The Effect of Formetanate Hydrochloride on the Glycated Human Hemoglobin

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ABSTRACT: *Glycation refers to the nonenzymatic glycosylation of the free amino groups of proteins and sugars. Advanced Glycation End-products (AGEs) are the final stage in the glycation process. AGEs cause many complications in diabetic patients. Fortunate hydrochloride is a highly effective pesticide widely used in agriculture. Hence, all human beings, both healthy and diabetic-affected patients, can be exposed to this toxin. Therefore, the purpose of the present research is to study, the effect of Formetanate hydrochloride upon the glycated human hemoglobin (GHb). To form glycated hemoglobin, Hb was incubated with glucose for 35 days under physiological conditions (dark, 37 °C, and pH 7.4). The effect of the toxin on GHb was investigated via docking studies, fluorometry, UV-Vis, and circular dichroism spectroscopy. Incubating Hb with glucose could degrade the structure of the protein. Samples containing GHb and formetanate hydrochloride showed remarkable changes in the structure; Heme-group degradation and an increase in β-sheet structures was also observed. The results of docking studies were consistent with these results. As diabetes is rapidly expanding in today's world and formetanate hydrochloride is widely used in agriculture, the impact of this toxin on these patients will be very important. According to the results obtained, this toxin can have a more destructive effect on the glycated Hb in these patients.*

KEYWORDS*: Hemoglobin glycation; Formetanate hydrochloride; Hb-AGE; Agricultural pesticide.*

INTRODUCTION

Formetanate hydrochloride [3-(dimethylaminomethylideneamino)phenyl] N-methylcarbamate; hydrochloride] is a highly effective acaricide and insecticide[1-3] widely applied to a variety of fruits and citrus, including grape, apple, pear, peach, mango, nectarine, orange, tangerine, watermelon, grapefruit, lime, pepper, bean, onion, and tomato [4,5]. The very high toxicity of Formetanate hydrochloride (effective dose (rats) 14.8: mg per kg) and the very high solubility of this substance in water may lead to its negative impact on non-target organisms such as humans and ecosystems[6]. This compound with two major functional groups is classified into formamidine acaricide and carbamate insecticide[7,8]. The formamidine group functions as an inhibitor of the octopamine neurotransmitter and the carbamate group act as an acetylcholinesterase regulatory enzyme inhibitor. Both groups trigger tension in insects by allowing constant impulse transmission at synapses[9–12].

Hemoglobin (Hb) is a hemoprotein with four subunits, including two alpha (α) chains and two betas (β) chains. The α chains contain 141 amino acids, whereas the β chains

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contain 146 amino acids[13]. Hb has three intrinsic fluorophores, namely, phenylalanine, tyrosine, and tryptophan. Tetrameric Hb possesses six tryptophan residues, i.e., two Trp α14 residues, two Trp β15 residues, and two Trp β37 residues [14].

Glycation refers to the nonenzymatic glycosylation of the free amino groups of proteins and carbonyl groups of sugars occurring when proteins are exposed to elevated concentrations of carbohydrates[15]. Glycation often occurs on the N-terminal valine of protein chains[16], although other amino acids on these chains can also be glycated[17]. During glycation, the amino group of a protein attaches to the carbonyl group of reducing sugar in order to produce a stable imine known as a Schiff base; rearrangement of this molecule forms an Amadori product. Finally, after several reactions, the process results in advanced glycation end-products (AGEs)[18–20]. AGEs cause various complications in diabetes and play a significant role in the development of the disease[21]. Hb is one of the most important proteins in red blood cells[22]. GHb presents in the forms of HbA1a, HbA1b, and HbA1c, which collectively make up HbA1[23]. Nearly 80% of HbA1 is HbA1c[24], which is a glycemic indicator playing a role in the control of diabetes[13].

Glycation causes many changes in the structure and function of proteins[25]. Given that no research has been done so far on the effect of the insecticide formetanate hydrochloride on glycated hemoglobin, this project we will examine whether diabetic patients will be more affected by this toxin or not.

EXPERIMENTAL SECTION

Materials

All stages of the present experimental design were approved by the Research Council of the Faculty of Biological Sciences of the Al-Zahra University of Tehran. In the current experimental study, human Hb was procured from Sigma Aldrich Company. Glucose, formetanate hydrochloride, sodium phosphate, ethylene diamine tetra acetic acid (EDTA), 8-aniline-1-naphthalene sulfonic acid (ANS), and sodium azide were purchased from Merck (Germany) Co.

The phosphate buffer solution was prepared by using sodium phosphate saline at a concentration of 50 mM and pH 7.4. Solutions of 0.16 mg/ml Hb, 40 mM D-(+)-glucose, and 0.0019 g/L formetanate hydrochloride were also prepared.

Hb (0.16 g/L) was incubated in the presence of glucose in 50 mM sodium phosphate buffer. All incubations were performed at 37 °C and 40 rpm for 5 weeks after sterile filtration through a 0.22 µm filter (Millex) in a shaker incubator[26]. In order to prevent the growth of fungi and bacteria, 1mM EDTA and 0.1mM Sodium Azide were added.

Methods

Fluorescence measurements

The fluorescence spectra of Hb were assessed by excitation at 280 nm and emission in the range of 300–600 nm. The tests were performed on a Varian Cary Eclipse spectrophotometer. The quartz cell path length was 1 cm, and the excitation and emission slit widths were 5 and 10 nm, respectively. The Hb concentration was kept at 3 µM, and the formetanate hydrochloride concentration varied from 52.5 µM to 120 µM.

The fluorescence emission spectra of the heme degradation products were detected by excitation at 321 and 460 nm and emission in the range of 350–700 and 490–700 nm, respectively.

A Varian Cary Eclipse spectrophotometer was used to collect Hb-AGE fluorescence spectra. Age-related fluorescence was investigated by excitation at 365, 375, and 380 nm and emission in the range of 390–600 nm. These wavelengths are related to some well-known AGE products such as vesperlysin, pyralin, crossline, and other products. All samples contained 0.16 g/L protein.

Absorption measurements

A Varian Cary 100 Bio spectrophotometer was used to collect UV-Vis spectra. The Hb concentration was maintained at 3 µM, and the formetanate hydrochloride concentration varied from 52.5 µM to 120 µM. The UV-Vis spectra of Hb were collected from 190 nm to 600 nm with a bandwidth of 1 nm.

Circular dichroism measurements

The Circular Dichroism (CD) spectra of Hb were collected from 190 nm to 250 nm with a quartz cuvette with a path length of 1 cm. In this test, the Hb concentration was kept at 3 µM, and the formetanate hydrochloride concentration varied from 52.5 µM to 120 µM.

Docking studies

Molecular docking is a computational method that could provide the feasible binding sites of the interaction between Hb and formetanate hydrochloride based on shape. Molecular docking was conducted by using Molegro Virtual Docker software (version 3.2.1, 2008). The crystalline structure of Hb with PDB Code 2d60 was obtained from RCSB PDB. The atomic structure of formetanate hydrochloride was achieved by applying the PubChem PDB. The docking results were then interpreted using LIGPLOT software. Minimization of the energy conformer was carried out to illustrate the optimized docked conformation[27].

RESULTS AND DISCUSSION

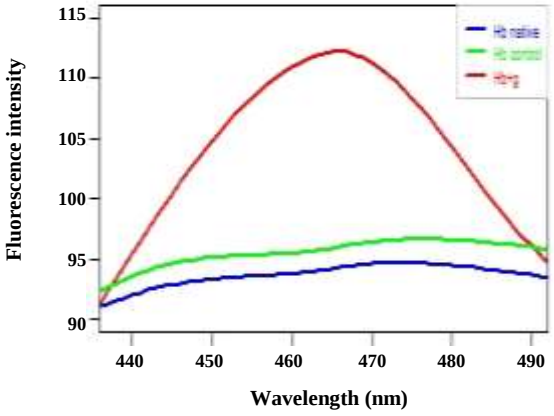
Evaluation of glycation hemoglobin

Fig. 1 A, B, and C revealed the fluorescence spectra of native Hb (Hb 0D(0 day)= native hemoglobin is Hb that does not contain formetanate hydrochloride toxin and the relevant tests were performed immediately after preparing the solution.), control Hb (Hb 35D(35-day =) Control hemoglobin is Hb that does not contain formetanate hydrochloride toxin and was incubated at 37 °C for 35 days.) and modified Hb (Hb + glucose= Hb was incubated with glucose for 35 days under physiological conditions after incubation at 37 °C for 35 days). The fluorescence spectra of the Hb samples were collected after excitation at 365, 375, and 380 nm. Increased fluorescence emission intensity was observed in the sample incubated with glucose, indicating that the said hemoglobin protein has been glycated after 35 days of glucose exposure.

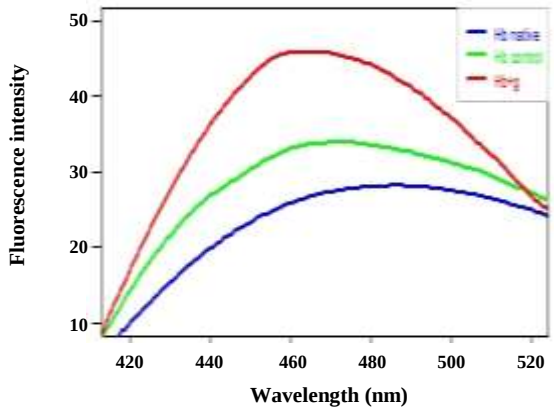
Evaluation of internal fluorescence

Fluorescence spectroscopy is a suitable technique to investigate the interaction between ligands and proteins. This method provides useful information on the conformational changes of Tyr and Trp [28]. Changes in the intrinsic fluorescence emission of GHb were investigated in the absence and presence of different concentrations of formetanate hydrochloride.

Glucose was selected as a glycation agent to evaluate diabetic conditions. Fig. 2 revealed the intrinsic fluorescence intensity of native Hb (Hb 0D), control Hb (Hb 35D), and modified Hb (Hb + glucose) in the presence of different concentrations of Formetanate hydrochloride after incubation at 37 °C for 35 days. As illustrated,



(A)



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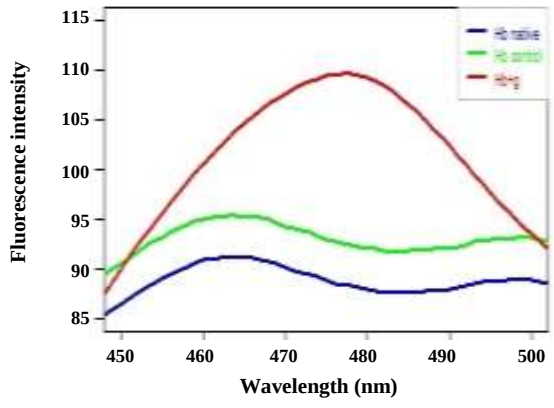

(C)

Fig. 1: Changes in the AGEs Fluorescence emission of fresh hemoglobin(Hb), control (Hb control), glucose-treated (Hb+g) at the concentration of 3 µM at 365 nm (A), 375 nm (B), and 380 nm (C) excitation wavelength for 35 days in phosphate buffer at 37 °C. sodium phosphate buffer at pH 7.4 contains 1mM EDTA and 0.1mM sodium azide.

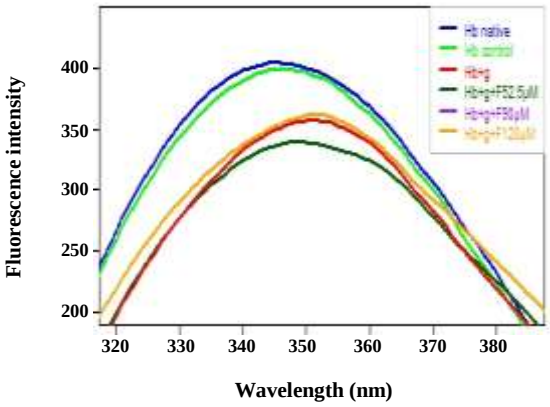

Fig. 2: Changes in the intrinsic emission intensity of fresh hemoglobin (Hb), control (Hb control), glucose-treated (Hb+g) at the concentration of 3 µM at 280nm excitation wavelength and in the presence of different concentrations of Formetanate hydrochloride (52.5, 90 and 120µM).

The native hemoglobin and control have high emission but during glycation, the structure of the protein opens and exposes it to the solvent and reducing its emission. In fact, a decrease in the intensity of glycated protein emission indicates the opening of its structure and the loss of the hydrogen bond. These results are in compliance with previous studies[29]. Addition of different concentrations of formetanate hydrochloride to the GHb solution while increasing the intensity of emission, the maximum peak of its emission moves to higher wavelengths (redshift).

The red shift signifies that the fluorescing aromatic residues buried in nonpolar hydrophobic cavities are moved to a more hydrophilic environment, This phenomenon indicates that the structure of hemoglobin has changed as a result of interaction with the formetanate hydrochloride toxin, and polarity around residues has increased.

The increase in the intensity of emission can be attributed to three reasons: The first reason could be due to the destruction of the heme prosthetique group. This group has a filtering property and absorbs the emission of the protein group and leads to the shutdown of the emission of GHb. If the degreasing substance destroys this part of the protein, its intensity of emission will increase.

The second reason is due to the interaction of formetanate hydrochloride toxin through its hydrophobic regions with GHb, which increases the hydrophobicity around fluorophore and leads to an increasing the intensity of emission. And the last reason could be due to changes in the structure of GHb as a result of interaction with the toxin. This change could be due to the fact that residues previously coated on tryptophan β37 were removed and leading to increased release intensity.

The extent of heme degradation

The binding of sugar to protein causes the degradation and production of fluorescent products. These products are non-protein and can be detected at excitation wavelengths of 321 and 460 nm[30]. In the heme degradation study, the fluorescence emission of the samples increased, thereby indicating heme destruction, as the incubation time increased. Glycation-induced oxidative stress is an initial factor of heme degradation[31–33]. *Behroozi et al.* similarly found that increases in incubation time enhance the rate of degradation of the heme group of Hb treated with glucose [34]. Fig. 3 A and B revealed the Change in the emission intensity of native Hb (Hb 0D), control Hb (Hb 35D), and modified Hb (Hb + glucose) in the presence of different concentrations of Formetanate hydrochloride after incubation at 37 °C for 35 days. As illustrated, The rate of heme destruction in GHb was higher than that of the fresh and control Hb. The fluorescence emission of the samples increased with the rise of the incubation period. during the interaction of GHb with formetanate hydrochloride, the intensity of emission increases more, indicating more destruction of heme. Moreover, as the concentration of formetanate hydrochloride increased, the fluorescence intensity and rate of heme destruction hiked.

Measurement of absorption spectra

The UV-Visible spectra of hemoglobin have an absorption peak in the region of 415 nm. This peak is due to the presence of a heme group in the protein called the soret band. UV-Vis spectroscopy is a suitable method to investigate structural changes in heme. Useful information on the secondary structure of heme can be obtained by examining the Soret band of the protein.[35] Fig. 4 reveals variations in the absorption spectra of fresh hemoglobin (Hb), control (Hb control), and glucose-treated (Hb+g) in the presence of different concentrations of Formetanate hydrochloride after incubation at 37 °C for 35 days. The absorption of the Soret band reduced in GHb. These results suggest that glycation changes the structure of Hb. By adding different concentrations of formetanate hydrochloride, this reduction in adsorption

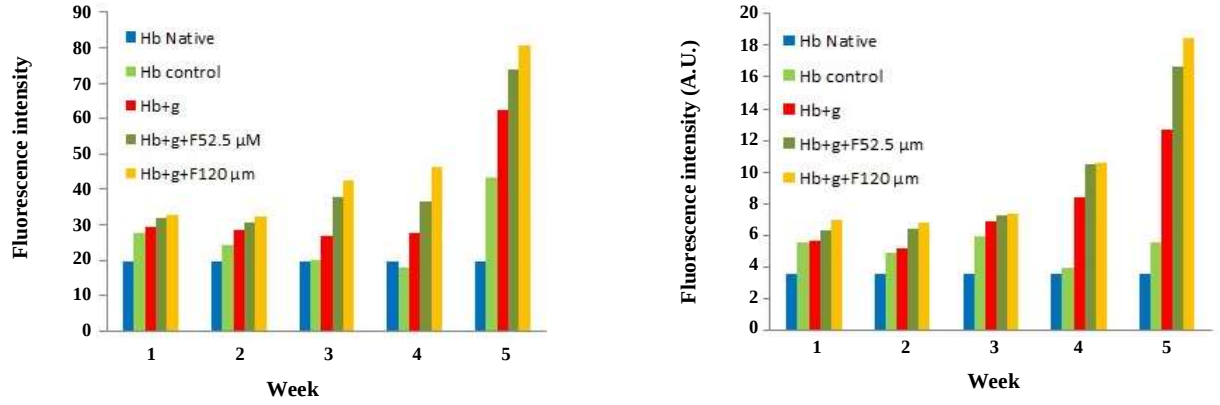

Fig. 3: Change in the emission intensity of fresh hemoglobin (Hb), control (Hb control), glucose treated (Hb+g) at the concentration of 3 µM at 321nm (A) and 460nm (B) excitation wavelength and in the presence of different concentrations of formetanate hydrochloride (52.5 and 120µM).

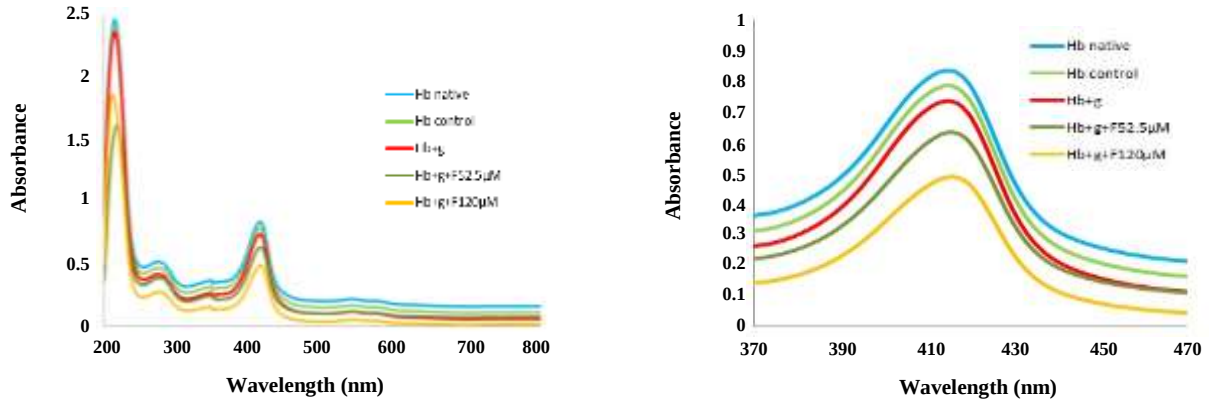

Fig. 4: Changes in the Absorption spectra of fresh hemoglobin(Hb), control (Hb control), glucose-treated (Hb+g) at the concentration of 3 µM and in the presence of different concentrations of Formetanate hydrochloride (52.5 and 120µM).

occurs more intensely. This means that the pesticide will change the structure to a greater extent. The absorption spectra in the Soret band region means the connection of the globin part and the heme group, so the reduction of absorption spectra in this region means the loss of the connection between these two parts, thereby reducing the absorption peak of sorts.

Measurement of circular dichroism spectra

Changes in the secondary structure of GHb were evaluated by CD measurements. Quantitative analyses of secondary conformational changes in the GHb samples were made in the absence and presence of various concentrations of formetanate hydrochloride. The far-UV CD spectrum of native Hb showed two negative peaks at 208 and 222 nm. The peak at 208 nm could be attributed to the α-helix (π→π*) transition, while the peak at 222 nm could be attributed to the α-helix and random coil (n→π*) transition, respectively[36]. Fig. 5 reveals changes in the far UV-CD spectra of fresh hemoglobin (Hb), control (Hb control), and glucose-treated (Hb+g) in the presence of different concentrations of Formetanate hydrochloride after incubation at 37 °C for 35 days.

As illustrated, glycation of Hb results in significant reductions in negative ellipticity in the region of 200–230 nm. The addition of formetanate hydrochloride to GHb enhanced the observed reduction in ellipticity. With due attention to Table 1, glucose and formetanate hydrochloride decrease the Hb tendency to form α-Helix and increase β-Sheet, β-Turn, and Random-coil.

The decrease in negative ellipticity of GHb reveals that the helical structure of the protein disappeared. Our results

Table 1: Percentage of second structures in samples of fresh hemoglobin (Hb), control (Hb control), glucose- treated(Hb+g) at the concentration of 3 µM and in the presence of different concentrations of Formetanate hydrochloride (52.5, 90 and 120 µm) in sodium phosphate buffer with PH7.4.

Secondary structures	Hb native	Hb control	Hb + g	Hb+g+F52.5µM	Hb+g+F90µM	Hb+g+F120µM
Alpha helix	19.52%	17.55%	16.30%	16.04%	15.59%	15%
Anti parallel	16.83%	18.695	18.79%	19.13%	19.21%	19.36%
parallel	10.84%	11.09%	11.335	11.46%	11.65%	11.75%
Beta turns	17.27%	17.29%	17.38%	17.48%	17.65%	17.86%
Random coils	35.54%	35.38%	35.75%	35.89%	35.905%	36%

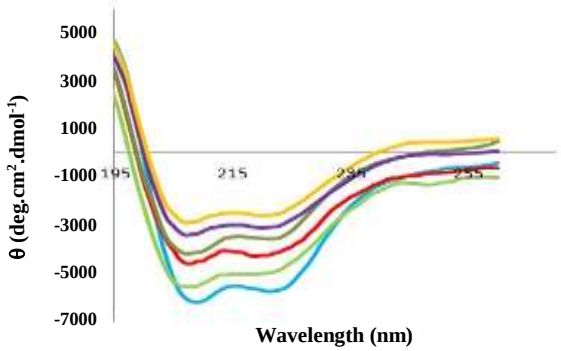

Fig. 5: Changes in the far UV-CD spectra of fresh hemoglobin(Hb), control (Hb control), glucose-treated (Hb+g) at the concentration of 3 µM and in the presence of different concentrations of Formetanate hydrochloride (52.5, 90 and 120µM).

are consistent with the results of Behroozi and colleagues, who stated that glycation results in a significant reduction in negative ellipticity in the 210–230 nm region[34]. Glycation clearly lowers the α-helix content and increases the random coil and β-sheet contents of Hb[37]. Our data show that the interaction of GHb and formetanate hydrochloride cause changes in the secondary structure of the protein.

Molecular docking simulation

The experiments were followed by docking studies. At this stage, formetanate hydrochloride was docked to Hb to determine the preferred binding sites of the former on the latter. Fig. 6 indicates that formetanate hydrochloride could bind to the hydrophobic pocket and central cavity of Hb. The lowest docking energy for the binding of formetanate hydrochloride to Hb is -87/19 kJ/mol,

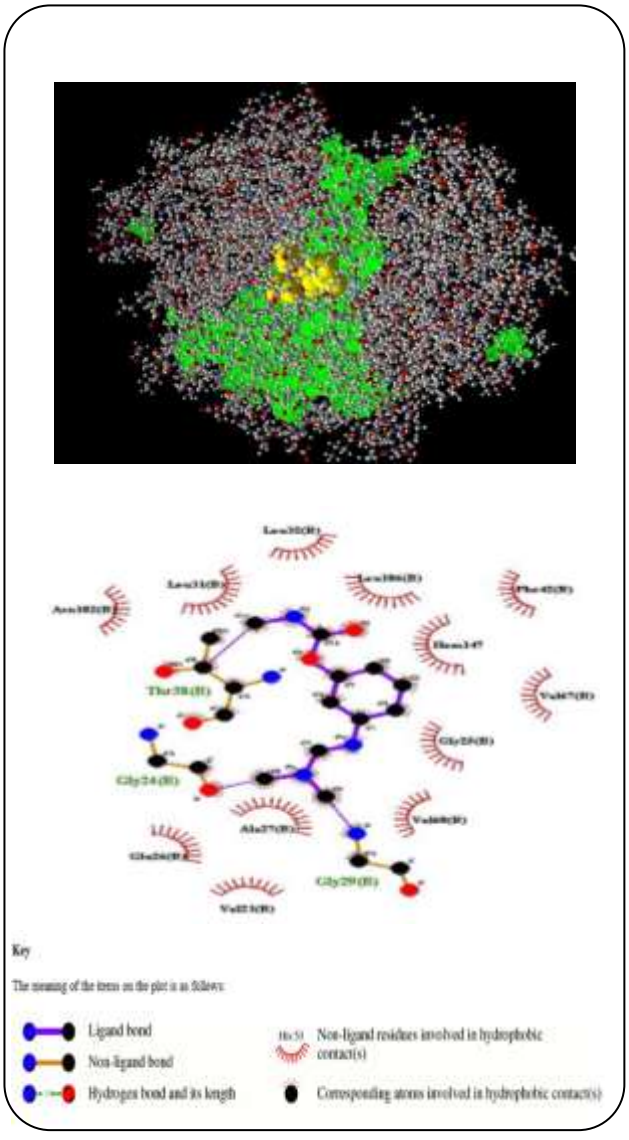

Fig. 6: Docking results of Hb complex with Formetanate hydrochloride. Formetanate hydrochloride is illustrated in yellow (A). LIGPLOT Results of Hb Complex with Formetanate hydrochloride (B).

a negative value means the interaction is energetically desirable. LIGPLOT calculations were performed from the interaction of Hb with formetanate hydrochloride as a ligand. In this case, the closest amino acids to formetanate hydrochloride are leucine, glycine, valine, phenylalanine, and alanine, and can easily interact with the prosthetic heme group. formetanate hydrochloride can cause conformational changes in the Hb and the competitive behavior of this insecticide for the oxygen site can subsequently reduce the oxy form of hemoglobin. The results of the molecular docking studies are in compliance with those obtained from fluorescence spectroscopy and heme degradation studies.

CONCLUSIONS

In the current study, the effect of glucose on GHb as well as the effect of formetanate hydrochloride on GHb was investigated. Our studies have shown that glycation causes the destruction of heme groups, forming AGE products and increasing the content of beta structure in Hb. This alteration of GHb structures occurs more strongly under the influence of formetanate hydrochloric. This means that diabetics will be more affected by the toxin.

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REFERENCES

[1] Stanley J., Preetha G., *Pesticide* Toxicity to Non-Target Organisms: Exposure, Toxicity and Risk Assessment Methodologies, *Pesticide Toxicity to Non-target Organisms: Exposure, Toxicity and Risk Assessment Methodologies* (2016). doi:10.1007/978-94-017-7752-0.

[2] Tingle C.C.D., Rother J.A., Dewhurst C.F., Lauer S., King W.J., Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns, *Rev. Environ. Contam. Toxicol.* **176**: 1–66 (2003).

[3] Childers C.C., Villanueva R., Aguilar H., Chewning, R., Michaud J.P., Comparative Residual Toxicities of Pesticides to the Predator Agistemus Industani (Acari: Stigmaeidae) on Citrus in Florida. *Exp. Appl. Acarol.* **25**: 461–474 (2001).

[4] Cockcroft D.W., et al. Reproduced with Permission of the Copyright Owner. Further Reproduction Prohibited Without, *J. Allergy Clin. Immunol,* **130**: 556 (2012).

[5] Cartwright R., Book Reviews: Book Reviews, *Perspect. Public Health*, **130**: 239–239 (2010).

[6] Bishop C.A., Collins B., Mineau P., Burgess N.M., Read W.F., Risley Ch., Reproduction of Cavity-Nesting Birds in Pesticide-Sprayed Apple Orchards in Southern Ontario, Canada, 1988-1994, *Environ. Toxicol. Chem.*, **19**: 588–599 (2000).

[7] Bakhti H., Hamida N. Ben, Kinetics and Mechanism of Degradation of Aqueous Promecarb Insecticide Studied by UV Spectrophotometry and HPLC, *Prog. React. Kinet. Mech.*, **42**: 145–153 (2017).

[8] Pesticides in the Modern World - Trends in Pesticides Analysis. *Pesticides in the Modern World - Trends in Pesticides Analysis* (2012).

[9] Ash-Bernal R., Wise R., Wright S.M., Acquired Methemoglobinemia: A Retrospective Series of 138 Cases at 2 Teaching Hospitals, *Medicine (Baltimore).* **83**: 265–273 (2004).

[10] Leonard S.T., Moerschbaecher J.M., Winsauer P.J., Estradiol Replacement in Gonadectomized Male Rats Alters Scopolamine-Induced Disruptions of Nonspatial Learning, *Exp. Clin. Psychopharmacol.* **16**: 532–546 (2008).

[11] Divito C.B., Davies S., Masoudi S., Muhoro C.N., Relative Stability of Formamidine and Carbamate Groups in the Bifunctional Pesticide Formetanate Hydrochloride, *J. Agric. Food Chem.*, **55**: 5377–5382 (2007).

[12] Al-Thani R.K., Al-Thani A.S., Elbetieha A, Darmani H., Assessment of Reproductive and Fertility Effects of Amitraz Pesticide in Male Mice, *Toxicol. Lett.*, **138**: 253–260 (2003).

[13] Sengupta B., Swenson J., Properties of Normal and Glycated Human Hemoglobin in Presence and Absence of Antioxidant, *Biochem. Biophys. Res. Commun.* **334**: 954–959 (2005).

[14] Venkateshrao S., Manoharan P.T., Conformational Changes Monitored by Fluorescence Study on Reconstituted Hemoglobins, *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.*, **60**: 2523–2526 (2004).

[15] Kameyama M., Okumiya T., Tokuhiro Sh., Matsumura Y., Matsui H., Ono Y., Iwasaka T., Hiratani K., Koga M., Estimation of the Hemoglobin Glycation Rate Constant, *bioRxiv* (2019), doi:10.1101/652818.

[16] Lledó-García R., Mazer N.A., Karlsson M.O.A., Semi-Mechanistic Model of the Relationship Between Average Glucose and Hba1c in Healthy and Diabetic Subjects, *J. Pharmacokinet. Pharmacodyn.* **40**: 129–142 (2013).

[17] Park S.Y., Yokoyama T., Shibayama N., Shiro Y., Tame J.R.H., 1.25 Å Resolution Crystal Structures of Human Haemoglobin in the Oxy, Deoxy and Carbonmonoxy Forms, *J. Mol. Biol.*, **360**: 690–701 (2006).

[18] Simm A., Protein Glycation During Aging and in Cardiovascular Disease, *J. Proteomics*, **92**: 248–259 (2013).

[19] Muthenna P. *et al.* Inhibition of Protein Glycation by Procyanidin-B2 Enriched Fraction of Cinnamon: Delay of Diabetic Cataract in Rats, *IUBMB Life*, **65**: 941–950 (2013).

[20] Awasthi S., Saraswathi N.T., Silybin, a Flavonolignan from Milk Thistle Seeds, Restrains the Early and Advanced Glycation end Product Modification of Albumin, *RSC Adv.*, **5**: 87660–87666 (2015).

[21] Dayanand K., HHS Public Access, *Physiol. Behav.*, **176**: 139–148 (2018).

[22] Khera P.K. et al. NIH Public Access, **90**: 50–55 (2016).

[23] Sacks, D. B. Measurement of Hemoglobin A1c: A New Twist on the Path to Harmony, *Diabetes Care*, **35**: 2674–2680 (2012).

[24] Clark S.L.D., Santin A.E., Bryant P.A., Holman R.W., Rodnick K.J., The Initial Noncovalent Binding of Glucose to Human Hemoglobin in Nonenzymatic Glycation, *Glycobiology*, **23**: 1250–1259 (2013).

[25] Singh V.P., Bali A., Singh N., Jaggi A.S., Advanced Glycation End Products and Diabetic Complications, *Korean J. Physiol. Pharmacol.*, **18**: 1–14 (2014).

[26] Vistoli G., De Maddis D., Cipak A., Zarkovic N., Carini M., Aldini G., Advanced Glycoxidation and Lipoxidation end Products (AGEs and ALEs): An Overview of their Mechanisms of Formation, *Free Radic. Res.* **47**, 3–27 (2013).

[27] DeLano W., Pymol: An Open-Source Molecular Graphics Tool, *Newsl. Protein Crystallogr*, **40**: 82–92 (2002).

[28] Elbaz N.M., Khalil I.A., Abd-Rabou A.A., El-Sherbiny I.M., Chitosan-Based Nano-in-Microparticle Carriers for Enhanced Oral Delivery and Anticancer Activity of Propolis, *International Journal of Biological Macromolecules*, **92**, Elsevier (2016).

[29] Kazemi F., Divsalar A., Saboury A.A., Structural Analysis of the Interaction Between Free, Glycated and Fructated Hemoglobin with Propolis Nanoparticles: A Spectroscopic Study, *Int. J. Biol. Macromol.*, **109**: 1329–1337 (2018).

[30] Nagababu E., Rifkind J.M., Formation of Fluorescent Heme Degradation Products During the Oxidation Of Hemoglobin by Hydrogen Peroxide, *Biochem. Biophys. Res. Commun.*, **247**: 592–596 (1998).

[31] Xie G., Timasheff S.N., Preferential Interactions of Urea with Lysozyme and their Linkage to Protein Denaturation, *Biophys. Chem.*, **105**: 421–448 (2003).

[32] Alayash A.I., Patel R.P., Cashon R.E., Abdu I.. Antioxid. Redox Signal. **3**, (2001).

[33] Nagababu E., Rifkind J.M., Heme Degradation During Autoxidation of Oxyhemoglobin, *Biochem. Biophys. Res. Commun.*, **273**: 839–845 (2000).

[34] Behroozi J., Divsalar A., Saboury A.A., Honey Bee Venom Decreases the Complications of Diabetes by Preventing Hemoglobin Glycation, *J. Mol. Liq.*, **199**: 371–375 (2014). http://dx.doi.org/10.1016/j.molliq.2014.09.034

[35] Vardapetyan H.R., Martirosyan A.S., Tiratsuyan S.G., Hovhannisyan A.A., Interaction between Hypericin and Hemoglobin, *J. Photochem. Photobiol. B Biol.*, **101**: 53–58 (2010).

[36] Gharib R., Khatibi A., Khodarahmi R., Haidari M., Husseinzadeh S., Study of Glycation Process of Human Carbonic Anhydrase II as Well as Investigation Concerning Inhibitory Influence of 3-Beta-Hydroxybutyrate on It, *Int. J. Biol. Macromol.*, **149**: 443–449 (2020).

[37] GhoshMoulick R., Bhattacharya J., Roy S., Basak S., Dasgupta A.K., Compensatory Secondary Structure Alterations in Protein Glycation, *Biochim. Biophys. Acta - Proteins Proteomics*, **1774**: 233–242 (2007).