Original Article

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Effects of Caffeine Mixed with Alpha Lipoic Acid in Preventing Streptozotocin-induced Diabetes in Rats: *In Silico* and *In vivo* Study

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ABSTRACT

Objective: This study aimed to investigate the combined effects of caffeine and Alpha Lipoic Acid (ALA) on oxidative stress, due to chronic hyperglycemia, in a model of diabetic rats induced with streptozotocin (STZ) (*in silico* and *in vivo* approaches).

Material and Methods: This *In silico* study investigated the interaction between caffeine and ALA against insulin receptors and enzymes of Glutathione Peroksidase-1 (GPx-1), with molecular docking. Male, Wistar rats were included using a quasi-experimental research design, with post-test only and a control group (*in vivo*). This study measured the end result of a 6-week-induction on body weight, fasting blood glucose, Malondialdehyde (MDA) and GPx-1 enzyme from 25 rats.

Results: Molecular docking found the interactions of caffeine and GPx-1 consisting of an energy bond of -5,06 kcal/mol, hydrogen and hydrophobic bond. Additionally, it showed the interaction of ALA and GPx-1 containing an energy bond of -5.16 kcal/mol, hydrogen bonding and hydrophobicity. However, there were no significant difference in body weight, fasting blood glucose, MDA and GPx-1 levels of the ALA-caffeinated diabetic rats compared to diabetic rats.

Conclusion: Caffeine and ALA have the potential to activate GPx-1 enzymes (*in silico* study). However, the use of a caffeine and ALA combination resulted in no significant difference in fasting blood glucose and oxidative stress conditions when compared to diabetic rats without additional induction (*in vivo* study).

Keywords: ALA, caffeine, diabetes, GPx-1, MDA

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Introduction

Most of the world's population clearly cannot avoid diabetes, due to various aspects of their life styles. By 2021, the number of cases globally could reach 537 million people with diabetes. Furthermore, this number is predicted to continue to increase to 643 million people in 2030, and 783 million people in 2045[1]. Known as a metabolic disease, diabetic is characterized by an increase of uncontrolled glucose levels in the blood (hyperglycemia)[2]. The causative factor is the lack of insulin production, which functions as an activator of protein transporters in capturing glucose into cells[3]. Both of these deficiency problems can be influenced by chronic hyperglycemia experienced by diabetics, in that it triggers oxidative stress problems in cells[4]. In this case, excessive blood sugar levels continuously trigger glucotoxicity, which results in various complications; especially at the system levels, such as macrovascular and microvascular[4].

Glucotoxicity will also trigger oxidative stress in cells within various, vital organs; one of which is the liver. Particular, anti-insulin hormones are released when there is severe stress, which then triggers a gluconeogenic response in the liver. Oxidative stress caused by chronic hyperglycemia will create the production of reactive oxygen species (ROS) through non-enzymatic glycosylation activity[5]. Elevated ROS levels can cause damage to the lipids that arrange the structure of cell membranes. This condition then produces many aldehydes, one of which is the most mutagenic, malondialdehyde (MDA)[6]. MDA is a general indicator of oxidative stress in the form of lipid peroxidation that occurs in cells[7]. In diabetic conditions, increased MDA levels are directly proportional to the increase in blood sugar levels[8]. Normally, there are enzymes in cells that work to control oxidative stress, such as Superoxide dismutase (SOD), Catalase (Cat) and Glutathione Peroxidase-1 (GPx-1)[9]. However, in hyperglycemic conditions, some enzymes will experience a deficiency; especially the GPx-1 enzyme; wherein, this Homozygous defect in the GPx-1 gene is a contributing factor to the problem[10].

The pathophysiological model of hyperglycemia can be applied to rats that are induced with streptozotocin (STZ). STZ is cytotoxic, with pancreatic beta cells being the main target of induction[11]. Briefly, STZ acts by crossing the membranes of glucose transporter 2 (GLUT-2) in pancreatic cells: STZ then results in deoxyribonucleic acid (DNA) alkylation. In addition, the release of nitric oxide (NO) into cells will inhibit aconitase activity; thereby, increasing DNA damage[12]. DNA damage in pancreatic cells will result in impaired insulin production; hence, the blood glucose levels in rats will increase[13]. When induced with STZ, at a dose of 50-65 mg/kg, rats would experience an increase in their eating and drinking frequency without gaining body weight[14].

Therefore, several methods can be used to reduce the effects of oxidative stress due to hyperglycemia. One of related studies, conducted by Abunasef and colleagues, on the harnessing caffeine in STZ-induced rats for 6 weeks, found that the induction of 100 mg/kg of caffeine was effective in reducing hyperglycemia, and could restore pancreatic beta cell function[15]. Similarly, another study, by Kagami et al., explained that the use of caffeine in the dose range of 10–100 mg/kg was protective against pancreatic beta cells in STZ-induced rats[16]. In addition, caffeine can repair cell damage, because it has an antioxidant effect; as found in the study of Devasagayam et al. that studied the effect of caffeine in vitro on liver oxidative stress[17].

In addition to caffeine, another active substance that can reduce the effects of hyperglycemia in diabetic rats is Alpha Lipoic Acid (ALA). ALA is the result of the enzymatic synthesis of octanoic acid and cysteine in the mitochondria of plant and animal cells[18]. Their study results explained that the induction of ALA 100 mg/kg was effective in reducing hyperglycemia in rats that were fed with a high-fat diet for

4 weeks[19]. Another study, by Maritim et al., found that the induction of ALA can also increase antioxidant enzymes. In that study, the induction of ALA at a dose of 50 mg/kg caused an increase in the antioxidant enzyme (GPx-1) in normal rats. Even a small dose of 10 mg/kg also resulted in an increase in the GPx-1 enzyme in STZ-induced rats[20].

ALA and caffeine contained in food sources, such as plants and animals, are generally consumed by humans. Although ALA is contained in both plants and animals, caffeine is generally contained in coffee[21]. Although people consume coffee and food sources containing ALA together to meet nutritional needs[22], it is not yet known whether the combination of the two has a positive effect on the body; especially in diabetics. Additionally, to the best of our knowledge, there are no studies that explain the effects of using caffeine and ALA on the problem of hyperglycemia. Therefore, this study investigated the effect of the combination of caffeine and ALA on the problem of hyperglycemia in STZ-induced rats.

Material and Methods

In silico study

An *In silico* study was conducted, by analyzing the interaction between caffeine and ALA on insulin receptors and antioxidant enzymes (GPx-1), using the molecular docking method. The computer equipment used in the interaction prediction test consisted of a Central Processing Unit (CPU) facility equipped with an Intel(R) Core (TM) i7-10700 processor, @ 2.90GHz 2.90 GHz, 8.00 GB RAM. The operating system (OS) installed on the CPU was Microsoft Windows 10 pro.

Ligand data preparation

Data on the compound of caffeine and ALA were obtained from the *Pubchem* site (https://pubchem.ncbi.nlm.nih.gov/). The caffeine used in this study was coded Pubchem CID_2519, while the ALA used was coded as Pubchem CID_864. Both the Caffeine and ALA ligand, downloaded from the *Pubchem* website, were prepared using ChemDraw 12.0 software and AutoDock Tools version 1.5.6. Both files were given additional gasteinger and hydrogen charges. In addition, to give the ligand a flexible effect, an additional torsion was added.

Receptor data preparation

The insulin receptor and GPx-1 enzyme data were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RSCB PDB) website (https://www.rcsb.org/). Prior to the utilization for docking, receptor preparation was carried out using the Biovia Discovery Studio 4.0 software. All other unused molecules, such as water molecules and unnecessary ligands were cleared from the receptor. The insulin receptor in PDB code was 4XST, DOI: http://doi.org/10.2210/pdb4XST/pdb, whilst the PDB enzyme code was GPx-1: 2F8A, DOI: http://doi.org/10.2210/pdb2F8A/pdb. Both of the insulin receptor and enzyme GPx-1 were prepared using AutoDock. Additionally both receptor files had added Kollman charges and were hydrogen polar.

Docking process

The prepared ligand and receptor data were continued into the process of docking using AutoDock. A grid map, with a size of 100x100x100, was placed at the center position of the ligand and receptor. Ligand conformation was added with Lamarckian Genetic Algorithm (LGA) consisting of 1,000 repetitions for each bond. The results of the docking conformation were then seen from their smallest to largest interaction energy values. The conformational energy was expressed in free energy binding utilizing units of kcal/mol. The other parameters analyzed were the hydrogen and hydrophobicity bonds. Docking results, in the form of interactions between ligands and receptors, were then displayed in 3D images using Biovia Discovery Studio software.

In vivo study

Animal preparation

This study obtained a permit to use experimental animals for research from the ethics commission of the Integrated Research and Testing Laboratory, Gadjah Mada University (Certificate Number: 00112/04/LPPT/IX/2017). This study measured the intervention results of 25 male Wistar rats. The rats were obtained from the Biopharmaceutical Laboratory. The average body weight of the rats was 140–200 g, and they were aged at 3–4 months. The rats were given time to adapt for 7 days. They were cared for at a temperature of ±250 C, 60.0% humidity, exposed to a light and dark cycle of 12 hours each, and had free access to food and water.

Diabetes induction

STZ induced in this study was of the brand Lacanai and Purebulk, Inc. was the brand of anhydrous caffeine. ALA (L5008) was purchased from Tokyo Chemical Industry (TCI). The rats that received STZ induction were given as much as 60 mg/kg in a single intraperitoneal (i.p) dose. The STZ was dissolved in sodium citrate, pH 4.5, to ensure that the rats had diabetes; early blood glucose levels were measured on the third day after STZ induction. The rats were then fasted for 16 hours. In this regard, blood glucose levels are said to be diabetic if >126 mg/dL[23]. Afterward, the final measurement of their blood glucose levels was carried out in the sixth week.

Caffeine and ALA induction

Induction of caffeine and ALA was performed in the positive diabetic group once a day, over 6 weeks. Caffeine was induced orally in rats at a dose of 60 mg/kg. Meanwhile, ALA induction was also carried out orally at a dose of 50 mg/kg; each was performed once a day.

Research design

The design used in this research was a quasi-experiment with a control group. A total of 25 rats used in this study were divided into 5 groups. The division of groups is shown in Table 1.

Table 1 Rats groups and applied interventions

Groups	Interventions	n
N	Normal (no treatments)	5
D	STZ 60 mg/kg BW i.p	5
DC	STZ 60 mg/kg BW i.p, Caffeine 60 mg/kg BW orally	5
DA	STZ 60 mg/kg BW i.p, ALA 50 mg/kg BW orally	5
DCA	STZ 60 mg/kg BW i.p, Caffeine 60 mg/kg BW orally, ALA 50 mg/kg BW orally	5

BW=body weight

Analysis of MDA and GPx-1 levels

Analysis of MDA and GPx-1 levels used the Sandwich Enzyme-Linked Immunosorbent Assay (Sandwich ELISA) method. From this, 100 mg of liver organ was homogenized into 900µl of cold Phospate-buffered Saline (PBS). Each sample concentration for MDA examination was 1:2 (without dilution) and GPx-1 1:100. The test results were measured by a curve equation, and presented in a unit of ng/0.1 g tissue.

Statistical analysis

Statistical analysis was performed by observing the normality of the data, based on the Saphiro Wilk test. For the parameters of MDA and GPx-1 enzyme levels, statistical tests were carried out using one way ANOVA. For the parameters of the rat's body weight and blood glucose, the researchers conducted a statistical test using Kruskal-Wallis dan Mann-Whitney, due to abnormal data distribution.

Results

In silico study

The results of molecular docking indicated that there were interactions in the form of negative energy values as well as hydrogen-hydrophobicity bonds between caffeine and ALA against the GPx-1 enzyme (Figure 1 and Table 2).

In vivo study

Effects of caffeine on body weight

The results of the Kruskal-Wallis test showed that there was a significant difference (p-value<0.05) in the body weight of all groups. Based on the results of the *Mann-Whitney* test, a significant difference in body weight was shown between groups N (234±25.09 g) and D (143 ±12.04 g), with a p-value of 0.04. Significant difference was also found between the groups D and DC (227.6 ± 35.92 g) with a p-value=0.04 (Figure 2).

Effects of caffeine on fasting blood glucose levels

Examination of blood glucose levels of rats, based on the Kruskal Wallis test, found significant differences (p-value<0.05) between all groups. Meanwhile, based on the results of the Mann-Whitney test, it was found that there was a difference between groups N (59.98±8.32 mg/dL) and D (471.96 ± 25.01 mg/dL), with a p-value of 0.04. Additionally, a significant difference in blood glucose levels was found between the N and DA groups (239± 179.513 mg/dL), with a p-value=0.04. However, the DCA group (251.72±222.94 mg/dL) in this study was found to be different from group D, with a p-value=0.01. Meanwhile, the DC group (149.26±128.3 mg/dL) was found to be significantly different from the D group, with a p-value=0.04

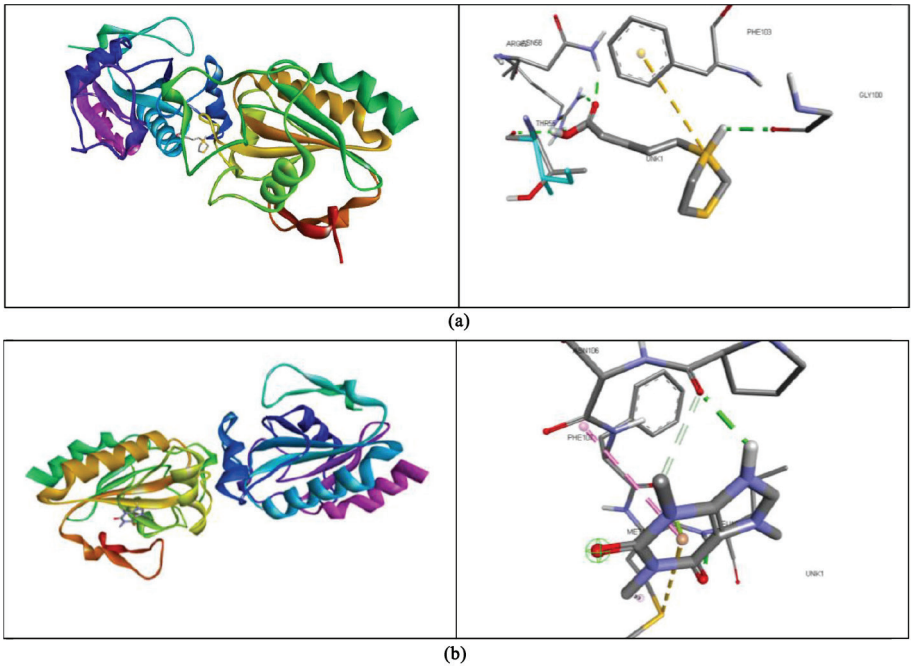

Figure 1 The results of the Molecular docking test, showing 3D interactions: (a) Alpha Lipoic Acid (ALA) and Glutathione Peroxidase-1 (GPx-1) (b) Caffeine and GPx-1 enzyme. ALA and caffeine did not form interactions; including hydrogen and hydrophobic bonds with insulin receptors

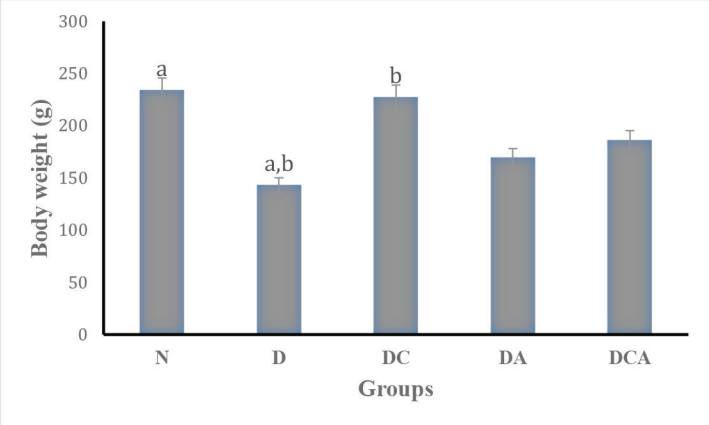

Figure 2 The results of the final examination, based on the Mann-Whitney test, there was significant difference between the body weights of the rats in group N: Normal – D: STZ 60 mg/kg; D-DC: STZ 60 mg/kg and Caffeine 60 mg/kg (a, b indicates p-value<0.05)

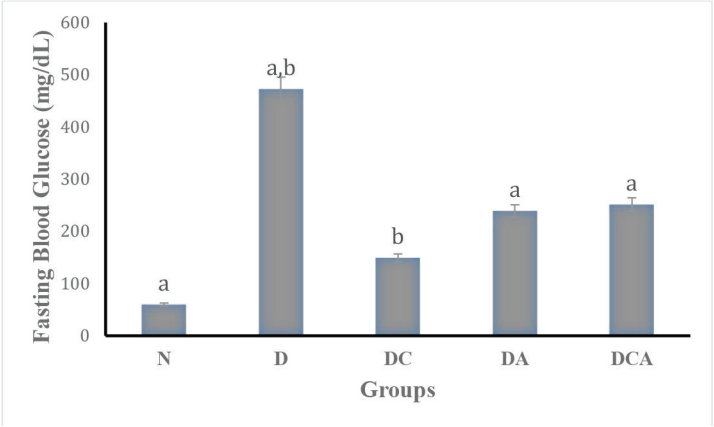

Figure 3 The results of the final examination of fasting blood glucose levels, based on the Mann Whitney test, there was a significant difference between the blood glucose levels of rats in groups N: Normal-D: Streptozotocin (STZ) 60 mg/kg, N-DA: STZ 60 mg/kg. kg and ALA 50 mg/kg, N-DCA: STZ 60 mg/kg, Caffeine 60 mg/kg and ALA 50 mg/kg; D-DC: STZ 60 mg/kg and Caffeine 60 mg/kg (a, b indicates p-value<0.05)

Table 2 The results of the Molecular Docking Test Prediction, between caffeine ligand (Pubchem CID_2519) and ALA (Pubchem CID_864) against insulin receptor (PDB ID:4XST) and enzyme Glutathione Peroxidase-1 (GPx-1) (PDB ID: 2F8A)

Molecules	Caffeine			ALA		
	Free Energy Binding (Kcal/mol)	Hydrogen Interactions	Hydrophobic Interactions	Free Energy Binding (Kcal/mol)	Hydrogen Interactions	Hydrophobic Interactions
Insulin Receptor	0.00	not detected	not detected	1.84	not detected	not detected
GPx-1	-5.06	Met108 A; Phe105 A; Asn106 A	Leu109 A; Pro105 A	-5.16	Thr55 B; Arg62 B; Asn58 B; Gly100 B	Phe103 B

Kcal/mol=kilocalorie per mole, ALA=alpha lipoic acid

The effect of caffeine on MDA levels and GPx-1 activities

The results of the *ANOVA* test found no significant difference (p-value>0.05) between all groups, both in MDA and GPx-1 levels (Table 3).

Table 3 The results of the examination for the status of oxidative stress: Malondialdehyde (MDA) and Glutathione Peroxidase-1 (Gpx-1). Based on the results of the ANOVA test, there was no difference between all groups N: Normal; D: Streptozotocin (STZ) induction 60 mg/kg; DC: induction of STZ 60 mg/kg and caffeine 60 mg/kg; DA: Induction of STZ 60 mg/kg and ALA 50 mg/kg BW; DCA: STZ induction 60 mg/kg, Caffeine: 60 mg/kg and ALA: 50 mg/kg

Groups	Oxidative stress measurements (ng/0,1 g tissue)	
	MDA	GPx-1
N	75.28±7.73	75.86±7.21
D	99.32±18.61	67.28±11.36
DC	90.34±8.61	79.52±4.03
DA	95.78±18.26	71.9±11.67
DCA	88.4±22.01	86.84±5.67

MDA=malondialdehyde, GPx-1=glutathione peroksidase-1
ng/0,1 g tissue=nanograms per 0,1 gram liver tissue

Discussion

The results of this study found that the body weight of STZ-induced rats was lower than that of normal rats, and in groups that received caffeine therapy (Figure 2). This weight loss could be caused by the activity of lipolysis. In other words, STZ can activate the performance of hormone -sensitive lipase (HSL) through the activation of adenylyl cyclase to stimulate the process of lipolysis in adipocytes[24]. Additionally, Chronic hyperglycemic concentrations cause adipocytes to increase their lipolytic activity. Hyperglycemia also maintains HSL concentrations, so that lipid droplet breakdown activities in cells remain active[25].

In this study, rats induced with STZ and caffeine had heavier body weight than decaffeinated STZ rats (Figure 2). Therefore, the results of this study are in line with the research of Naidoo et al. Although, there were no differences compared to the decaffeinated STZ group, the STZ and caffeine groups experienced weight gain over 13 weeks. It should be noted that in their study, Naidoo et al. used caffeine doses of 20 mg and 40 mg/kg[26]. Meaning, this can be caused because the caffeine is controlling oxidative stress, due to STZ induction through improving the function of antioxidant enzymes[27,28].

Meanwhile, based on the docking molecular test in this study, it was found that there was a bond between

caffeine and the GPx-1 enzyme (Table 2). Caffeine was found to interact to form hydrophobic bonds by binding to Leu109A and Pro105 A. Leucine (Leu) is an amino acid that plays an important role in protein synthesis and metabolic function[29]. The amino acid leucine can increase the activity of the GPx-1 enzyme in neutralizing free radicals, such as anti-superoxide anion (ASA) and anti-hydroxyl radicals (AHR)[30]. Proline (Pro) is an amino acid that has a function similar to that of Leu, such as regulating gene expression and cellular mechanisms for disease prevention through stimulation of antioxidant enzymes[3]. While other amino acids form hydrogen bonds between caffeine and GPx-1, such as Met108 A, Phe105 A and Asn106 A (Table 2). These three amino acids also have a role as a stimulator of antioxidant enzymes; especially in the liver[32,33].

On the other hand, the *in vivo* test results in this study did not find significant difference in the activity of the GPx-1 enzyme in the decaffeinated STZ rats group, nor in the caffeine-induced STZ rats (Table 3). Additionally, the activity of the GPx-1 enzyme did not differ when compared to the STZ group induced by the combination of caffeine and ALA. This could be due to the absence of a synergistic effect between caffeine and ALA on the GPx-1 enzyme. Thus, the synergistic combination can increase the therapeutic benefits; especially in providing more precise biological control[34]. Besides GPx-1 enzyme levels, the combination of caffeine and ALA also did not produce significant differences in fasting blood glucose (Figure 3) and MDA (Table 3).

As found in this study, the effect of caffeine on glucose resulted in lower fasting blood glucose than that of the decaffeinated STZ rats groups (Figure 3). In other words, lower blood glucose levels are thought to be due to the cytotoxic oxidative stress of STZ rectified by the GPx-1 enzyme. This finding is supported by the results of docking studies that have been carried out previously, in that is there was no interaction between caffeine and amino residues on insulin receptors (Table 2). Hence, the results of this study affirm the research conducted by Abunasef et al. In their study they explained that blood glucose levels in STZ rats induced by caffeine at a dose of 100 mg/kg per day had improved[15].

The pancreatic beta cells in the STZ rats group that received induced caffeine improved, based on microscopic observations. Thus, it is possible that caffeine inhibits the performance of the enzyme poly ADP-ribose polymerase-1 (PARP-1), which is activated when DNA damage occurs due to STZ-induced[15].

Based on the *in silico* study, ALA in this study, also had an interaction with the GPx-1 enzyme. However, as to the *in vivo* study of caffeine, the researcher found no significant difference in GPx-1 levels of STZ rats with ALA induction compared to those without ALA induction (Table 3). ALA, tested using molecular docking in this study, was found to interact with GPx-1 through the amino acids Thr55 B, Arg62 B, Asn58 B and Gly100 B forming hydrogen bonds; whereas, the hydrophobic bonds were formed by ALA by binding to the amino acid Phe103 B (Table 2). Threonine (Thr) is an amino acid that plays a role in assisting in the protection of lipid metabolism in the liver[35]. In addition, the interaction with Thr can activate the expression of thermogenic genes associated with lipolysis activity[35]. This is predicted to be a factor as to why the bodies of the STZ rats induced with ALA or caffeine and ALA in this study were not significantly different from the group of STZ rats without caffeine or ALA induction (Figure 2). ALA binding to the amino acid Glycine (Gly) that has been shown, based on *in silico* studies, is predicted to trigger the antioxidant activation process that can prevent damage to cells, due to 4-hydroxynonenal (4-HNE)[36].

Previous studies have also explained that ALA has antioxidant properties. The mechanism of the action of ALA studied is similar to that of caffeine, which is able to repair pancreatic beta cell damage due to cytotoxicity

from STZ induction. This was evidenced in the study of Budin et al., who found that there was an improvement in the pancreatic beta cells of diabetic rats models after STZ induction[37]. Accordingly, ALA reduces ROS by increasing enzyme activity, so that pancreatic beta cells can once again function in the production of insulin to control blood glucose levels[37].

Conclusion

To conclude, the use of a combination of caffeine and ALA did not produce any difference in fasting blood glucose parameters, nor oxidative stress status when compared to STZ rats without additional induction (*in vivo* study). However, as a result from molecular docking caffeine and ALA have the potential to activate the GPx-1 enzyme, because there are interactions that form hydrogen and hydrophobic bonds (*in silico* study). Nevertheless, further exploratory studies are required in terms of the use of the combination of caffeine and ALA on the effect of STZ in rats; especially to identify the effects of synergistic performance as well as antagonistic induction.

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Conflict of Interest

The authors declare there are no conflict of interests.

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