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Role of zinc along with ascorbic acid and folic acid during long-term *in vitro* albumin glycation

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The present study aimed to investigate the role of Zn alone and in the presence of ascorbic acid (AA) and folic acid (FA) in albumin glycation. Glycation was performed by incubations of bovine serum albumin with glucose at 37°C along with Zn, AA or FA separately and Zn + AA or Zn + FA for 150 d. Glycation-mediated modifications were monitored as fluorescence of advanced glycation endproducts, carbonyl formation, β aggregation (thioflavin T and Congo red dyes), albumin-bound Zn, thiol groups and glycated aggregate's toxicity in HepG2 cells. Zn inhibited glycation and β aggregation, probably due to observed higher protein-bound Zn. It also protected protein thiols and increased cell survival. AA and FA enhanced glycation, which was lowered in Zn-co-incubated samples. FA increased albumin-bound Zn and showed maximum cell survival. Although these results warrant further *in vivo* investigation, the present data help in the understanding of the interplay of Zn with micronutrients in albumin glycation.

Ascorbic acid: Bovine serum albumin: Glycation: Folic acid: Zinc

Chronic hyperglycaemia causes the Maillard reaction in which reducing sugars, such as glucose, react non-enzymically with amino groups of proteins through a series of reactions, ultimately forming advanced glycation endproducts (AGE), triggering several non-communicable diseases[1–5]. Glycated albumin comprises about 6–15 % of the total albumin in normal individuals and rises to 32–40 % in hyperglycaemia. These modifications affect the properties of albumin in several ways, including altered conformation and consequently altered binding. Diabetes mellitus, liver diseases and nephropathy are just a few disorders in which altered albumin functions have been described[6,7].

In diabetes and Alzheimer's disease, an imbalance in the concentration of Zn and micronutrients is suggested as a causative factor[8–10]. Though the antioxidant role of Zn and ascorbic acid (AA) is well established, there are many contradictory reports on beneficial or deleterious effects of Zn, AA or folic acid (FA) supplementation in Alzheimer's disease and AGE-related diseases[11–13]. Our initial experiments have suggested that Zn may directly inhibit the glycation of bovine serum albumin (BSA). Hence, detailed investigations were carried out to understand the role of Zn alone and Zn along with AA and FA in the long-term glycation of BSA.

Experimental methods

Chemicals

BSA (fraction V, catalogue no. A-7906, initial fractionation by heat shock, purity 98 % (electrophoresis), remainder mostly globulins and fatty acids depleted), thioflavin T and Congo-Red were obtained from Sigma Chemical Company (St Louis, MO, USA). AA, FA, $ZnSO_4$, glucose and other chemicals were from Hi-Media (Mumbai, India).

Glycation of bovine serum albumin

For preparation of glycated BSA samples, five different sets, containing BSA (10 mg/ml), glucose (0·5 M) in PBS (140 mM-sodium chloride, 2·7 mM-potassium chloride, 10 mM-disodium hydrogen phosphate, 1·8 mM-potassium di-hydrogen phosphate, pH 7·3) were incubated in the dark at 37°C for 150 d in sealed tubes. Before incubation, Zn (375 μM), AA (400 μM) and FA (270 μM) were added to first three sets separately, whereas binary mixtures of Zn + AA and Zn + FA were added in the fourth and fifth set, respectively. Reaction mixtures were filtered through 0·22 μm Millipore membrane filters (Millipore Corp., Billerica, MA, USA) into sterile plastic-capped vials to maintain sterility. After the incubation period, it was ensured that all the solutions were free of microbiological contamination. All the experiments were performed in triplicate and appropriate controls (only BSA, BSA + glucose, i.e. sets no. 6 and 7) were maintained under similar conditions.

After incubation, the reaction mixtures were extensively dialysed against distilled water and stored at 4°C for subsequent experiments. The final volumes of the dialysates were made to 3 ml and the protein concentrations were determined by Lowry's method.

Abbreviations: AA, ascorbic acid; AGE, advanced glycation endproducts; BSA, bovine serum albumin; FA, folic acid; HbA1c, glycosylated Hb; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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Quantification of advanced glycation endproducts

The relative degree of glycation was assessed by measuring intrinsic fluorescent signals from AGE using a spectrofluorometer (F-2500; Hitachi, Tokyo, Japan). Emission scans were taken as arbitrary units/mg protein from 400 to 550 nm (slit, 10 nm) with an excitation wavelength of 370 nm (slit, 10 nm) to assess the overall effect of micronutrients on levels of AGE formation with correction for background fluorescence of PBS.

Carbonyl group estimation

Protein carbonyl groups were estimated by the method of Uchida *et al.* [14]. Briefly, 0·5 ml protein samples were mixed with an equal volume of 2,4-dinitrophenylhydrazine (0·1 %) in 2 M-HCl and incubated at room temperature for 1 h. After incubation, protein was precipitated by 20 % TCA (0·5 ml) and washed three times with 1 ml ethanol + ethyl acetate (1:1, v/v) mixture. Finally, the precipitate was solubilised in 6 M-guanidium hydrochloride and absorbance was read at 365 nm (UV1; Thermo Spectronic Corp., New York, USA). Protein carbonyl concentration was calculated by using the molar extinction coefficient ($\varepsilon 365\,\text{nm} = 21$ per mM per cm). The results were expressed as nmol carbonyls/mg protein.

Thioflavin T assay

For determination of β aggregation, thioflavin T, a marker for amyloid cross β structure, was used[15]. Hence, glycated samples (100 μl) were incubated with 32 μM-thioflavin T in triplicate. Fluorescence was measured after 1 h incubation at room temperature. Excitation and emission wavelengths were 435 nm (slit, 10 nm) and 485 nm (slit, 10 nm), respectively, with correction for background signals from buffer without thioflavin T.

Binding of Congo red

Congo red binding to cross-β structure was estimated by measuring absorbance at 530 nm of amyloid structures[16]. For this purpose, glycated samples (500 μl) were incubated with 100 μl of 100 μM-Congo red in PBS with 10 % (v/v) ethanol for 20 min at room temperature. Absorbance was recorded for the Congo red-incubated samples as well as for Congo red background.

Cell-culture assays

Human hepatocyte carcinoma cell line (HepG2) was kindly provided by Dr M. S. Patole (National Centre for Cell Sciences, India). HepG2 cells were grown in Eagle's minimum essential medium (MEM; Sigma Chemical Co., St Louis, MO, USA) containing 10 % fetal bovine serum (Sigma Chemical Co.) and incubated in a humidified chamber (85 % humidity) containing 5 % CO_2 at 37°C in a CO_2 incubator (Thermo-Forma, Marietta, OH, USA). Cell viability was indirectly measured as a function of the percentage of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduced.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

HepG2 cells were seeded onto a ninety-six-well plate (Corning Inc., Corning, NY, USA) at a final cell count of 20 000 cells per well. For the assay, MEM containing the glycated samples (200 μl) was transferred into each well. After 4 h incubation at 37°C, medium was removed and MTT (150 μl/well) was added to each well and the plate was kept in a CO_2 incubator for an additional 2–4 h. The cells were lysed by the addition of a lysis solution (50 % dimethylformamide, 20 % SDS, pH 4·7) and were incubated for 1 h. The degree of MTT reduction in each sample was subsequently assessed by measuring absorption at 570 and 630 nm using a microplate reader (Bio Kinetics EL340; Bio-Tek Instruments, Winooski, VT, USA). The net difference $= A_{570} - A_{630}$ was used to express the viability of the cells. Results were expressed as percentage cell viability relative to unglycated control (% BSA control).

Protein-bound zinc estimation

To determine the protein-bound Zn in glycated samples, dialysed samples (1 ml) free of unbound Zn were digested using concentrated HNO_3 for 1 h. The final volume of 3 ml was made with metal-free water (Millipore Q; Billerica, MA, USA) and readings for Zn were taken on an atomic absorption spectrometer (AA 800; PerkinElmer, Shelton, CT, USA). All determinations were carried out in triplicate and the final result was the mean of three estimations. Values were expressed as μmol bound Zn/mg protein.

Protein thiol estimation

Thiol groups of native or modified BSA were measured according to Ellman's assay[17] using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Briefly, the samples (3·5 mg/ml) were mixed with 0·05 M-PBS (pH 7·6) and incubated for 15 min with 2·5 mM-DTNB. The absorbance was measured at 410 nm. The free thiol concentration of samples was calculated with the help of a standard curve performed with various native BSA concentrations (0·8 to 4 mg/ml, corresponding to 19–96 nmol total thiols).

Statistical analysis

Statistical analysis was performed in Microsoft Excel (Windows XP version; Microsoft Corp., Redmond, WA, USA). Data were expressed as the mean values and standard deviations of triplicate values. The significance of the results was determined by comparison with positive control glycated samples using one-way ANOVA for all sets of experiments.

Results

Formation of amyloid albumin in presence of different factors

To determine the modifications in the glycated albumin due to co-incubation with Zn, AA and FA, we measured two different glycation biomarkers.

Advanced glycation endproduct fluorescence. Emission fluorescence scans indicated progressive accumulation of

AGE in the positive control (BSA + glucose) samples. However, in Zn-co-incubated samples (BSA + glucose + Zn), the overall fluorescence was reduced significantly ($t = 15{\cdot}11$; $P < 0{\cdot}001$), indicating lowered glycation as compared with the positive control (Fig. 1(a)). When compared with FA alone, Zn exhibited a significant inhibitory effect ($t = 26{\cdot}49$; $P < 0{\cdot}001$) on the extent of AGE formation during its co-incubation with FA (Fig. 1(b)). A similar accompanying decrease

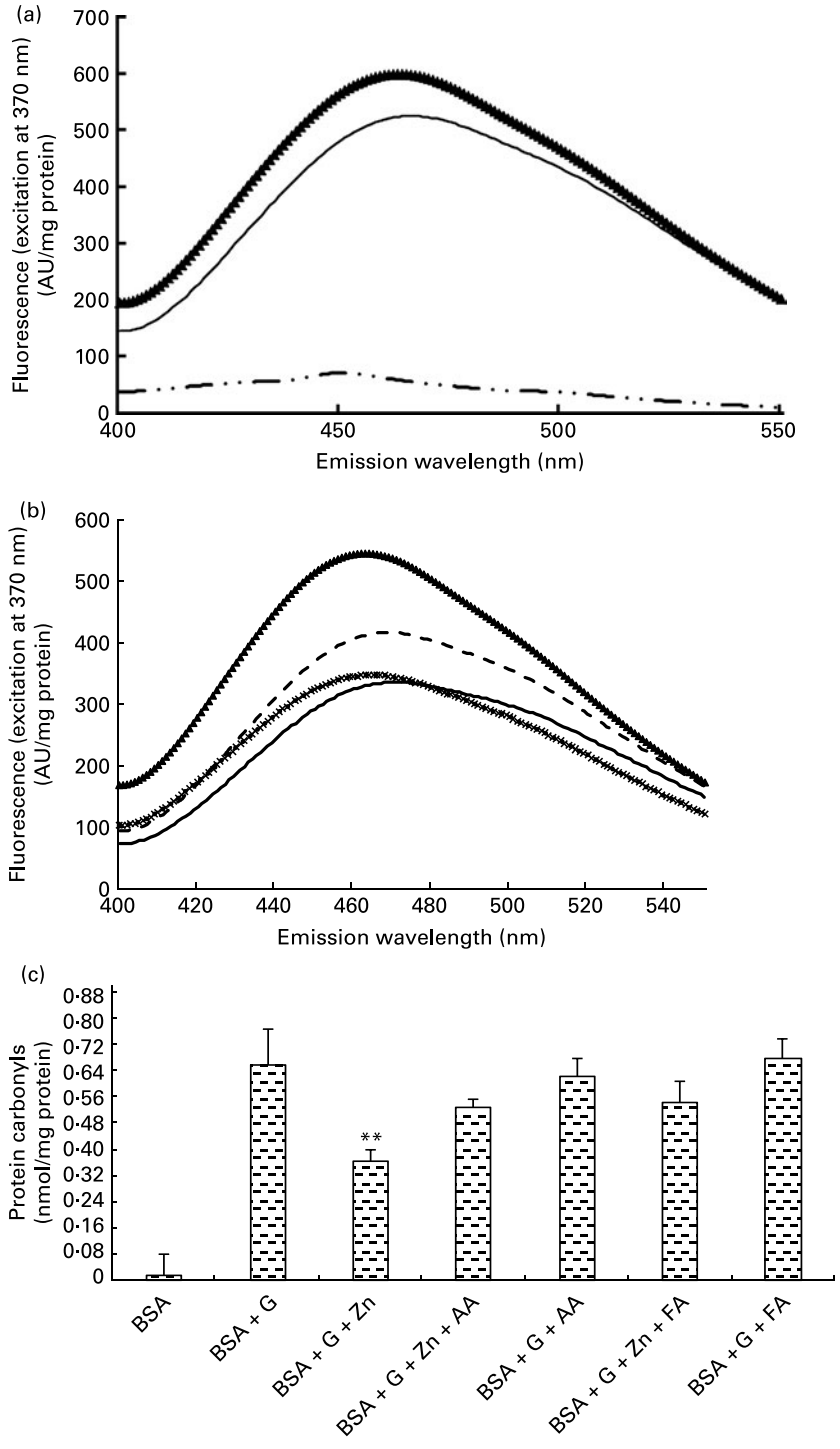

Fig. 1. Glycation of bovine serum albumin (BSA) samples as measured with advanced glycation endproducts (AGE) spectra and protein carbonyl assay. Emission scans from 400 to 550 nm were taken with an excitation wavelength of 370 nm for AGE. (a) AGE fluorescence in the presence of Zn along with controls. AU, arbitrary units; (–···–), BSA; (–▲–), BSA + glucose (G) (positive control); (—), BSA + G + Zn. (b) Spectra of glycated samples by co-incubation of Zn along with ascorbic acid (AA) or folic acid (FA). (·· × ··), BSA + G + Zn + AA; (- - -), BSA + G + AA; (–▲–), BSA + G + FA; (—), BSA + G + Zn + FA. (c) Protein carbonyls expressed as nmol carbonyls/mg protein. Values are means (n 3), with standard deviations represented by vertical bars. ** Mean value was significantly different from that of the BSA + G treatment (positive control) ($P < 0{\cdot}01$; one-way ANOVA).

was also observed in Zn + AA samples as compared with AA samples ($t = 17{\cdot}99$; $P<0{\cdot}001$) (Fig. 1(b)).

Carbonyl estimation. Since AGE measurement is non-specific; the carbonyl estimations by the dinitrophenylhydrazine assay are generally used to resolve ambiguities. Results indicated that for the samples incubated with Zn, the protein carbonyls were reduced significantly ($P<0{\cdot}01$). Zn showed a marginally significant decrease ($P>0{\cdot}05$) when incubated along with AA and FA (Fig. 1(c)). The difference in the results of AGE and dinitrophenylhydrazine assays (for Zn-co-incubated AA or FA samples) could be attributed to the different specificity in these two measurements.

Albumin advanced glycation endproduct condensates as β-fibrils during glycation

We determined the level of β aggregation of BSA in the presence of various factors using thioflavin T, a fluorescent dye that specifically binds with fibrous structures (Fig. 2(a)). β Aggregates formed were significantly different between the treatments as shown by one-way ANOVA ($F = 17{\cdot}66$; $P=0{\cdot}001$). In Zn-co-incubated samples, aggregates were marginally reduced as compared with positive control samples ($P=0{\cdot}08$). The maximum aggregation occurred in AA-co-incubated samples, yet not significantly different from positive control samples. AA + Zn-co-incubated samples showed strong reduction in aggregation, whereas in the presence of FA, Zn did not show considerable inhibition in β-sheet formation ($P>0{\cdot}05$).

To confirm the observation that Zn inhibits β-sheet formation of albumin experimentally, Congo red, another β-sheet-specific dye, was used (Fig. 2(b)). With this assay, the positive albumin control exhibited the strongest absorbance as expected and all the treatment groups showed significant reduction in absorbance ($F = 41{\cdot}83$; $P<0{\cdot}001$). On the other hand, aggregates from Zn-co-incubated albumin samples displayed significantly lower absorbance ($P<0{\cdot}001$). Samples with FA illustrated lower absorbance that was further reduced slightly with Zn co-incubation. While samples with AA elicited weaker absorbance, the samples with AA + Zn had significantly decreased absorbance as compared with AA alone.

Data of thioflavin T and Congo red together suggest that β-sheet conformations were prevented by Zn alone or Zn in the presence of the two vitamins. One way of explaining

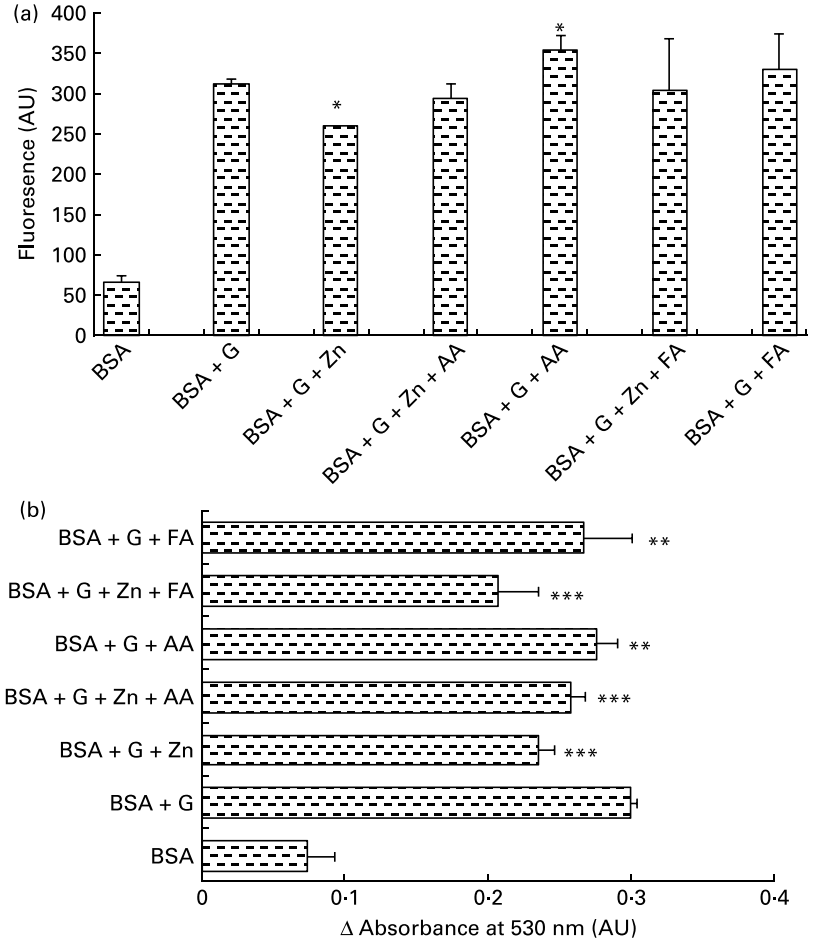

Fig. 2. Transition of amino acid residues to β-sheet conformation as indicated by amyloid-specific dyes. (a) Effect of Zn along with ascorbic acid (AA) or folic acid (FA) on thioflavin T fluorescence. AU, arbitrary units; BSA, bovine serum albumin; G, glucose. Values are means (n 3), with standard deviations represented by vertical bars. * Mean value was marginally significantly different from that of the BSA + G treatment (positive control) ($P=0{\cdot}08$; one-way ANOVA). (b) Effect of Zn along with AA or FA on Congo red absorbance at 530 nm. Values are means (n 3), with standard deviations represented by horizontal bars. Mean value was significantly different from that of the BSA + G treatment (positive control): ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ (one-way ANOVA).

these results might be that during the glycation reaction, Zn prevented the β-sheet formation in albumin by promoting the native α-sheet conformation. The affinities of Congo red and thioflavin T for AA and FA samples were nearly analogous to the positive control. During glycation when Zn was present along with AA or FA, Zn could restrain the native conformation that was comparable with glucose + Zn samples.

Toxicity of aggregates of albumin in HepG2 cells

We found a greater extent of β aggregation in positive control samples, which further showed cytotoxicity and decreased cell viability to 1·05 % (Fig. 3). Zn seemed to have effectively suppressed β aggregation, since 54 % of the cells treated by glycated protein co-incubated with Zn survived after 4 h incubation ($P<0·01$). With FA alone, only 18 % of cells were viable, but the presence of Zn along with FA significantly protected cells against the aggregate toxicity (75 % viability; $P<0·001$). In contrast to FA, aggregates formed in the presence of AA were less toxic to cells (32 % viability; $P<0·05$) and co-incubation with Zn marginally increased cell survival (38 % viability). Overall, against glycation-induced β-amyloid toxicity, Zn + FA treatment exhibited a maximum protective effect in HepG2 cells followed by Zn alone, though a similar synergistic effect was not manifested by Zn + AA samples.

Zinc affinity of albumin modified by different factors

The unglycated and glycated albumin controls contained about 9 μmol bound Zn/mg protein (Fig. 4(a)). When Zn (375 μM) was available during glycation, the level of albumin-bound Zn increased to 11·07 μmol/mg protein ($P<0·001$) and probably this increased bound Zn had an attenuating effect on BSA glycation. Parallel to our previous findings[18], maximum albumin-bound Zn (11·4 μmol/mg protein; $P<0·001$) was revealed in Zn + FA samples that also elicited the highest cell survival (75 %). On the other hand, co-incubation of Zn with AA significantly inhibited Zn binding to albumin (8·7 μmol/mg protein), which might be the reason for lower cell survival with Zn + AA samples. This inhibitory role of AA on Zn–albumin binding was also observed in AA samples, where bound Zn was withdrawn, as Zn levels went down to 5·2 μmol/mg protein compared with controls ($P<0·01$). The correlation between albumin-bound Zn and cell viability (r 0·61; $P>0·05$) indicated a possible role of protein-bound Zn in reducing aggregate toxicity in HepG2 cells.

Effect of zinc, ascorbic acid and folic acid on protein thiols during glycation

Albumin is considered as the major source of thiols in plasma. Glycation modifies thiol group(s) to form disulfide bonds and intermolecular aggregates in albumin[19]. We therefore examined whether thiol groups were altered under our experimental conditions. In positive control samples, the thiols were significantly decreased by 50 % (Fig. 4(b)). Results indicated a prominent beneficial effect of Zn for protein thiols ($P<0·05$). AA showed a protective role on thiol group modification with no obvious change in the presence of Zn. FA alone manifested a similar response to AA, but with Zn it gave very strong shielding from denaturation, as thiol concentrations were almost similar to native BSA ($P<0·01$). These results are on par with other reported studies and collectively suggest that when there is more protein-bound Zn, the thiol groups are less likely to be affected by glycation-induced changes[20,21].

Discussion

It is known that albumin could be modified by the factors present in its plasma environment. In the present study we have investigated the *in vitro* modification of BSA by glycation in the presence of Zn, AA and FA and their binary combinations. The long-term incubation was performed for maximum induction of β aggregation with associated cytotoxicity observations. The present study demonstrates the antiglycation role of Zn, which was additionally influenced by other molecules such as AA and FA.

Several lines of evidence strongly suggest that Zn being in a single oxidation state of Zn^{2+} is a redox inert metal and

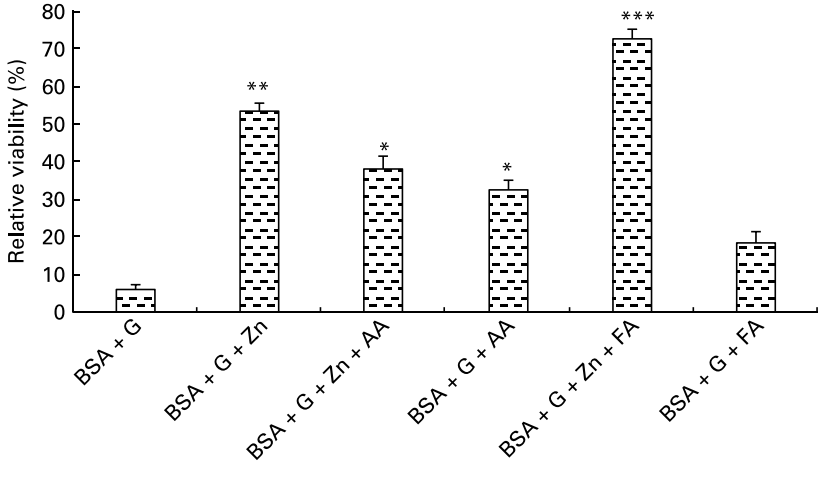

Fig. 3. Effect of Zn alone or along with ascorbic acid (AA) or folic acid (FA) upon glycated albumin cytotoxicity. The results were expressed as percentage relative viability to the unglycated bovine serum albumin (BSA) control. G, glucose. Values are means (n 5–6), with standard deviations represented by vertical bars. Mean value was significantly different from that of the BSA + G treatment (positive control): * $P<0·05$, ** $P<0·01$, *** $P<0·001$ (one-way ANOVA).

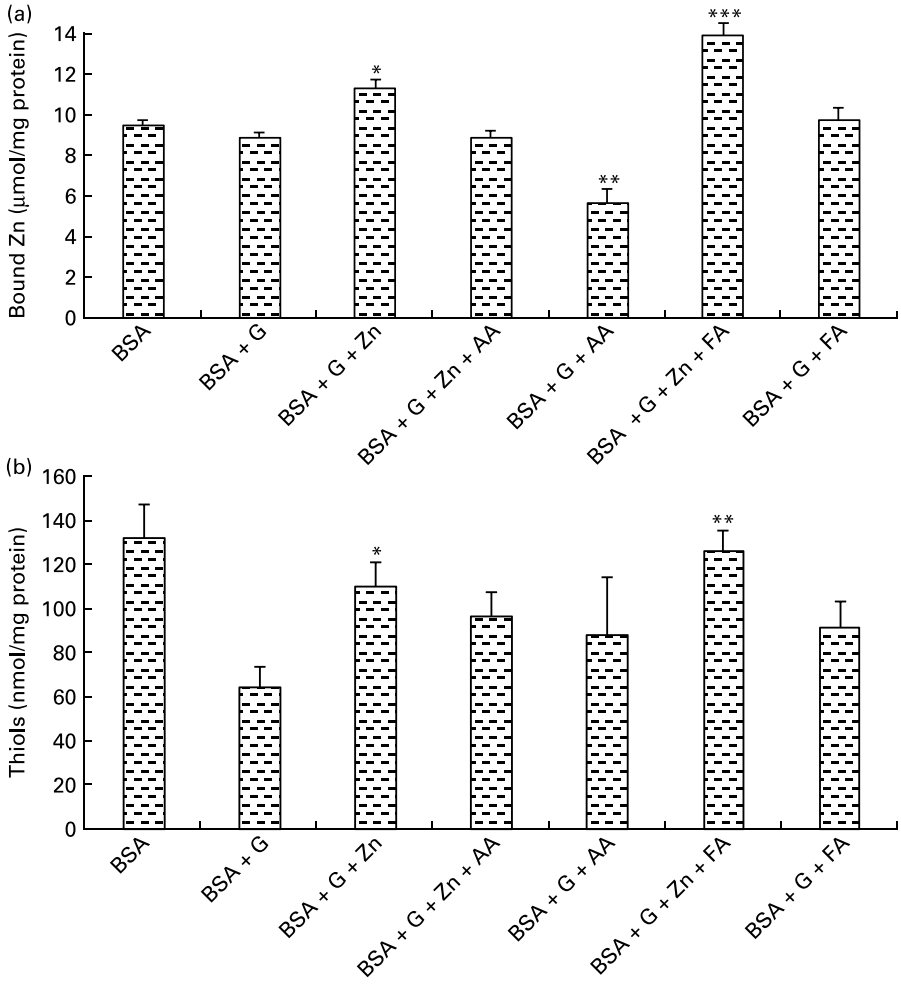

Fig. 4. Effect of different factors on (a) albumin–Zn binding and (b) thiol groups of albumin during glycation. BSA, bovine serum albumin; G, glucose; AA, ascorbic acid; FA, folic acid. Values are means (n 3), with standard deviations represented by vertical bars. Mean value was significantly different from that of the BSA + G treatment (positive control): * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ (one-way ANOVA).

hence does not directly participate in oxidation–reduction reactions[22]. It is also known to possess an indirect antioxidant property, particularly during protein oxidation[23]. In the literature, we find no direct evidence for a role of Zn in albumin glycation. However, there exists some debate about the role of Zn in the aetiology of Alzheimer's disease, which is a glycation-induced disease. Zn has been thought to act as a key mediating factor in Alzheimer's disease pathophysiology as (a) Zn^{2+} triggers Aβ aggregation[24,25] and (b) abnormally high levels of Zn have been found within amyloid deposits in Alzheimer's disease patients[26]. Conversely, a protective role of Zn in amyloid deposition in Alzheimer's disease has also been suggested since (a) Zn acts as an antioxidant and protects the brain from extensive redox chemical reactions that contribute to Alzheimer's disease-related oxidative stress[27,28] and (b) Zn supplementation in combination with a low-Cu diet significantly decreases ($P<0{\cdot}01$) amyloid precursor protein expression in platelets[29].

We found reduced β aggregation as well as protected thiols in the Zn-containing samples. According to Powell[23], Zn protects thiol groups against oxidation, through one of the three mechanisms: (1) direct binding of Zn to the thiols, (2) steric hindrance as a result of binding to some other protein site in close proximity to the thiol group or (3) a conformational change from binding to some other site on the protein. Further, during glycation, levels of protein-bound Zn were sufficiently elevated to decrease β-amyloid cytotoxicity. Our findings of effective protective action of Zn in HepG2 cells are in agreement with other reports stating that Zn protects against the Aβ-generated oxidative stress and related cytotoxicity in primary neuronal cells and human embryonic kidney cells[30,31]. There is no direct *in vivo* evidence about the role of Zn in albumin glycation; however, there are reports indicating an inhibitory effect of Zn in the glycation of other proteins. For example, in type 2 diabetic patients, a decrease in glycosylated Hb (HbA1c) was reported with Zn supplementation[32]. Also, results from our laboratory indicated that low plasma Zn levels were associated with the formation of cataract with and without diabetes[33,34].

In normal blood plasma, AA is bound to the albumin ($K = 1200$ per M)[35]. Zn also binds to albumin ($K = 7{\cdot}28$ per M) where histidine residues are involved. In the covalent attachment of glucose and AA to human serum albumin, participation of histidine residues has been proposed, where

the modification of histidine groups enhances ascorbate-mediated protein fluorophore formation[36]. Formation of AA-attributed protein fluorophores might be the reason for the observed increase in AGE fluorescence. With respect to the positive control, there was similarity in protein carbonyl formation and β aggregation in AA-co-incubated samples. Second, Zn binding to albumin was reduced in the presence of AA, which may be due to the competition of AA with Zn for albumin histidine residues. This explains the lowered cell viability and thiol levels in Zn + AA samples as compared with Zn samples.

During glycation, FA is glycated to form N2-[1-(carboxyethyl)] folic acid, which elucidates higher AGE fluorescence in FA-containing samples. With FA, the protein carbonyls and β aggregates formed were similar to the positive control which showed elevated HepG2 toxicity. On the other hand, the addition of FA along with Zn showed enhanced Zn–albumin binding and the same samples showed maximally protected thiol groups with the highest cell viability. FA has chelation activity and it has been reported previously that the presence of FA causes marked improvement in levels of albumin-bound Zn[18]. This increase in albumin-bound Zn may be offering additional protection to albumin during glycation. This indicates the dual role of FA (in the presence and absence of Zn) in albumin glycation. There are reports on beneficial effects of FA supplementation in Alzheimer's disease[37], where Alzheimer plaque formation is shown to be inhibited by FA through detoxification of homocysteine in the liver and kidneys. The present results suggest another protective role of FA other than homocysteine control, in glycation.

The role of micronutrients in albumin glycation is scarcely reported. Only Vinson & Howard[38] demonstrated an antiglycation role of AA in BSA glycation. In the case of other proteins of physiological importance, AA has been shown to be a potent glycation factor of lens crystallins. AA is known to act as a pentosidine precursor in the glycation reaction and plays an important role in crystallin browning[9,39]. Contrasting to these, Krone & Ely[40] have studied HbA1c in subjects supplemented with up to 20 g AA daily and found that for each 30 μM increase in plasma AA, HbA1c was reduced by approximately 0·1 g/dl (1 g/l). Vitamin C supplementation can decrease insulin glycation and ameliorate aspects of the obesity–diabetes syndrome in ob/ob mice[41]. A significant decrease in fasting blood sugar, TAG, LDL, HbA1c and serum insulin was seen in the group supplemented with 1000 mg vitamin C[42]. Increased levels of homocysteine have been associated with type 2 diabetes and FA is reported to reduce some complications. However, in the case of FA, there are no reports of its direct effects on protein glycation.

To conclude, we found a marked decline in BSA glycation with increased cell viability in the presence of Zn during long-term incubation. This gives a new dimension to the protective function of Zn in glycation reactions. *In vivo* studies relevant to albumin metabolism are hitherto required to further confirm the physiological implications of these *in vitro* results on the interplay of Zn, AA and FA.

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R. S. T. was involved in data collection, data analysis, data interpretation, the literature search and manuscript preparation. V. V. A. was involved in study design, data analysis, data interpretation, the literature search, manuscript preparation and review of the manuscript.

Both the authors have no financial or commercial conflicts of interest.

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