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An investigation of acute effects at various doses of malathion on glucose homeostasis and insulin resistance in rat liver, pancreas and serum

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ABSTRACT

Objective. This study investigates acute effects of various doses of Malathion on glucose homeostasis and insulin resistance in rat.

Methods. Rats were randomly divided into four groups of 6 animals each. Corn oil was given orally to Group 1. Group 2, Group 3, and Group 4 received malathion dissolved in corn oil via oral administration at the doses of 100, 200 and 400 mg/kg, respectively. 24 hours later the rats were sacrificed.

Results. Acute administration of Malathion led to a decrease in serum butryl cholinesterase (BChE) levels at all doses tested. It also caused a significant increase in serum advanced glycation end products (AGEs), insulin, and TNF-α levels at all doses. Moreover, Malathion administration raised the liver ALT, AST and LDH, TNF-α, and glycogen levels in a dose dependent manner. It also led to a remarkable increase in pancreatic insulin levels at all doses.

Conclusions. Acute administrations of Malathion affect glucose homeostasis in a dose dependent manner through its effects on the liver, serum, and pancreas.

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Introduction

Pesticides, which are widely used all over the world, are serious threats to the environment and public health. In many countries, pesticide poisoning has been reported to be the primary cause of morbidity and mortality. Organophosphates constitute 50% of the total recorded cases of pesticide poisoning amongst the pesticide family [1]. Malathion is one of the earliest developed organophosphate insecticides. Malathion (2-(dimethoxythiophosphorylthio) succinic acid diethyl ester) is a non-systemic, broad-spectrum organophosphate insecticide [2]. Its LD50 value for rat is 1375 mg/kg; NOEL value is 25 mg/kg/day for both rats and humans; ADI value is 0,2 mg/kg/day, and its LEL value is 0,34mg/kg/day for humans [3]. Malathion, like other organophosphates, inhibits acetyl cholinesterase, which can be life-threating for many species of insects, animals, and humans [4]. Severe cholinesterase inhibition has been observed in people exposed to Malathion both acutely and chronically [2].

By affecting carbohydrate metabolism, Malathion may lead to inflammation, oxidative stress, and tissue damage. Earlier studies have suggested that Malathion could induce hyperglycemia through its impacts on glucose metabolism in the liver as well as skeletal muscle. It is also known to enhance glycogenolysis in the liver and skeletal muscle and gluconeogenesis in the liver [4]. It has been shown that acute Malathion poisoning results in enhanced glycogen deposition in liver within 6-24 h. [5].

Literature indicates that chronic hyperglycemia affects insulin release and this pathophysiologic process, which is

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called glucotoxicity, leads to insulin resistance [6]. Moreover, studies show that Malathion causes oxidative stress both in vivo and in vitro [7, 8]. In addition, organophosphates cause the formation of pro-inflammatory cytokines, which are responsible for the activation of the Janus kinase and I-kappa B kinase beta (IKKβ) pathways [9].

Subchronic administration of Malathion has been shown to influence glucose metabolism in rat muscle [4] and liver [10], but its acute administration influences hormonal control of glucose metabolism [11]. In addition, experimental studies have shown that chronic administration of Malathion increases glycogen [4, 12].

The purpose of this study was to investigate the acute effects of Malathion administration on glucose metabolism at various doses and determine the mechanisms of insulin resistance as well as to illuminate the pathway of liver glycogenesis and gluconeogenesis.

Materials and Methods

This study was approved by University Local Ethics Board (code number G.U. ET. 14.015). All chemicals used in the study were purchased from SIGMA. In the study, 24 Wistar albino (weight ~ 230 g) female rats were used. Animals were randomly divided into four groups of 6 animals each. Group 1 (Control group) was given corn oil. Group 2, Group 3, and Group 4 were administered 100, 200 and 400 mg/kg Malathion, respectively. Malathion was dissolved in corn oil and administered by oral gavage. All rats had access to standard rat feed and tap water ad libitum. The rats were sacrificed 24 hours after Malathion administration. Blood samples were taken via cardiac puncture under anesthesia and transferred to regular biochemistry tubes for serum tests. Blood samples were centrifuged at 3500 rpm at 4 ^{0}C for 10 minutes to separate serum. The liver and pancreas tissues were removed and washed with serum physiologic solution. Tissue samples were kept at -80 ^{0}C until homogenization. Then tissue samples were homogenized in ten volumes of Tris-HCl buffer using a homogenizer. The homogenates were then centrifuged at 3500 rpm and supernatants were separated and kept at -80 ^{0}C until analysis.

Blood Glucose Test measured with Roche Diagnostics brand Cobas E411 autoanalyzer. As a test principle, hexokinase catalyzes formation of glucose-6-phosphate by using ATP. Glucose-6-phosphate is oxidized to gluconate-6-phosphate in the presence of NADP. During the reaction, the rate of NADPH formation is directly proportional to the glucose concentration and is measured photometrically. Results are expressed as mg/dL. Serum BChE activity was measured with Roche Diagnostics brand Cobas E411 model autoanalyzer. ChE catalyzes the hydrolysis of butyrylthiocholine to thiolcholine and butyrate. Thiolcholine reduces yellow (III) hexacyanoferrite to colorless hexacyanoferrate (II). This reduction is measured photometrically. Results are expressed as U/L.

Serum and pancreatic tissue insulin levels, serum and liver tissue TNF-α levels, and serum AGEs levels were measured using commercially available kits (Shanghai YH Biosearch). These kits use an enzyme-linked immune sorbent assay (ELISA) method based on biotin double antibody sandwich technology to assay rat insulin, rat TNF-α, and rat AGEs. We added samples to the appropriate wells, which were pre-coated with insulin monoclonal antibodies, TNF-α monoclonal antibodies or AGEs monoclonal antibodies, respectively. After the incubation period, anti-insulin, anti-TNF-α or anti-AGEs antibodies labelled with biotin and streptavidin-HRP were added to form an immune complex. Unbound enzyme molecules were removed after incubation via automated washing, followed by the addition of substrate A and B. The solution turned blue during the incubation and changed to yellow with the addition of acid solution in order to stop the reaction. Developed color intensity and concentrations are positively correlated. Results are expressed as mIU/L for insulin, ng/L for TNF-α and ng/mL for AGEs.

The protein content of the liver tissues was measured by using the method described by Lowry et al [13]. Under alkaline environment, copper ion (Cu^{+2}) forms a complex with the peptide bonds in the proteins and is reduced to Cu^{+1}. Reduced copper and tyrosine, tryptophan, and cysteine amino acids in the side chain of proteins cause color formation by reducing the Folin-Phenol reagent. The intensity of the color formed is directly proportional to concentration of proteins, which was measured spectrophotometrically at 660 nm. Results are calculated as U/mg protein.

Liver tissue supernatant's ALT, AST and LDH activities were studied with Roche Diagnostics brand Cobas E411 model autoanalyzer at 1:50 dilution ratio. ALT catalyzes the reaction between L-alanine and 2-oxoglutarate leading to pyruvate. Pyruvate is reduced by NADH to L-lactate. The rate of NADH oxidation is directly proportional to catalytic ALT activity. ALT activity is determined by measuring decrease in absorbance. Results are expressed as U/mg protein.

AST catalyzes the reaction between L-Aspartate and 2-oxoglutarate leading to formation of L-glutamate and oxaloacetate. Oxaloacetate reacts with NADH in the presence of malate dehydrogenase (MDH) to form NAD^+. The rate of NADH oxidation is directly proportional to the

catalytic AST activity, and AST activity is determined by measuring the decrease in absorbance. LDH catalyzes the conversion of L-lactate to pyruvate. In this reaction, the rate of NADH formation is directly proportional to the catalytic activity of LDH, and LDH activity is determined by measuring the increase in absorbance.

Liver tissue weighing 500 mg was extracted with 1.5 ml of 30% KOH by incubating for 45 min at 95 ^{0}C and cooled. 2 ml H2SO4 were added to the liver extract to adjust its pH, then 0.2 % anthrone solution was added to the mixture. The developed color was measured spectrophotometrically at 620 nm. Results are expressed as mg/100 mg wet tissue [14].

Data Analyses

Statistical analysis of the data was carried out using Kruskal Wallis test and the $p \leq 0.05$ among the four groups. Mann-Whitney U test with Bonferroni correction was used to identify the significance level of the differences between pairs of two groups. Since 6 pairwise comparisons are possible for 4 groups, p value (0.05) was divided by 6 according to Bonferroni correction (0.05 / 6 = 0.0083). Differences between two groups were considered significant when $p<0.008$. Correlation analysis was performed using the Spearman correlation.

Results

Acute administration of Malathion led to a significant decrease in serum BChE levels in Group 2, 3 and 4 compared to Group 1 (p=0.002, p= 0.002, and p=0.002, respectively) (Figure 1). At 24h after administration of Malathion at doses of 100, 200 and 400 mg/kg, glucose levels did not change significantly (p= 0.352) in Groups 2, 3 or 4 compared with Group 1 (Figure 2).

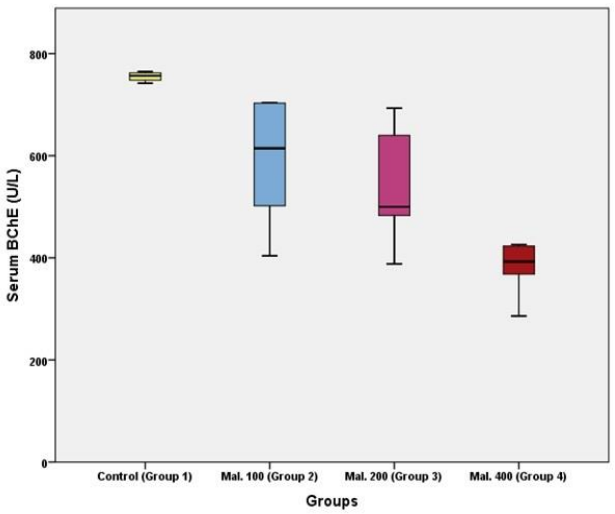

Figure 1. Serum BChE Levels

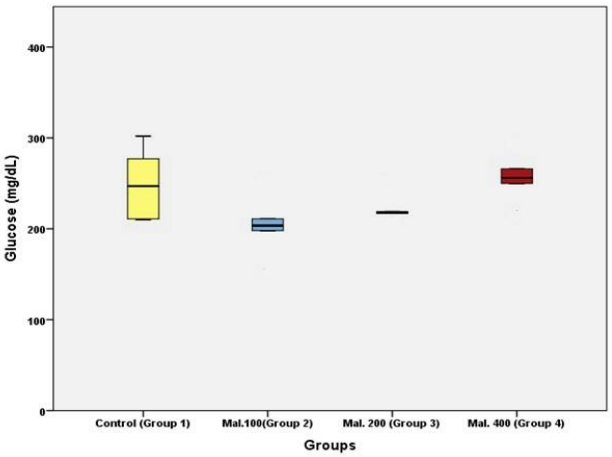

Figure 2. Serum Glucose Levels

There was a significant increase in serum insulin levels in Group 4 compared to Group 1 (p= 0.002) (Figure 3). In addition, serum TNF-α levels of Groups 3 and 4 were remarkably higher compared with Group 1 (p=0.002 and p=0.002, respectively) (Figure 4).

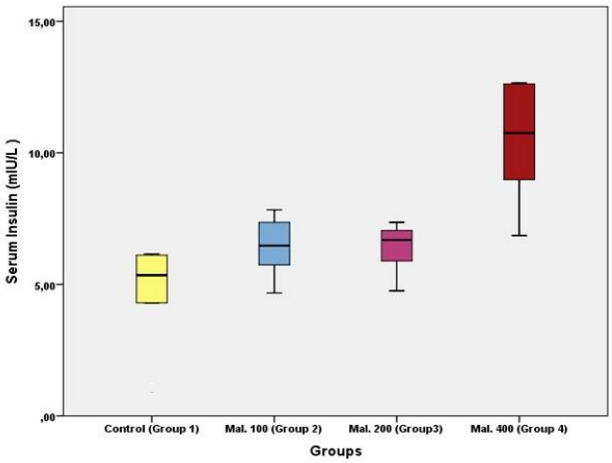

Figure 3. Serum Insulin Levels

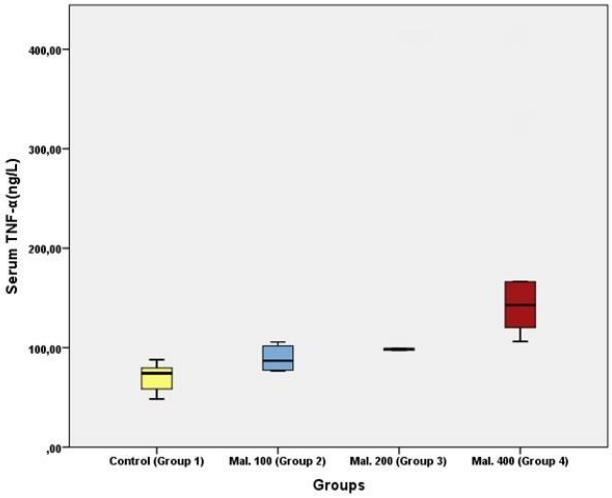

Figure 4. Serum TNF-alpha Levels

We found significant increases in serum AGEs levels in Groups 2 and 4 compared to Group 1 (p= 0.008, p=0.002) (Figure 5). Numeric results and significant differences of serum parameters are presented in Table 1.

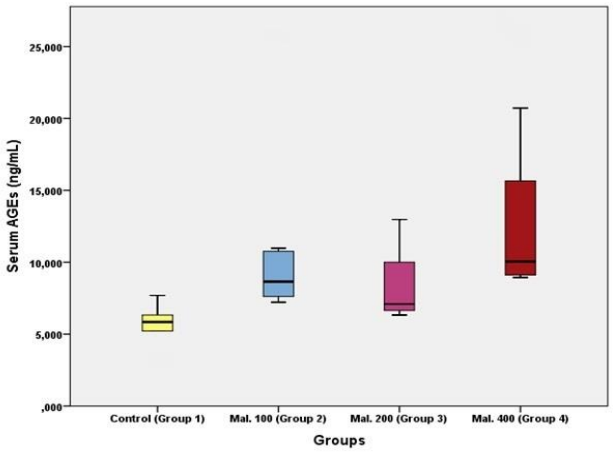

Figure 5. Serum AGEs Levels

Pancreas insulin levels of Group 3 and Group 4 were significantly higher compared with Group 1 (p=0.008 and p=0.004, respectively) (Figure 6) (Table 2).

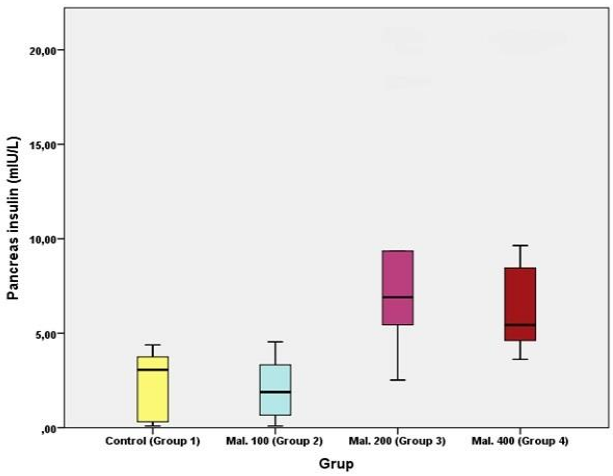

Figure 6. Pancreas Insulin Levels

There is a significant increase in the liver ALT (Figure 7), AST (Figure 8) and LDH (Figure 9) in Group 3 and Group 4 compared to Group 1 and Group 2 (p<0.008).

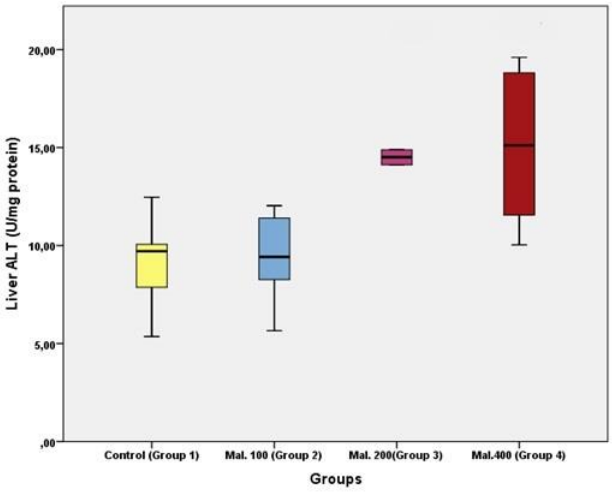

Figure 7. Liver ALT Levels

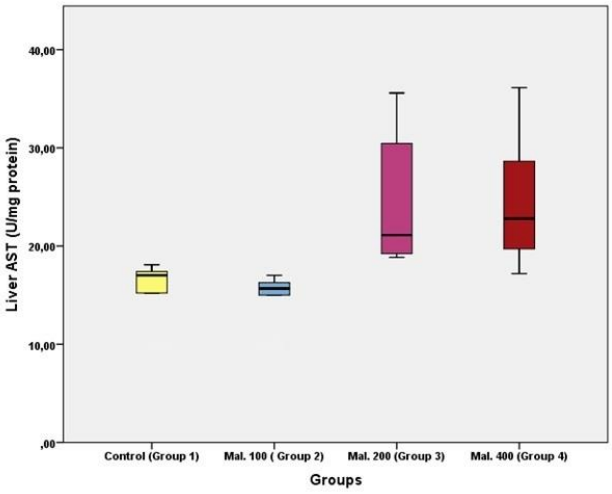

Figure 8. Liver AST Levels

Table 1. The numeric results and significant differences of serum parameters

Groups/Parameters	Group 1 (n=6) Mean ± SD	Group 2 (n=6) Mean ± SD	Group 3 (n=6) Mean ± SD	Group 4 (n=6) Mean ± SD
BChE (U/L)	755±9.1	590.3±118.8 [a]	534±112.8 [b]	381.3±53.7 [c]
Glucose (mg/dL)	249.00±36.6	203.83±31.6	223.6±17.99	256.33±25.2
Insulin (mIU/L)	4.71±1.9	6.42 ±1.1	6.40 ±0.9	10.4±2.4 [c]
TNF-α (ng/L)	70.5 ±14.5	89.2 ±12.1	97.9 ±4.8 [b]	167.7±81.3 [c]
AGEs (ng/mL)	5.71 ± 1.44	8.98 ± 1.60 [a]	8.35 ± 2.63	11.42 ± 4.79 [c]

BChE (Butryl Cholinesterase), TNF-α (tumor necrosis factor-alpha), AGEs (serum advanced glycation end products), SD (standard deviation), n (number of animals), a (significance p<0.008; difference between Group 1 and Group 2), b (significance p<0.008; difference between Group 1 and Group 3), c (significance p<0.008; difference between Group 1 and Group 4)

Table 2. The numeric results and significant differences of pancreas insulin level

Groups/Parameters	Group 1 (n=6) Mean ± SD	Group 2 (n=6) Mean ± SD	Group 3 (n=6) Mean ± SD	Group 4 (n=6) Mean ± SD
Insulin (mIU/L)	2.44 ± 1.82	2.07 ± 1.67	8.24 ± 5.42[b]	6.20 ± 2.35[c]

SD (standard deviation), n (number of animals), a (significance p<0.008; difference between Group 1 and Group 2), b (significance p<0.008; difference between Group 1 and Group 3), c (significance p<0.008; difference between Group 1 and Group 4)

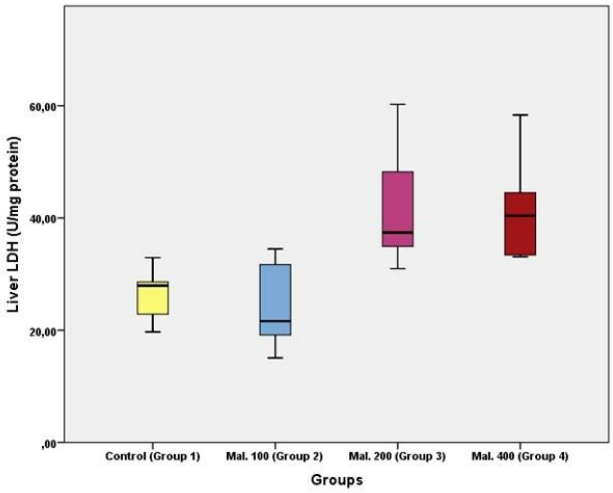

Figure 9. Liver LDH Levels

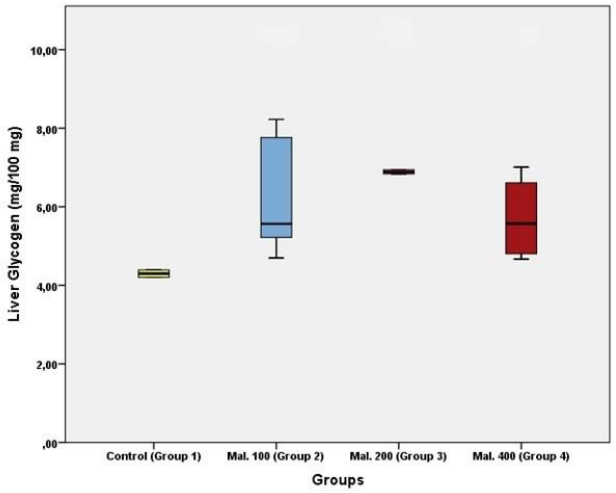

Figure 11. Liver Glycogen Levels

A statistically significant increase in TNF-α levels was found in Group 4 compared to Group 1 (p=0.008) (Figure 10).

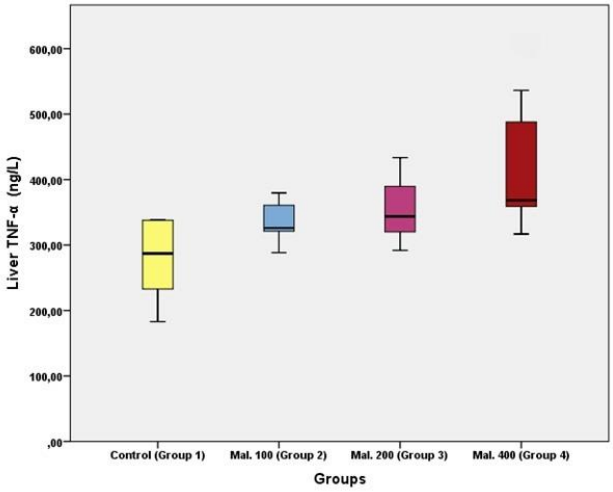

Figure 10. Liver TNF-alpha Levels

There was a statistically significant increase in glycogen levels in Group 2, Group 3, and Group 4 compared to Group 1 (p= 0.004, p= 0.002, p= 0.004 respectively) (Figure 11). Numeric results and significant differences of liver parameters are presented in Table 3.

According to the Spearman test, strong correlations were found between serum AGEs and insulin levels, BChE and serum insulin, insulin and TNF-α levels, and BChE and TNF-α ($p<0.01$) (Table 4). There was a positive correlation between serum insulin and pancreas insulin levels ($p<0.05$) and a strong negative correlation between BChE and pancreas insulin ($p<0.001$). We also found a positive correlation between serum insulin and liver glycogen ($p<0.01$) (Table 5).

Discussions

Malathion is an organophosphorus compound widely used in agriculture. The widespread use of organophosphate-based pesticides is often criticized for the risks associated with their neurotoxicity [15]. Neurotoxicity results in mitochondrial damage, ROS production, and DNA fragmentation in the cell [16]. Malathion induces toxicity via its bioactive analogue malaoxon [17]. There are accidental and/or suicidal exposure cases to high doses in the literature [18, 19]. In addition, workers in agriculture may also be exposed to such pesticides [20]. Studies indicate that acute and/or chronic exposure to organophosphates induces hyperglycaemia [4, 5].

Table 3. The numeric results and significant differences of liver parameters

Groups/Parameters	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)	Group 4 (n=6)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
ALT (U/mg pro.)	9.2 ± 2.4	9.3 ± 2.3	15.1 ± 2.4 [b,d]	15 ± 3.8[c,e]
AST (U/mg pro.)	15.8 ± 2.9	15 ± 2.5	24.4 ± 7 [b,d]	24.5 ± 7.1[c,e]
LDH (U/mg pro.)	26.7 ± 4.7	23.9 ± 7.6	41.5 ± 11[b,d]	41.7 ± 9.3 [c,e]
TNF-α (ng/L)	277 ± 60.8	333.7 ± 32.2	353.8 ± 50.6	406.2 ± 85.7[c]
Glycogen(mg/100mg)	4.3 ± 0.4	6.2 ± 1.5[a]	7 ± 0.8[b]	5.7 ± 1[c]

ALT (Alanine Aminotransferase), AST (Aspartate Aminotransferase), LDH (Lactate Dehydrogenase, TNF-α (tumor necrosis factor-alpha), SD (standard deviation), n (number of animals), a (significance $p<0.008$; difference between Group 1 and Group 2), b (significance $p<0.008$; difference between Group 1 and Group 3), c (significance $p<0.008$; difference between Group 1 and Group 4), d (significance $p<0.008$; difference between Group 2 and Group 3), e (significance $p<0.008$; difference between Group 2 and Group 4)

Table 4. Correlation analyses among serum parameters

Parameters		Glucose	Insulin	BChE	AGEs	TNF-α
Glucose	**r**	1.000	0.273	-0.043	-0.212	0.092
	p	.	0.208	0.845	0.332	0.678
	n	24	24	24	24	24
Insulin	**r**	0.273	1.000	-0.529	0.542	0.613
	p	0.208	.	0.008**	0.006**	0.001**
	n	24	24	24	24	24
BChE	**r**	-0.043	-0.529	1.000	-0.586	-0.843
	p	0.845	0.008**	.	0.003**	0.000**
	n	24	24	24	24	24
AGEs	**r**	-0.212	0.542	-0.586	1.000	0.695
	p	0.332	0.006**	0.003**	.	0.000**
	n	24	24	24	24	24
TNF-α	**r**	0.092	0.613	-0.843	0.695	1.000
	p	0.678	0.001**	0.000**	0.000**	.
	n	24	24	24	24	24

BChE (Butryl Cholinesterase), AGEs (serum advanced glycation end products), TNF-α (tumor necrosis factor-alpha), r (Correlation coefficient), p (significance), n (number of animals), ** (significance correlation at the 0.01 level (2-tailed))

Table 5. Correlation analyses between some serum parameters and pancreas insulin, liver glycogen

Parameters		Serum insulin	BChE	Pancreas insulin	Glycogen
Serum insulin	**r**	1.000	-0.529	0.485	0.472
	p	.	0.008**	0.016*	0.020*
	n	24	24	24	24
BChE	**r**	-0.529	1,000	-0.527	-0.367
	p	0.008**	.	0.008**	0.078
	n	24	24	24	24
Pancreas insulin	**r**	0.485	-0.527	1,000	0.274
	p	0.016*	0.008**	.	0.196
	n	24	24	24	24
Glycogen	**r**	0.472	-0.367	0.274	1,000
	p	0.020*	0.078	0.196	.
	n	24	24	24	24

BChE (Butryl Cholinesterase),), r (Correlation coefficient), p (significance), n (number of animals), ** (significance correlation at the 0.01 level
(2-tailed)), * (significance correlation at the 0.05 level (2-tailed))

Subchronic administration of Malathion significantly decreases serum BChE activity. Consistent with this, we showed that acute administration of Malathion at 100, 200 and 400 mg/kg to rats significantly inhibited BChE enzyme activity. Mentioned doses of Malathion were chosen due to their acute toxic effects, 100 mg/kg, which is known as toxic dose, 400 mg/kg, plateau level (70% inhibition of cholinesterase), and 200 mg/kg, considered an intermediate value [21].

Like fructose-induction [22], many studies have suggested that organophosphate pesticides have effects on glucose homeostasis. For example, glucose homeostasis has led to changes in liver glucose production, which is thought to have been altered by gluconeogenesis in Malathion toxicity [23]. In our study, differences between glucose levels of groups were found to be insignificant ($p>0.008$). Although the differences between the groups in our study was not statistically significant, we believe further investigation is required, as other studies have concluded that hyperglycemia might occur as a result of Malathion treatment [24]. In this context, a slight increase in blood glucose levels of Group 4 and no significant changes in blood glucose levels of Group 2 and Group 3 may be due to elevated insulin levels. We think that hyperglycemia may occur if the dose and/or exposure time were increased.

In our study, there was a significant increase in serum insulin levels in Group 4 compared to the control group. Hyperinsulinemia has been reported with impaired glucose homeostasis after acute exposure to organophosphate [25]. It is emphasized that reactive oxygen species (ROS) play an active role in the pathophysiology of insulin resistance. Some researchers have also mentioned that ROS led to insulin resistance by preventing threonine phosphatase activity [26]. In this study, the levels of serum AGEs were examined and differences were observed between the control group and the test groups. However, a statistically significant change was found in Group 2 and Group 4 compared to Group 1. Recent studies demonstrated that AGEs have a crucial role in complications developing in

diabetes, liver disorders, neurodegenerative disease, and even in some types of cancer [8]. It is proposed that enhanced levels of AGEs may lead to insulin resistance or Type 2 Diabetes by increasing oxidative stress, inflammation, and/or affecting insulin receptor substrate 1 (IRS-1) [27]. In line with this, we found a positive correlation between serum insulin and AGEs levels ($p<0.01$).

Many studies have hypothesized that insulin resistance is caused by inflammation and oxidative stress on glucose metabolism. According to these hypotheses, ROS leads to activation of serine/threonine kinases which inhibits of phosphorylation IRS-1 and this may lead to insulin resistance [8].

In addition to an increase in serum insulin, we found that pancreatic insulin levels were also significantly elevated in Group 4 compared to Group 1. Furthermore, the insulin levels of Group 3 and Group 4 were significantly higher than those of Group 2 ($p<0.008$). These results suggested that insulin production may increase to maintain glucose homeostasis. This study revealed that there is a correlation between serum insulin levels and pancreatic insulin levels ($p<0.05$). It is suggested that subchronic exposure of Malathion stimulates insulin secretion by pancreatic beta cells through inhibition of AChE activity [28]. Inhibition of AChE activity causes accumulation of acetylcholine that activates cholinergic receptors leading to an increase in insulin secretion. In a similar way, we observed that there are strong negative correlations between serum BChE and serum insulin levels as well as pancreatic insulin levels ($p<0.01$). Thus, our study revealed this relationship using acute doses of Malathion.

Several studies have shown that organophosphate compounds cause an increase in levels of TNF-α and IL-6, which in turn have a significant role in inflammation. For example, organophosphate compounds such as Malathion and diazinon have been shown to increase these cytokines [29, 30]. In this study, a statistically significant increase in both serum and liver TNF-α levels were observed in Group 4 compared to the control group ($p <0.008$). Although, there were increases in TNF-α levels in Group 2 and 3 as well, they were not significant.

Literature has emphasized that Malathion intoxication increases insulin secretion, parallel to an increase in blood glucose levels [31, 32]. Subchronic Malathion exposure studies have shown that insulin resistance is related to oxidative damage and inflammation [33]. In this study we found a strong correlation between serum TNF-α levels and serum insulin levels ($p<0.01$), consistent with previous studies.

Experimental studies have shown that chronic administration of Malathion modifies liver carbohydrate metabolism and increases the rate of liver glycogen synthesis [4, 12]. 24 hours after administration of 50 and 100 mg/kg Malathion, the amount of liver glycogen was increased [5]. In another study, Malathion increased liver glycogen synthesis within 2 hours after an acute dose of 400 mg/kg, the synthetic process peaked after 12 hours, and remained the same for the following 24 hours [34]. This finding demonstrates that Malathion inhibits the activity of glycogen phosphorylase enzyme and subsequently activates the enzyme glycogen synthase, which acts as an antagonist of glycogen phosphorylase enzyme [12]. It is also well known that insulin activates the synthesis of glycogen both in muscle and liver tissues. This synthesis of glycogen is catalyzed by glycogen synthase, which is the key enzyme in the pathway of glycogen synthesis [35].

In this study a statistically significant increase in glycogen in Groups 2, 3, and 4, compared to group 1 ($p<0.008$) was observed, in accordance with previous studies [5, 34]. We also found a positive correlation between serum insulin and liver glycogen ($p<0.05$)

It has been observed that a chronic dose of diazinon, which is a member of the organophosphate chemical family, decreased liver ALT, AST and LDH activities and enhanced lipid peroxidation. This finding suggests that lipid peroxidation damages the integrity of cellular membranes, resulting in leakage of cytoplasmic enzymes into the blood [36]. In contrast, in our study, acute doses of Malathion significantly increased liver ALT, AST, and LDH activities in all groups. Our results showed that liver ALT, AST, and LDH activities of Groups 3 and 4 were higher than that of Groups 1 and 2 ($p<0.008$). This could be due to differences in dosage administration and also the period of exposure. This suggests that acute doses of organophosphates may not change the integrity of the cellular membrane.

In another study, chlorpyryfos, a member of the organophosphate family, caused hyperglycemia and resulted in the activation of the gluconeogenesis pathway. Tyrosine aminotransferase (a glucogenic enzymes) also increased when an acute dose of chlorpryfos was administered [37]. Similarly, we propose that ALT, AST, and LDH (also glucogenic enzymes) could increase due to activation of the gluconeogenesis pathway.

Conclusions

According to our results, acute administration of Malathion influences glucose homeostasis, affecting the liver, serum, and pancreas in a dose dependent manner. Therefore, we suggest that necessary precautions be taken to protect the public from the damaging effects of organophosphates even after an acute exposure.

We recommend that further studies be conducted to elucidate the exact mechanisms of actions of Malathion on glucose metabolism. After acute administration of

Malathion, it is also important to analyze the levels of all glycogenic enzymes, cytokines, and other biomolecules. Investigation of the relationship between insulin resistance, inflammation, oxidative stress and gluconeogenesis is also thought to be essential.

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