ORIGINAL ARTICLE

In Vitro, D-Ribose and Formaldehyde Glycating Effects on Hen Egg White Lysozyme

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ABSTRACT

Background: Glycation causes severe damage to the protein structure, instigating different diseases like cataracts, nephropathy, vasculopathy, retinopathy, atherosclerosis, neurodegenerative disease, diabetes, and age-dependent complications. Formaldehyde, a pollutant present in human habitation, is produced endogenously or exogenously during cooking or incinerating wood, paints, furniture, chipboards, fabric etc. Its higher concentrations can cause cell damage that promotes the formation of DNA/Protein cross-links. The present study aimed to evaluate the glycating effects of formaldehyde on hen egg white lysozyme in comparison with known glycating agent D-ribose.

Methods: In this, *in-vitro* study, hen egg white lysozyme (HEWL) glycation with different concentrations of formaldehyde (0.25mM, 0.5mM, 1mM and 2mM) and D-ribose (0.01mM, 0.05mM, 0.1mM and 0.5mM) was examined using two different experimental conditions: concentration and time duration. Further cross-linking of protein was also analysed using SDS-PAGE technique.

Results: Glycation of HEWL treated with formaldehyde increased with increasing concentrations (0.25mM, 0.5mM, 1mM and 2mM) and time duration (1, 3, 7 and 15 days). Cross linking of HEWL showed visible glycation at 2mM concentration. Cross-linked HEWL products gave dimer at 0.25mM and 0.5mM and trimers at 1mM and 2mMat 3, 7 and 15days. However, compared to formaldehyde, D-ribose glycation at different concentrations (0.01mM, 0.05mM, 0.1mM and 0.5mM) did not show the prominent cross linking of protein.

Conclusion: Formaldehyde was found to be a more potent glycating agent compared to D-ribose. Compared to D-ribose, formaldehyde can produce protein misfolding and can be used in clinical research to establish the role of formaldehyde in patients with diseases.

Keywords: Formaldehyde; D-Ribose; Lysozyme; SDS PAGE.

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INTRODUCTION

Glycation is a general term that covers the progression of complex and unconstrained responses between sugars and amino protein groups producing Schiff bases[1], which are being rearranged and form Amadori products. This product is stable and degraded to highly reactive dicarbonyl compounds, which can interact with sulfhydryl, amino and guanidine groups of protein to generate cross-linked product called advanced glycation end products (AGEs)[2], linked to pathophysiological complications, such as cataracts, nephropathy, vasculopathy, retinopathy, atherosclerosis and neurodegenerative disease[3].

Among the protein glycation, hen egg white lysozyme (HEWL) has attracted the greatest concern and interest because HEWL is structurally like human lysozyme, and in vitro fibrils derived via

HEWL are comparable to fibrils produced in patients[4]. The ability of HEWL fibrils to cause apoptosis in neuroblastoma cells enhances the HEWL a reasonable model to examine the amyloid development in vitro[5]. Possible glycation sites of HEWL are the a-amino group at N-terminus, also the lysine or hydroxylysine residue group[6]. Some research has shown the inclusion of specific proteins in the development of AGEs and correlated with amyloid formation[7]. In view of the above data and its role in the regular immune system, we choose HEWL to inspect the glycation effects.

D-ribose, a glycating agent, and reducing monosaccharide, is predominantly active in protein glycation, leading to the production of AGEs[8], which leads to cell dysfunction and death[9]. It is present in every living cell and is a key component of many biomolecules that play an important role in metabolism[10]. Research shows that glycation with D-ribose produces AGEs faster than glucose glycation, which takes a longer time[11].

Formaldehyde (FA), a product of cell metabolism, is naturally present in human habitation along with its endogenous production, we are also exposed to FA exogenously during cooking, incinerating wood, paints, furniture, chipboards, fabric etc. FA can cause tau protein misfolding and globular aggregation that is harmful to hippocampal neurons[12]. It may also be exposed to certain neurodegenerative diseases[13]. FA at 1mM, increases apoptotic activity and reduces mitosis in tumour cells and endothelial cultured cells, while its high concentration of approximately 10mM causes cell damage and necrotic cell deaths[14] and leads to the formation of cross-links between DNA and Protein, which cause damage to DNA. It is also considered a cross linking agent and can respond with thiol and amino groups of various proteins, which can lead to protein polymerization[15].

HEWL along with sugars has the capacity to generate cross-linking oligomers. Our examination compared the characteristics of lysozyme ribosylation with those of FA in vitro glycation, to determine the potential for aggregate and polymer formation. This study can be beneficial to understand the impacts of FA glycation on the structure of HEWL, its cross linking of protein and its effect on human health. This study aimed to evaluate the glycating effects of formaldehyde at 37°C on HEWL at pH 7.4 in comparison with known glycating agent D-ribose.

METHODS

In this in-vitro study Hen egg white lysozyme (HEWL) (10mg/ml) was added in 0.1M sodium phosphate buffer of pH 7.4 at 37°C for 1, 3, 7, and 15 days in the presence and absence of 0.01mM, 0.05mM, 0.1mM and 0.5mM D-ribose. To prevent bacterial growth, sodium azide (1mM) has been used and incubated under sterile laboratory conditions. At certain times, aliquots were withdrawn and stored at -20°C for further analysis. In this research, native HEWL alone incubated at 37°C in the absence of D-ribose is used as a control.

Also, incubation of HEWL (10mg/ml) was carried out in 0.1M sodium phosphate buffer (containing 1mM sodium azide to prevent bacterial growth) in the absence and presence of formaldehyde (FA) of concentratons: 0.25mM, 0.5mM, 1mM and 2mM, the reaction mixture was incubated at 37°C at different time intervals (1, 3, 7, and 15 days). After incubation Eppendorf tubes were removed and reacted solutions were immediately placed at -20°C for further evaluation. The corresponding HEWL without FA was used as the control.

Glycation of HEWL with D-ribose and FA were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots collected at different time intervals were combined in the ratio of 1:1 with sample-diluting buffer (SDB, 2X) containing 10% SDS (4ml), bromophenol blue (few crystals), β-mercaptoethanol (1ml), glycerol (2ml), and tris-HCL (1M, 1.25ml). The Eppendorf tube containing SDB sample was capsized capped and boiled for 2 min in a water bath at 100°C. After heat treatment, 10µl of the solution was loaded into a well of prepared SDS PAGE gel (10%) and electrophoresis was carried out with the help of mini-protean 3 system, Bio-Rad, Hercules, CA according to standard Laemmli method[16]. For staining, Coomassie Brilliant Blue G-250 (CBBG) was used and destaining was accomplished with aqueous acetic acid/methanol solution (100ml of acetic acid and 200 ml of methanol per litre). The gel was scanned using a gel documentation system (Uvitec, UK).

RESULTS

At first day incubation, the expression of HEWL did not change when the concentration of FA increased compared to the control on SDS-PAGE (Figure 1a). When HEWL was incubated with FA for 3, 7 and 15 days and compared with the control, increased expression of HEWL products were observed (Figure 1b-d), and more prominent glycation was seen at 15 days of incubation (Figure 1b-d), proving the glycating effect of FA increases with the increase in days.

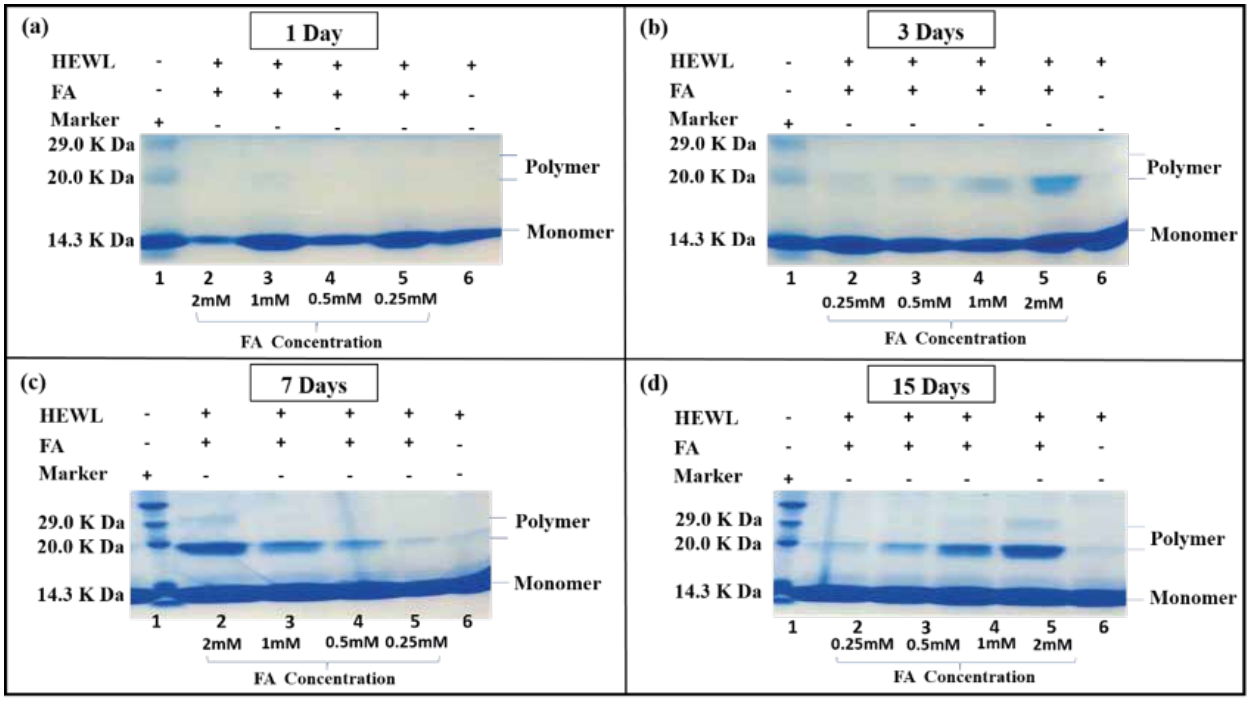

Figure 1: (a) 10% SDS PAGE of the product of Hen egg white lysozyme (HEWL) incubated with different concentration of formaldehyde(FA) (0.25mM, 0.5mM, 1mM and 2mM) at 37°C for 1 day (b) 3 days (c) 7 days. (d) 15 days.

Moreover, cross linking of HEWL increased with the increase of concentration as well as incubation days compared to control (Figure 2a-d), a more visible glycation was noted at 2mM concentration. Cross-linked HEWL products represented dimer and trimer of HEWL compared to the size of un-glycated HEWL (~14 K Da) and these products were observed at the high molecular weight. The presence of cross linking products increased from 0.25mM - 2mM over time (Figure 2a-d). The only dimer was observed at 0.25mM and 0.5mM (Figure 2a-b), but when the concentration increases to 1mM and 2mM, trimers were also seen at 3, 7 and 15 days (Figure 2c-d).

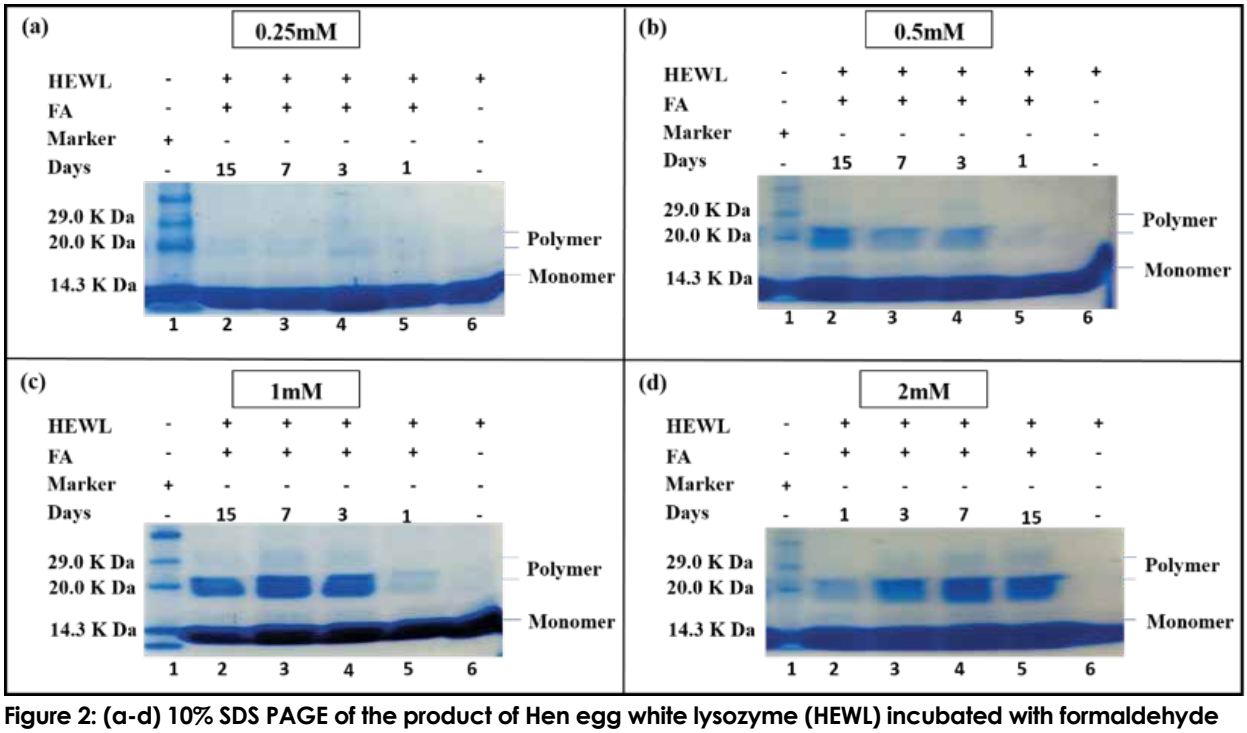

Figure 2: (a-d) 10% SDS PAGE of the product of Hen egg white lysozyme (HEWL) incubated with formaldehyde (FA) 0.25mM, 0.5mM, 1mM, 2mM for 1, 3, 7, and 15 days at 37°C.

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HEWL was treated at 37°C during 1, 3, 7 and 15 days at different concentrations of D-ribose. The results show that the expression of HEWL does not change on first day when the concentration of D-ribose increases compared to the control (Figure 3a). With 3 days of incubation, protein glycation increases with the increase of concentration, but no significant change occurs (Figure 3b). At 7 days of incubation, more prominent glycation was seen only at 0.5mM D-ribose concentration (Figure 3c). Moreover, the cross-linking in HEWL increases with the increase in the concentration of D-ribose compared to the control at 15 days of incubation (Figure 3d), proving that glycation of HEWL with D-ribose increases with the increase of days.

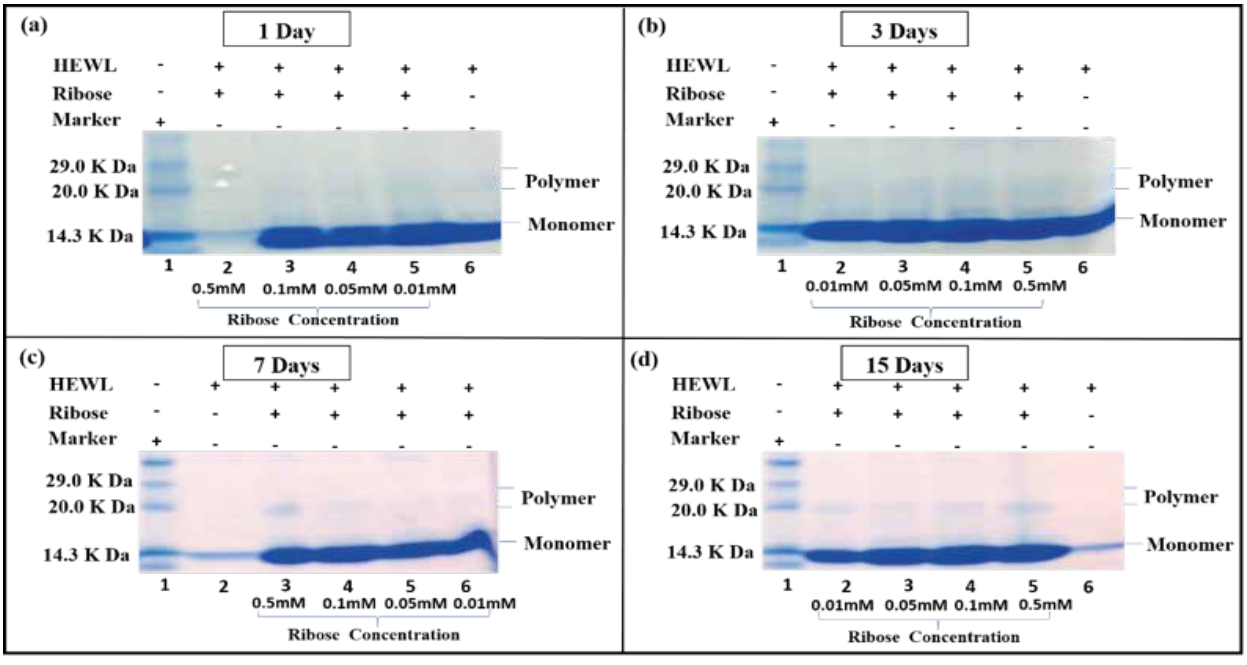

Figure 3: (a) 10% SDS PAGE of the product of Hen egg white lysozyme (HEWL) incubated with different concentration of D-Ribose (0.01mM, 0.05mM, 0.1mM and 0.5mM) at 37°C for 1 day. (b) 3 days. (c) 7 days. (d) 15 days.

The increase of concentration (0.05-0.5mM) of D-ribose, cross linking of HEWL increases while at 0.01mM concentration no significant change has been observed (Figure 4a-d).

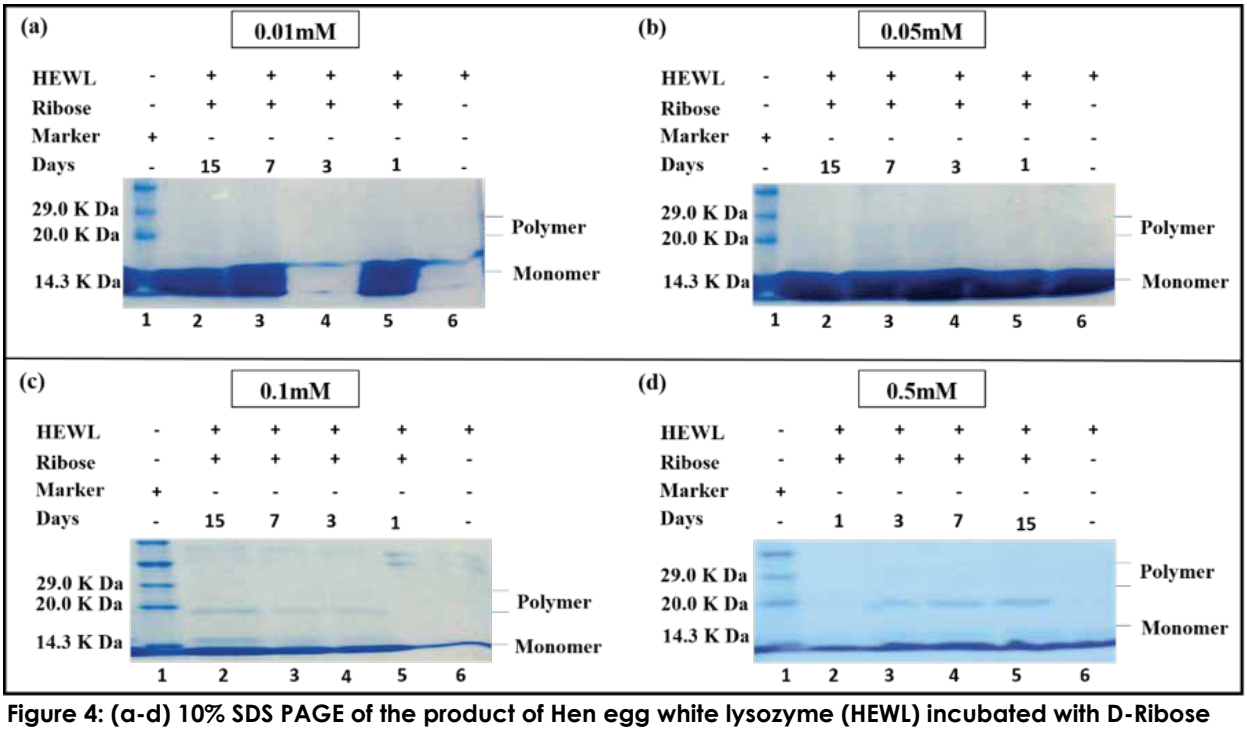

Figure 4: (a-d) 10% SDS PAGE of the product of Hen egg white lysozyme (HEWL) incubated with D-Ribose at 0.01mM, 0.05mM, 0.1mM, 0.5mM for 1, 3, 7, and 15 days at 37°C.

DISCUSSION

Disordered proteins, have previously been reported, and are involved in complicated disorders like cancer, cardiovascular disease[17], neurodegenerative diseases[18], or diabetes[19]. Previously research shows that formaldehyde (FA) of different concentrations (FA ≥ 0.1mM) when reacted with BSA protein, results in the neuronal cell lines (SH-SY5Y) being cytotoxic[20]. Another study revealed that BSA glycation with ribose sugar leads to the production of advanced glycation end products (AGEs), which also results in the neuroblast cells cytotoxicity[21].

To examine either glycation was faster with FA or D-ribose, we separately incubated HEWL with different concentrations of FA and D-ribose and analysed the results using SDS-PAGE. Formaldehyde is a known pollutant present in human habitation, exogenous exposure of FA is motor vehicle exhaust, power plants, petroleum refineries, cooking operations, incinerating wood burning, paints, furniture, chipboard, fabric, smoke etc. so it proven that humans are continuously exposed to FA. Along with its exogenous exposure, it is also endogenously produced as a metabolic by-product. The safe concentration of formaldehyde exposure is 0.1 mg/m^3 [22], but a survey showed that its concentration in home and workplaces exceed the above guideline value[23], that are responsible to increase the risk of various diseases. Under the highlights of the previous study, we designed our goal to find out the glycating effects of FA and D-ribose at the different concentrations on lysozyme and their cross-linked product. In the first part of our, study HEWL was incubated at 37°C for 1, 3, 7 and 15 days with different FA concentrations. With the passage of days (1-15days incubation), high molecular HEWL products were formed, showed that the glycating effect of FA increases with the increase of days. In Addition, at 37°C, HEWL was incubated at different FA concentrations for different intervals. It was observed that with the increase of FA concentration (0.25-2mM), cross-linking of HEWL increased and more prominent glycation was observed at 2mM concentration.

However, in the second part, HEWL was incubated at 37°C for 1, 3, 7 and 15 days at various concentrations of D-ribose. The study results illustrated that glycation of HEWL with D-ribose increases with concentration and time dependent manner. D-ribose is a monosaccharide that is naturally present in the mitochondria of cells that is responsible for energy production[24]. In the healthy individual, the concentration of D-ribose in cerebrospinal fluid and blood are 0.01-0.1mM[25]. To the best of our knowledge, we treated HEWL with different concentrations of D-ribose for various intervals. Out of these, three concentrations of D-ribose are normal in a range that is present in healthy individuals, but the 0.5mM is increased concentration than normal and we had compared the glycating effect of all these concentrations. We also found that at the concentration of 0.01mM D-ribose, the structure of the HEWL does not change compared to the control. While the cross-linking of HEWL at 0.05mM, 0.1mM and 0.5mM increase with the time of glycation compared to the control. Our result indicated that cross linking of HEWL was more prominent at 0.5mM concentration of D-ribose sugar, which further proved that an increase of concentration of D-ribose directly increased the glycating effects on lysozyme and its dimers and trimers[25].

In the comparison of the glycating effects of both FA and D-ribose at its physiological concentrations, it has beenproved that polymerization of HEWL increases with the concentration and time dependent manner and FA was more reactive glycating agent than D-ribose. FA at its physiological concentrations of 0.5mM and 1mM also showed glycation, which was more prominent at 2mM. During glycation, 2-fold increase expression of HEWL was observed at 2mM FA concentration. However, HEWL bands incubated with D-ribose was not as prominently observed as HEWL incubated with FA under similar conditions. In this study, we found that compared to FA, D-ribose did not show the prominent polymerization of protein. These findings concluded that HEWL is more prone to FA glycation compared with D-ribose. In addition to the above results, this research will be beneficial to understand the impacts of FA glycation on the structure of HEWL as well as its results in cross linking of protein. Moreover, this study will be beneficial for future studies, which will help in pre-clinical and clinical research to establish the role of FA in patients with diabetes and neurodegenerative diseases.

CONCLUSION

The glycation promoted the cross linking of HEWL. Compared to D-ribose, FA is a potent glycating agent, can produce protein misfolding and cross-link HEWL product, as observed by SDS-PAGE. FA showed more glycation of HEWL with the increasing concentration and days of incubation, compared to D-ribose.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

FN and UZ performed all experimental work and acknowledged valuable material inside these articles. RK and UZ contributed equally in writing the manuscript. All authors read and approved the final version of the manuscript.

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