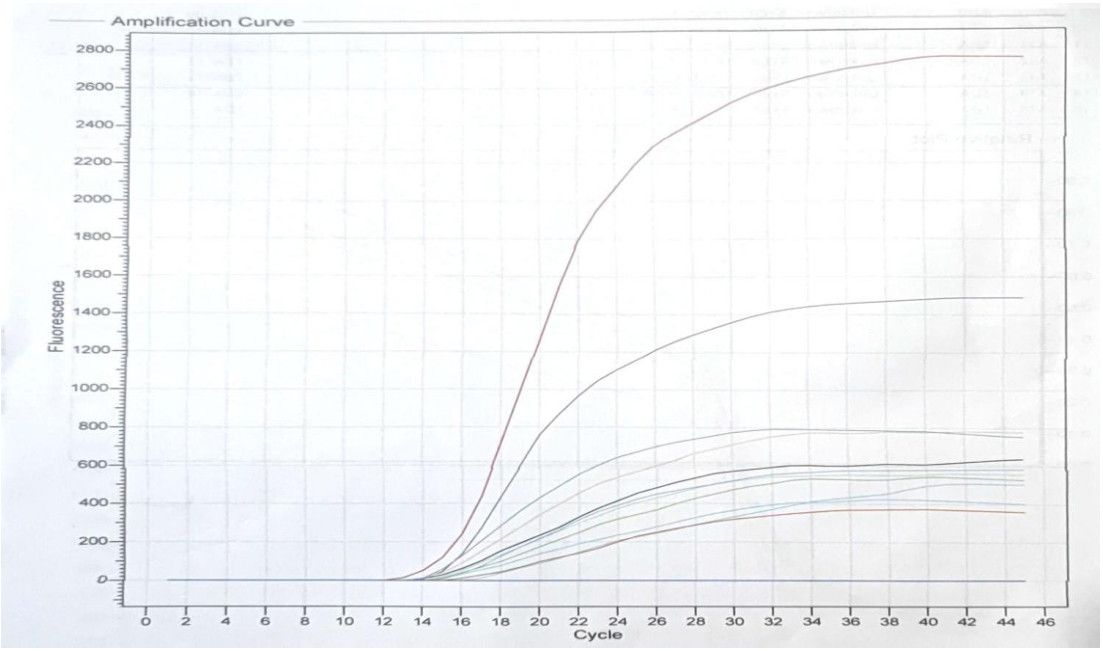

Figure 1: Curves (Ct) of Gene LDA-1 By qRT PCR

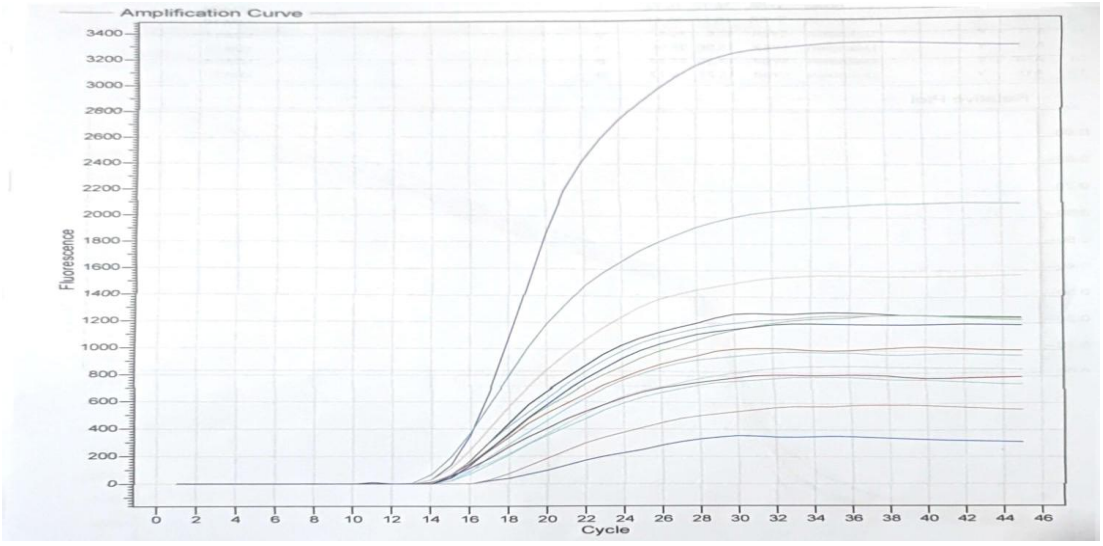

Figure 2: Curves Ct of Gene LDA-2 By qRT PCR

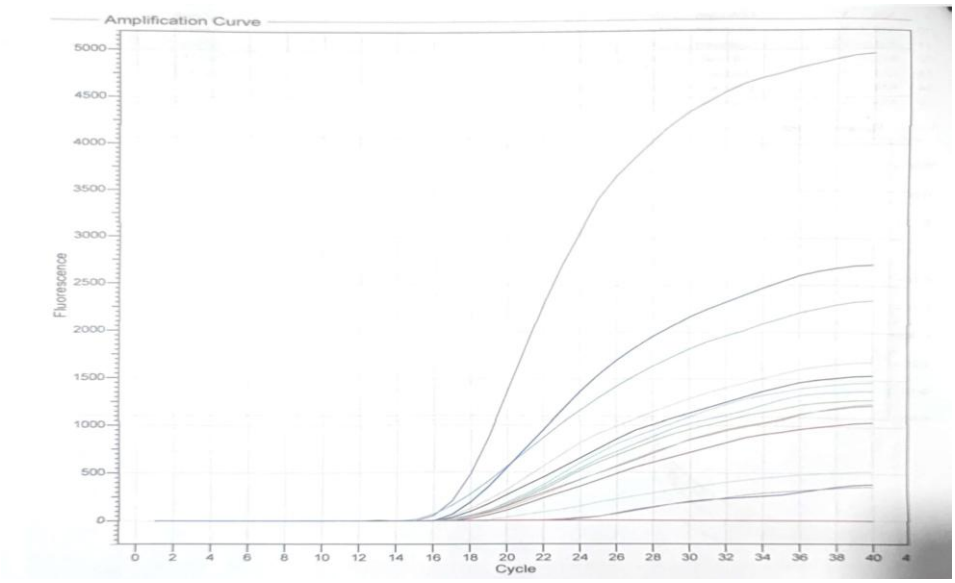

Figure 3: Curve (Ct) of Gene LDH By qRT PCR

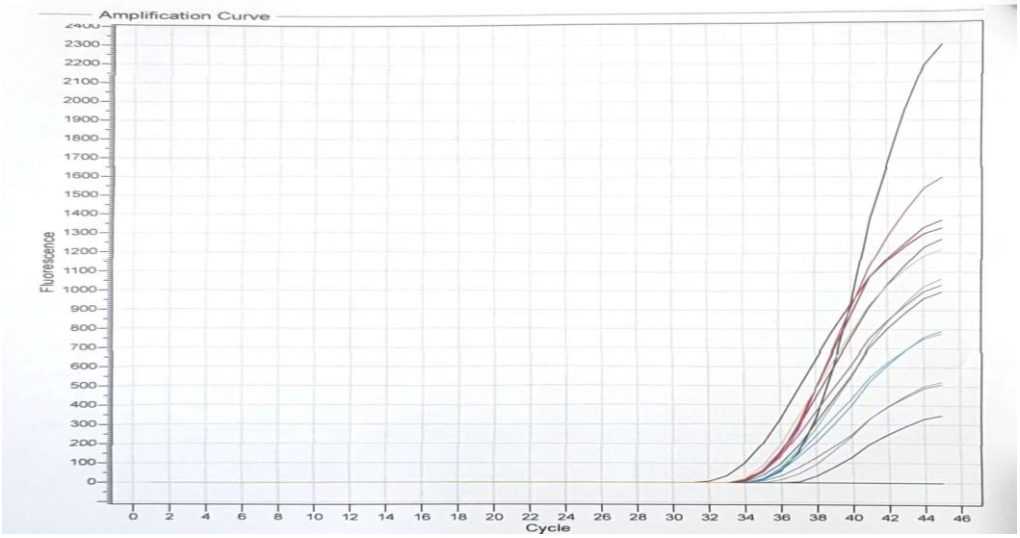

Figure 4: Curve (Ct) of Gene LDB By qRT PCR

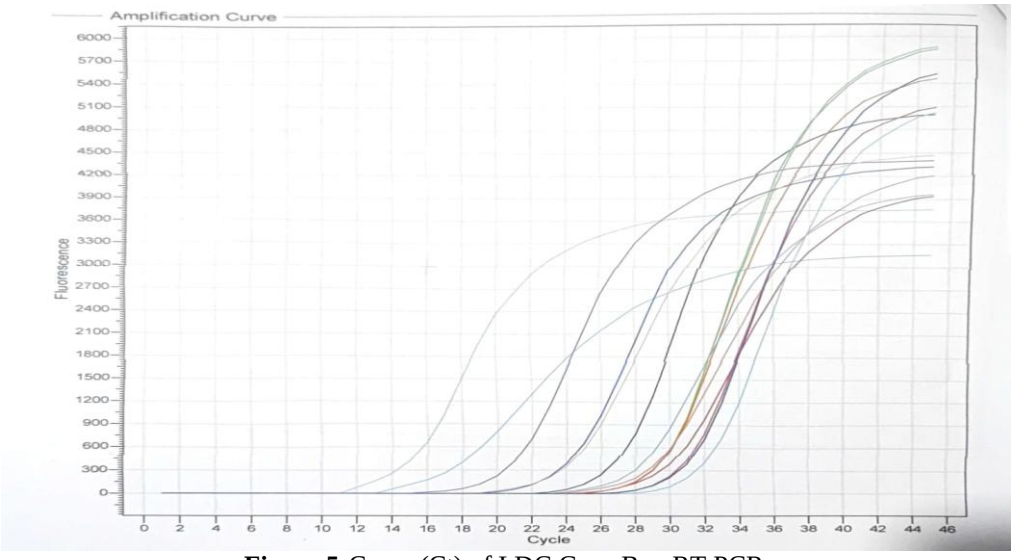

Figure 5: Curve (Ct) of LDC Gene By qRT PCR

Calculate the value of Ct using the Livak equation

The equation below was used to calculate the Cycle Threshold (Ct) values, as they appear in the form of Dissassociation Curves, where the Fluorescence is at its highest, and represent the gene expression values of the LDH isotopes, as the Act for each sample is calculated as follows:

ct Gene-ct H.K=A ct (Treated sample)

Δct(Control)=ct Gene-ct H.K

ΔΔ ct = Δ ct (Treated) Δct (con.) Folding = 2^{-}ΔΔct

Treated Sample refers to the treated isolate, Control refers to the untreated isolate, Ct Gene: refers to the ct value of the target gene, Ct H.K refers to the Ct value of the calibration gene.[24]

Chemical and immunological tests to measure the level of the transpeptidase enzyme GGT were carried out using the Sandwich-ELISA method kit (Elabscience, Korea), as the following steps- :

Reagents and samples brought to room temperature (18-25°C) before use, concentrated washing solution diluted to 1x working solution, The standard solution was diluted to concentrations of 1.65, 6.25, 12.5, 13.3, 25, and 50, also the Biotinylated detection Ab/Ag solution was diluted to 1x the working solution 15 minutes before the end of the step, and the conjugated HRP diluted to 1x the working solution 15 minutes before use

Performing the examination

Samples and standard added to each well (100 microliters of the standard or sample), and incubated for 90 minutes at 37°C. Then 100 µl of biotinylated detection Ab/Ag was added. Placed in the incubator for 1 hour at 37°C. Samples dried and wash 3 times. Conjugated HRP (100 µl) added Incubated for 30 minutes at 37°C. Dried and washed 5 times with washing solutionlater 90 µl of substrate reagent added and incubated for 15 minutes at 37 °C, reaction stop solution (50 microliters) added and read the OD value at 450 nm immediately then results calculated.

RESULTS AND DISCUSSION

Table 4: Effect of difference treatments of BABA on LDA-1 gene expression

Treatments ( )	2^{-}ΔΔct ±SD	Fold change ±SD	Conclusion
100	0.33 ±0.08 c	3.030 ±0.62 b	Down-regulation
150	0.57 ±0.19 c	1.754 ±0.35 c	Down-regulation
200	0.86 ±0.12 bc	1.163 ±0.41 c	Down-regulation
250	5.12 ±0.76 a	4.120 ±0.73 a	Up-regulation
Control (without)	1.00 ±0.00 b	0.00 ±0.00 d	--
LSD	0.8156 **	0.9971 **	--
P-value	0.0001	0.0002	--
Means having with the different letters in same column differed significantly, ** (P≤0.01).			

Table (4) shows the highest value of gene expression at the concentration of D (5.12)This means that this concentration of the amino acid activated this enzyme LDA-1, which is consistent with the study by [12]. the results of which showed that (Emamectin benzoate) significantly increase the lactate dehydrogenase activity, and lactate level, which indicates organ damage in treated animals. and the lowest concentration was in treatment A(0.33) That inhibited this enzyme and that is consistent with the same studyTo try it on the drug called "nootropic" Peracetam is mainly used to treat dementia and brain injury It also causes significant inhibition of lactate hydrogenase activity in the heart, kidneys and liver. while treatments B and C were close to the value of the control treatment.

Table 5: Effect of difference treatments of BABA on LDA-2 gene expression

Treatments ( )	2^{-}ΔΔct ±SD	Fold change ±SD	Conclusion
100	0.54 ±0.12 b	1.852 ±0.48 a	Down-regulation
150	0.49 ±0.07 b	2.041 ±0.61 a	Down-regulation
200	0.92 ±0.15 b	1.087 ±0.26 b	Down-regulation
250	2.2 ±0.34 a	1.20 ±0.32 ab	Up-regulation
Control (without)	1.00 ±0.00 b	0.00 ±0.00 c	--
LSD	0.739 **	0.815 **	--
P-value	0.0087	0.0092	--
Means having with the different letters in same column differed significantly, ** (P≤0.01).			

The results of the statistical analysis (Table 5) showed that there were statistically significant differencesfor amino acid BABA on LDHA-2, and that the highest value was in treatment D(2.2), which was reached. Due to their composition of three subunits of LDHB and one unit of LDHA [6]its activation controls the flow of basal autophagy. For oxidative cancer cells, according to a study [13], and its lowest value is in treatment B(0.49), which means inhibiting the gene expression of this enzyme, and silencing LDHB prevents basal autophagy and the proliferation of cancer cells, and also leads to Apoptosis [13] followed by treatment A, while treatment C is close to the control.

Table 6: Effect of difference treatments in Fold change of LDH gene expression

Treatments ( )	$2^{-}\Delta\Delta$ct ±SD	Fold change ±SD	Conclusion
100	1.06 ±0.42 bc	0.06 ±0.02 c	Up-regulation
150	1.74 ±0.57 b	0.74 ±0.19 c	Up-regulation
200	0.62 ±0.28 c	1.612 ±0.37 b	Down-regulation
250	8.41 ±1.02 a	7.41 ±0.84 a	Down-regulation
Control (without)	1.00 ±0.00 bc	0.00 ±0.00 c	--
LSD	1.206 **	0.817 **	--
P-value	0.0001	0.0001	--
Means having with the different letters in same column differed significantly, ** (P≤0.01).			

However (Table 6), LDH consists of two subunits of LDHA and two subunits of LDHB [6]Our results showed activation of this enzyme at three treatments, namely A, B, and the highest value at the concentration of D(8.41), which indicates, especially considering that high LDHB expression is considered An important predictor of shorter survival in patients with lung adenocarcinomas [14]
As for treatment C(0.62), it had the opposite effect, as it inhibited the enzyme, and this is consistent with a study conducted by [15] who showed that taking LDHA inhibitors in combination with chemotherapy agents, they give anti-tumor and anti-cancer effects .

Table 7: Effect of difference treatments of BABA on LDB gene expression

Treatments ( )	$2^{-}\Delta\Delta$ct ±SD	Fold change ±SD	Conclusion
100	0.003 ±0.0002 c	333.33 ±62.17 c	Down-regulation
150	0.0001 ±0.00003 c	10000 ±257.02 a	Down-regulation
200	0.0002 ±0.00007 c	5000 ±66.41 b	Down-regulation
250	9.6 ±0.84 a	8.6 ±0.72 d	Up-regulation
Control (without)	1.00 ±0.00 b	0.00 ±0.00 d	--
LSD	0.892 **	271.263 **	--
P-value	0.0001	0.0001	--
Means having with the different letters in same column differed significantly, ** (P≤0.01).			

LDB consists of three subunits of LDHA and one subunit of LDHB [6] Its highest value occurred when treated with a concentration of D(9.6) As it appeared in (Table 7), thus leading to the activation of this enzyme, whose activation indicates the involvement of these proteins in early carcinogenesis and tumor development in Cancer patients, according to a study[15], as for parameters A, B, and C(0.0002), have an inhibitory effect. Several reports have shown that suppressing or inhibiting LDHA can prevent metastasis of human cancer, including renal cell carcinoma . Experiments found in vivo and in vitro demonstrated that downregulation of LDHA suppresses RCC cell migration and invasion by inhibiting EMT. In addition, the results indicated that LDHA could enhance the Warburg effect. These results demonstrated that LDHA mediates tumor metastasis by promoting EMT in RCC, suggesting that LDHA could be a promising therapeutic target for the treatment of RCC [16].

Table 8: Effect of difference treatments of BABA on LDC gene expression

Treatments ( )	$2^{-}\Delta\Delta$ct ±SD	Fold change ±SD	Conclusion
100	0.40 ±0.11 b	2.50 ±0.37 b	Down-regulation
150	0.19 ±0.06 b	5.263 ±0.74 a	Down-regulation
200	0.92 ±0.15 b	1.089 ±0.22 cd	Down-regulation
250	3.00 ±0.41 a	2.00 ±0.25 bc	Up-regulation

Control (without)	1.00 ±0.00 b	0.00 ±0.00 d	--
LSD	0.891 **	1.259 **	--
P-value	0.0073	0.0006	--
Means having with the different letters in same column differed significantly, ** (P≤0.01).			

BABA affected LDC as it consists of four subunits of LDHA[6], it is activated at D(3.0) concentration, according to this study According to (Table 8). Many genetic and phenotypic studies have demonstrated the potential role of lactate dehydrogenase (LDHA) or LDH5, which is a single homodimeric tetramer. Subtype A, in cancer development and metastasis. One of the important enzymes in aerobic glycolysis, as it catalyzes the final step of glycolysis, by converting pyruvate to lactate. Most cancer cells have an enhanced rate of tumor glycolysis to ensure the energy demand of rapidly growing cancer cells resulting in increased lactate production. Excess lactate induces extracellular acidosis, which facilitates invasion, angiogenesis, and proliferation and affects the immune response [17] Its lowest concentration was in treatment B(0.19), which led to inhibition of the enzyme, and it is possible, according to a study[15], that when taking LDHA inhibitors in combination with other chemotherapy agents, they have synergistic anti-tumor effects by restoring the sensitivity of resistant cells to cancer cells. This also corresponds to treatment with concentration.

Gamma GlutamylTransferase

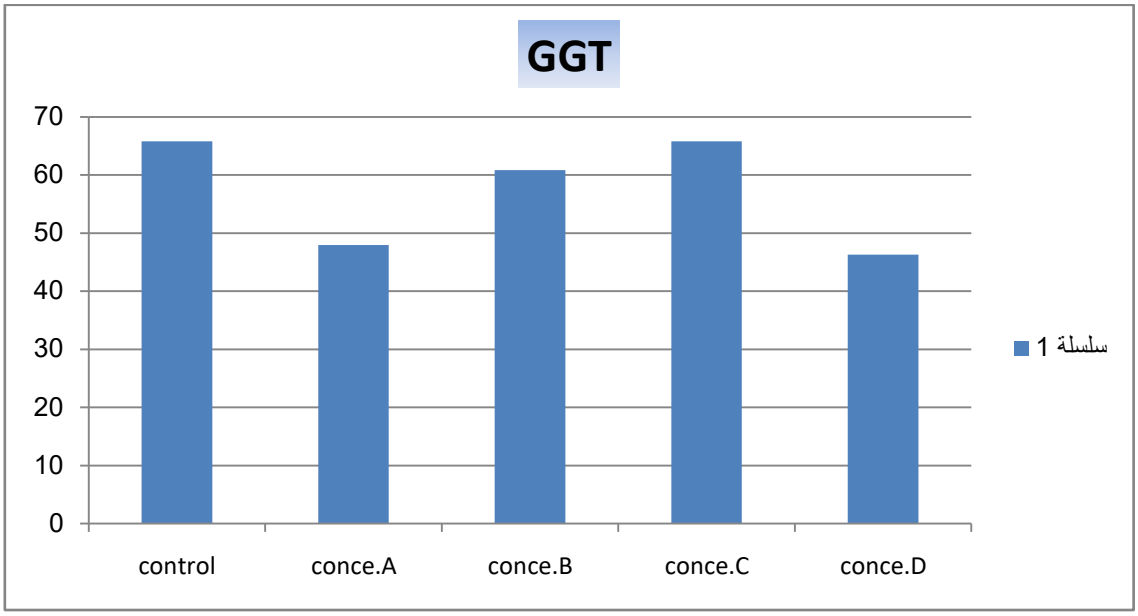

Figure 6: Effects of BBA on GGT

The results of the statistical analysis showed that there were statistically significant differences compared to the control values, and the highest value was in the fourth treatment at C(65.82). This may indicate the effect of this acid at this concentration on the arteries and heart disease, as a previous study conducted by[18] that the activity of γ-glutamyltransferase (GGT) in the blood is associated with the development of atherosclerosis and cardiovascular events, and another study by [19] demonstrated that an increase in GGT concentration is associated with oxidative stress and metabolic disorders.

Conditions that increase GGT in the blood, such as obstructive liver disease, high alcohol consumption, and the use of enzyme-inducing medications, lead to increased production of free radicals and threaten to deplete glutathione in patients with STEMI who `undergoing primary PCI [20], high GGT levels on admission have been found to be associated with no remission phenomena and increased long-term mortality [21].

This increase is consistent with a study[22] to study the effect of GABA, an anticonvulsant drug, which also caused an increase in GGT levels.

As for treatments B, C, and E, we find the lowest level of enzyme concentration, and this may cause a defect in the control of the processes of oxidation, reduction, and glutathione metabolism, and thus difficulty in recovering cystine, because its functions, when present in a natural ratio, are to control oxidation, reduction, and glutathione metabolism to ensure the recovery of cystine, according to the study [8].

This inhibition is consistent with a study [23], whose results indicate that GGT1 inhibitors, such as L-α-aminobutyric acid (L-ABBA), have the ability to reduce the hormone GSH and thus stimulate oxidative stress in cancer cells and reduce their resistance to many chemotherapy agents

CONCLUSION

1- The high concentration of 250 mg/kg of BABA led to an increase in the gene expression levels of the five isoenzymes, while the lower concentrations had the opposite effect to the high concentrations of the amino acid, that is, they led to the inhibition of the isoenzymes below the normal level represented by the control sample.
2- The increase in BABA concentration caused the possibility of cardiovascular diseases, especially the enzymes LDA1 and LDA2, because they possess the LDHB subunit in a greater proportion than the rest of the enzymes.
3- High levels of LDH, LDB, and LDC indicate tissue damage and cell degeneration, as they are secreted into the blood in abundance because they contain the LDHA subunit, which is found in tissue cells of the liver, kidneys, and skeletal muscle
4- Treatment with the amino acid BABA at a concentration of 250 mg/kg increased the level of the enzyme Gamma glutamyltranspeptidase, which is responsible for oxidizing glutathione, and restored the amino acid cysteine, which is necessary to reduce damage to the body.

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Conflicts of interest
The authors declare that they have no potential conflict of interest concerning the authorship or publication of this article.

Significance Statement of the Research
Due to the importance of the amino acid β-amino butytic acid (BABA) as it stimulates the resistance of plants against biotic and abiotic stresses, and also because it is widely used in the preparation of anti-tumor, anti-AIDS, and other drugs, and the results of previous research for its anti-diabetic effect and to increase the body's immunity at some concentrations, and also because of the importance of Lactate dehydrogenase (LDH) enzyme in its effect on the activity of cancer cells. Its analysis is used to determine the extent of the development of cancer, and it is also used to predict Corona disease Covid 19. As for choosing the enzyme Gamma glutamyletransferase (GGT), it is one of the important liver enzymes for predicting liver diseases and because it rids the body of free radicals and restores Cysteine.

Research Objectives
1- Identify the effect of the amino acid (BABA β-amino butyric acid) on the gene expression of lactate dehydrogenase isotypes.
2- Identify the effect of the amino acid BABA on the enzyme Gamma GlutamyleTransferase in rats injected with the acid.
3- Knowing the effect of the amino acid BABA on the enzymatic and genetic aspects of the animal
4- Giving importance to studying the effect of the amino acid BABA on animals because of its extremely important effects in using it as a new treatment option for many diseases.

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