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Original Article

Beneficial effect of vitamin E supplementation on the biochemical and kinetic properties of Tamm–Horsfall glycoprotein in hypertensive and hyperoxaluric patients

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Abstract

Background. This study aimed to assess the therapeutic efficacy of oral vitamin E supplementation on the biochemical and kinetic properties of Tamm–Horsfall glycoprotein (THP) in hypertensive and hyperoxaluric patients.

Methods. Newly detected hypertensives ($n = 200$) and stone formers ($n = 200$) were each subdivided into two groups. One group ($n = 100$) was administered the antioxidant vitamin E at 400 mg/day given as an oral supplement along with standard therapeutic drugs for hypertension and hyperoxaluria and the patients were followed for a period of 9 months. The other group ($n = 100$) did not receive vitamin E (placebo controls). Age and sex-matched controls ($n = 100$) were monitored simultaneously. THP was isolated from 24 h urine samples before and at the end of every third month during a period of 9 months from the vitamin E-treated hypertensive and hyperoxaluric groups. THP samples were also collected from control subjects, and at the end of the ninth month from placebo controls. The isolated protein was assessed for purity by SDS–PAGE. The purity-checked proteins were subjected to spectrophotometric crystallization assay, calcium oxalate (CaOx) crystal interaction studies, and biochemical analysis of sialic acid, thiol and carbonyl content. Plasma superoxide, hydroxyl radical, hydrogen peroxide and vitamin E levels as well as superoxide dismutase and catalase activities were also monitored.

Results. The THP from the hypertensive and hyperoxaluric subjects exhibited a significant promoting effect on the nucleation and aggregation phases and caused a concomitant increase in CaOx crystal interaction. The altered kinetic properties of THP in these subjects were strongly associated with increased carbonyl content and with decreased thiol and sialic acid contents. Oral administration of vitamin E to these patients caused near normalization of these biochemical alterations and satisfactorily restored the kinetic properties of THP to near normal activity. At the end of 9 months, THP isolated from placebo controls (hypertensive and hyperoxaluric) showed highly aggregated calcium oxalate monohydrate crystals as observed by light microscopy. In contrast, vitamin E-supplemented patients showed CaOx dihydrate crystals that were similar to control THP. There was an imbalance in the oxidant and antioxidant levels. For the oxidants, superoxide, hydrogen peroxide and hydroxyl radical levels were increased, and for the antioxidants, there was loss of antioxidant enzyme activities and a decline in plasma vitamin E level in both hypertensive and hyperoxaluric patients. Supplementary antioxidant (vitamin E) corrected this imbalance to near normal conditions.

Conclusion. We hypothesize that the loss of THP inhibitory activity in the hypertensive and hyperoxaluric patients in a crystallizing medium is mediated primarily by oxidative damage to this protein. The possible occurrence of renal stones in essential hypertensive subjects, and the risk of recurrence in hyperoxaluric subjects, may be explained by oxidative damage to renal tissues that remained unchecked by standard drug therapies. The normalization of the kinetic properties of THP following vitamin E supplementation is in support of our hypothesis.

Keywords: lipid peroxidation; pathogenetic link between hypertension and urolithiasis; Tamm–Horsfall glycoprotein

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Introduction

The prevalence of nephrolithiasis has been reported to be 79% higher in hypertensive than in non-hypertensive subjects [1]. Alterations in calcium metabolism may play an important role in the pathogenesis of both hypertension and nephrolithiasis and may provide a plausible linking mechanism between the two disorders [2]. Supersaturation with calcium, oxalate and lithogenic salts *per se* cannot provide the sole explanation for the occurrence and retention of renal stones. There must be at least one other operating factor that causes the progression from the precipitation of calcium oxalate (CaOx) crystals to their subsequent growth and retention within the renal collecting system. Composition analysis of renal stones has indicated that organic matrix accounts for ~2.5% of the weight of each renal stone. Matrix is a poorly defined mishmash of uncharacterized macromolecules, of which Tamm–Horsfall glycoprotein (THP) is a part [3].

Research to date suggests that THP plays a dual role in modifying crystal aggregation both *in vivo* and *in vitro*. In solutions with high pH, low ionic strength and low concentrations of calcium and THP, this glycoprotein acts as a powerful inhibitor of CaOx crystal aggregation. Conversely, the combination of low pH, high ionic strength and high concentrations of calcium and THP causes the aggregation of the monomeric THP molecule, which lowers its inhibitory activity against CaOx crystal nucleation and aggregation [4].

In addition to these properties, the kinetic properties of THP could be altered by free radical-mediated oxidative injury. Prior studies by Cao *et al.* [5] demonstrated that oxidatively modified THP has a tendency to lose its zeta potential. Further research from our laboratory has shown that *in vitro* nitrated THP tends to lose its inhibitory activity in crystal growth system [6].

If free radical-mediated damage is considered to be a plausible factor in bridging the link between nephrolithiasis and hypertension by modulating the kinetic properties of inhibitory macromolecules, the quenching of these radical-mediated reactions is an essential step in curbing the occurrence and recurrence of renal stones. The present study aimed to assess the effect of oral antioxidant vitamin E supplementation in modulating the kinetic properties of THP in both hypertensive and hyperoxaluric subjects.

Patients and methods

This study was approved by the institutional ethics committee, and the patients gave informed consent for participation in the study. They were also given a clear picture of the beneficial properties of vitamin E before starting the vitamin E supplementation.

Collection of samples

The hypertensive patients were out-patients from the hypertensive clinic at the Department of General Medicine, Stanley Medical College and hospitals, an urban family welfare centre, Guild of Service, the Indian Red Cross Society and the Sri Ramachandra Medical College and Hospitals, Chennai, Tamilnadu, India. We included patients who exhibited a systolic pressure $\geq$160 mmHg and/or a diastolic pressure of $\geq$95 mmHg. The hypertensive patients selected for these studies were newly detected and were identified as essential hypertensives using the criteria listed below. We excluded patients who presented hypertension as a secondary cause due to diabetes mellitus, acute respiratory distress syndrome, acute myocardial infarction, chronic renal failure, bronchial asthma or cerebrovascular accidents, and those who were exposed to insecticides, drug thyroid replacements, antidepressants, non-steroidal anti-inflammatory drugs (NSAIDS) or allopurinol.

Kidney stone patients (seventh day following surgical calculus removal) were from the Urology post-operative ward, Department of Urology, Stanley Medical College and Hospitals, Chennai.

The patients and control subjects were grouped as follows. Group I were age- and sex- matched control subjects ($n=100$). Group II were essential hypertensive subjects ($n=200$) who were given antihypertensive drugs, such as angiotensin-converting enzyme (ACE) inhibitors ($n=80$) and β-blockers ($n=80$); 40 were males and 40 were females on each drug. The remaining 40 patients were administered calcium channel blockers; of these, 20 were males and 20 were females. Group II subjects were subdivided into groups III and IV. Group III subjects underwent supplementary antioxidant therapy and were followed-up at the end of the third (group IIIa), sixth (group IIIb) and ninth months (group IIIc), respectively. Group IV patients did not receive antioxidant supplementation (placebo controls) and were followed-up at the end of 9 months following the initial screening (group II before subdivision).

Group V were hyperoxaluric subjects ($n=200$) that included 100 males and 100 females. They were on the seventh day of the post-operative ward after surgical removal of renal stones and were on diuretic supplementation. Group V subjects were subdivided into groups VI and VII. Group VI underwent antioxidant supplementation and were monitored at the end of the third (group VIa), sixth (group VIb) and ninth months (group VIc). Group VII did not receive antioxidant supplementation (placebo controls) and were followed-up at the end of 9 months following the initial screening (group V before subdivision).

Samples of 24 h urine were collected from each of the groups at the respective months using toluene as preservative. Blood was collected by vein puncture from the antecubital vein and was immediately transferred to heparinized tubes to prevent coagulation; the blood was analysed for oxidants, such as superoxide radical [7], hydroxyl radical [8] and hydrogen peroxide [9]. Antioxidant status was monitored as the enzymatic activities of superoxide dismutase (SOD) [10] and catalase [11], and as levels of plasma vitamin E [12]. All experiments were performed immediately after procuring the samples.

The 24 h urine was utilized for the isolation of THP by the methods of Serafini-Cessi [13] and Gokhale *et al.* [14]. SDS–PAGE was utilized to check the purity of the isolated THP [14]. The purity assessed THP was utilized for biochemical parameters such as thiol [15], sialic acid [16] and carbonyl content [17].

A spectrophotometric crystallization assay was carried out with the THP isolated from all the groups by the method of Hess [18]. The percentage inhibition in the presence of THP from various groups were calculated as:

$$[1 - [S_{Nm}/S_{Nc}] \times 100 \text{ for the rate of nucleation}$$

$$[1 - [S_{Am}/S_{Ac}] \times 100 \text{ for the rate of aggregation}$$

respectively, where m is the modulator, and c the control. Negative inhibition values showed the promotion of the respective crystallization process. CaOx crystal interaction was carried out according to the method of Laxmanan *et al.* [19]. The [^{14}C]oxalate-labelled CaOx monohydrate crystals used in the experiment were synthesized by previously standardized procedures in other laboratories. Briefly, 0.53 mmol of unlabelled plus 0.01 mmol of [^{14}C]oxalic acid was dissolved in water titrated to pH 6.6 with 1.6 M potassium hydroxide to convert oxalic acid fully to its dipotassium salt, $K_2{}^{14}C_2O_4$, without excess K^+ ions. The CaOx salt was then prepared by adding, alternately every 30 s, 40 µl aliquots of the $K_2{}^{14}C_2O_4$ solution and 0.4 M $CaCl_2$ $2H_2O$ to 4.0 ml of water in a conical flask with constant agitation provided by a magnetic stirrer. The temperature of the reaction was thermostatically maintained at 75°C. Nitrogen gas was bubbled through the solution to exclude CO_2. The suspension of $Ca_2{}^{14}C_2O_4$ was then digested for 5 h under the same conditions. After cooling, the crystals were collected by centrifugation and were washed seven times with 30 ml of deionized water to remove the potassium and chloride ions. The crystals were then dried to constant weight at 70°C for 4 days to convert the calcium trihydrate and dihydrate to the most stable monohydrate form. The CaOx* crystals (72 268 c.p.m.) were stored at 22°C in a polyethylene container in a vacuum desiccator over anhydrous $CaSO_4$ until use. When needed, the required amounts were dissolved in 0.05 M HCl. Protein–crystal interactions were assessed as follows: 0.1 ml of the protein (100 µg) was incubated in 0.8 ml of acetate buffer (200 mM, pH 4.5] and 0.1 ml of 50 nmol of labelled CaOx* crystals (5000 c.p.m.). Non-specific binding was determined by adding 100 µM cold oxalate (100 µM sodium oxalate made ice cold before use) to the incubation solution and subsequently decreasing the buffer of the incubation mixture to 0.7 ml and incubating the solution for the same time period as that of the specific binding solution. The total incubation time was 20 min for both the specific and non-specific binding. At the end of the incubation period, the mixture was filtered through 0.45 µm membrane filters with the aid of constant vacuum, and the filters were washed twice with buffer. The filters were placed in mini vials (7.0 ml) to which 2 ml of scintillation fluid [toluene 200 ml, Triton X-100 100 ml, 2,5 diphenyl oxazole (PPO) 1.5 g and 1,4-(2-(phenyl oxazolyl)-benzene) (POPOP) 0.015 g] was added and the CaOx* was measured in a Kontron Betamatic IV liquid scintillation counter.

Light microscopic studies to assess the protein interaction with CaOx crystals were performed as previously described [20].

Statistics

Data are presented as means ± SD from six determinations. Statistical analysis was carried out using analysis of variance (ANOVA; SPSS for Windows Release 9.05)

Results

Oxidant and antioxidant status

Table 1 summarizes the oxidant and antioxidant status before and after vitamin E supplementation. The levels of superoxide, hydroxyl radical and hydrogen peroxide were increased by 47, 35 and 39%, respectively, in the hypertensive (group II) subjects and by 48, 38 and 40%, respectively, in the hyperoxaluric (group V) subjects. A 50% decline in the antioxidant SOD (haemolysate) and catalase (erythrocyte membrane) levels was observed in both group II and group V patients ($P < 0.001$). Plasma vitamin E levels were depleted by 76 and 79% in group II and group V, respectively. Vitamin E supplementation restored oxidant and antioxidant status markers to near normal values. The percentage restoration of the oxidants, including superoxide, hydroxyl radical, and hydrogen peroxide, was significant and was 44, 33 and 37%, respectively, in group IIIc (hypertensive subjects after 9 months vitamin E supplementation); and 46, 34 and 36%, respectively, in group VIc (hyperoxaluric subjects who had undergone vitamin E supplementation for a period of 9 months). However, the percentage normalization was only 18, 2 and 4% in group IV (hypertensive placebo controls) patients; and only 15, 1 and 2% in group VII patients, respectively. Antioxidant status makers also showed a similar trend during normalization.

Electrophoretic mobility

SDS–PAGE depicts the purity of isolated THP; Figure 1 shows an overall representation of the five groups excluding the placebo controls. This figure demonstrates that the isolated THP had a high level of purity.

CaOx crystal interaction studies*

Table 2 summarizes the interaction between ^{14}C-labelled CaOx monohydrate crystals and THP. The interaction between CaOx monohydrate crystals and THP increased by 73% in group II (hypertensives) and by 74% in group V (hyperoxalurics); co-supplementation of vitamin E to group III (hypertensive) and group VI (hyperoxaluric) patients for a period of 9 months rapidly reduced the CaOx crystal interaction to ~72% in group IIIc (hypertensive subjects who had undergone supplementation for a period of 9 months) and to 73% in the group VIc patients; in contrast, normalization was not as significant in placebo controls, and values were 1.2% in group IV (hypertensive placebo controls) and 1% in group VII (hyperoxaluric placebo controls).

Crystallization studies

Table 2 summarizes the percentage inhibition or promotion of nucleation and aggregation phases in

Table 1. Oxidant and antioxidant status among the various groups

		Superoxide radical[1]	Hydroxyl radical[2]	Hydrogen peroxide radical[2]	SOD[4]	Catalase[5]	Vitamin E[6]
Control							
Group I ($n=100$)		$42.9\pm4.7^{b***c***}$	$17.5\pm2.3^{b***c***}$	$61.8\pm3.5^{b***c***}$	$0.051\pm0.005^{b***c***}$	$7.81\pm0.86^{b***c***}$	$1.35\pm0.03^{b***c***}$
Hypertensive subjects before treatment							
Group II ($n=200$)		$80.5\pm5.7^{a***}$	$27.0\pm2.2^{a***}$	$101.1\pm6^{a***}$	$0.029\pm0.002^{a***}$	$3.95\pm0.64^{a***}$	$0.31\pm0.03^{a***}$
Hypertensive subjects after treatment group III ($n=100$)							
3 months	Group IIIa	$64.5\pm4.4^{a***b***}$	$25.7\pm2.1^{a***b***}$	$86.8\pm2.4^{a***b***}$	$0.047\pm0.003^{a***b***}$	$5.90\pm0.80^{a***b***}$	$0.82\pm0.04^{a***b***}$
6 months	Group IIIb	$45.0\pm5.0^{a***b***}$	$20.6\pm2.5^{a***b***}$	$64.7\pm9.0^{a**b***}$	$0.049\pm0.005^{a**b***}$	$7.47\pm0.88^{a**b***}$	$1.32\pm0.12^{a*b***}$
9 months	Group IIIc	$43.7\pm3.8^{a*b***}$	$17.8\pm2.2^{a*b***}$	$61.2\pm10.8^{aNSb***}$	$0.049\pm0.003^{a*b***}$	$7.61\pm1.06^{a*b***}$	$1.31\pm0.16^{aNSb***}$
	Group IV	$65.7\pm7.8^{a***b***}$	$26.5\pm2.4^{a***b***}$	$97.2\pm\ 7.9^{a***b*}$	$0.036\pm0.002^{a***b***}$	$4.03\pm0.70^{a***bNS}$	$0.33\pm0.05^{a***b**}$
Hyperoxaluric subjects before treatment							
Group V ($n=200$)		$82.0\pm5.5^{a***}$	$28.2\pm2.7^{a***}$	$103.4\pm8^{a***}$	$0.023\pm0.002^{a***}$	$3.91\pm0.66^{a***}$	$0.30\pm0.02^{a***}$
Hyperoxaluric subjects after treatment group V ($n=100$)							
3 months	Group VIa	$65.1\pm4.2^{a***c****}$	$26.0\pm3.2^{a***c***}$	$88.1\pm12.4^{a***c***}$	$0.045\pm0.005^{a***c***}$	$5.41\pm0.49^{a***c***}$	$0.80\pm0.01^{a***c***}$
6 months	Group VIb	$51.0\pm3.9^{a***c***}$	$21.1\pm2.7^{a***c***}$	$65.3\pm8.5^{a***c***}$	$0.046\pm0.004^{a***c**}$	$7.43\pm0.86^{a**c***}$	$1.30\pm0.12^{a**c***}$
9 months	Group VIc	$68.2\pm8.1^{a***c***}$	$27.3\pm3.0^{a***c**}$	$99.2\pm7.9^{a***c***}$	$0.035\pm0.002^{a***c***}$	$3.99\pm0.66^{a***bNS}$	$0.33\pm0.02^{a**c***}$
	Group VII	$44.5\pm3.6^{a**c***}$	$18.1\pm2.0^{a**c***}$	$63.2\pm9.2^{a*c***}$	$0.048\pm0.003^{a**c***}$	$7.54\pm0.99^{a*b***}$	$1.29\pm0.15^{a*c***}$

Values are means ± SD of six experiments; the comparisons include a = control *vs* various groups, b = hypertensives *vs* various hypertensive groups; c = hyperoxalurics *vs* various groups.
[1]mmol of NBT reduced/ml/min; [2]nmol of MDA/ml/min; [3]μmol/ml/min; [4]SOD activity as the amount of enzyme activity required to give 50% inhibition of pyrogallol; [5]μmol of hydrogen peroxide converted/mg protein/s; [6]mg/dl.
$^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

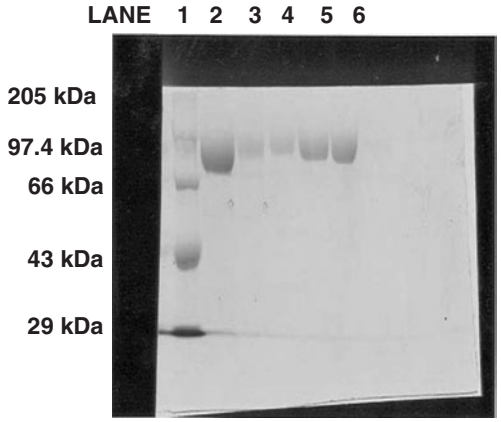

Fig. 1. Electrophoretic mobility of Tamm–Horsfall glycoprotein isolated from control and various experimental groups.

the presence of THP isolated from the various groups. The THP isolated from the normal subjects (group I) inhibited both the nucleation and aggregation phases by 13.52 and 25.32%, respectively. In contrast, THP isolated from the group II and group V patients promoted the nucleation and aggregation phases. The percentage promoting effect of the nucleation phase was −11.60% in group II and −12.36% in group V subjects; the promoting effect on the aggregation phase was −37.81% in group II and −43.49% in group V subjects.

Following 9 months of oral vitamin E supplementation, the percentage promoting effect was transformed into a percentage inhibiting effect for both the nucleation and aggregation phases. The inhibiting effect was 13.24% in group IIIc and 12.78% in group VIc subjects for the nucleation phase and 24.94% in group IIIc and 24.73% in group VIc subjects for the aggregation phase. The placebo controls, who still showed a percentage promoting effect rather than a percentage inhibiting effect compared with their vitamin E-treated counterparts, did not show significant normalization.

Biochemical parameters

THP isolated from the various groups was assessed for thiol, sialic and carbonyl contents, and these data are presented in Table 2. Thiol depletion was 39% in group II and 51% in group V subjects. Normalization following 9 months of vitamin E supplementation was rapid and the percentage normalization was 38% in group IIIc and 36% in group VIc subjects. In contrast, the percentage normalization was less significant among the placebo controls, being 10% in the group IV and 3% in the group VII subjects.

Depletion of sialic acid was prominent and was 35% in group II subjects and 43% in group V subjects; normalization of sialic acid levels following vitamin E supplementation was rapid and was 33% in group IIIc and 40% in group VIc subjects compared with placebo controls, who showed only 8 and 24% normalization in group IV and VII subjects, respectively.

Significant ($P < 0.001$) increases in protein carbonyl content were observed in group II (61%) and group V (63%) subjects compared with control subjects. Oral supplementation with vitamin E rapidly returned the carbonyl content to near normal values, and the percentage restoration was 60% in group IIIc and 62% in group VIc subjects. Placebo controls, who showed only 13 and 4% restoration in groups IV and VII, still had a significantly high carbonyl content in the THP that was isolated from these subjects ($P < 0.001$).

Light microscopic studies

Figure 2 depicts the representative fields of photographs at 400 × magnification. In the crystal growth system (Figure 2A), only CaOx monohydrate crystals were seen when calcium chloride and potassium oxalate reacted. When THP isolated from group I (normal) subjects was added as a modulator, only CaOx dihydrate crystals formed (Figure 2B).

Significant aggregates of CaOx monohydrate crystals were observed in THP isolated from group IV (placebo hypertensives) and group VII (placebo hyperoxalurics) subjects (Figure 2C and D). Supplementation with vitamin E restored the normal modulating property in group IIIc and group VIc, who had CaOx dihydrate crystals similar to those in control (group I) subjects (Figure 2E and F).

Discussion

Proteins such as THP and nephrocalcin act as modulators of crystal nucleation and aggregation [21]. High levels of calcium, the concentration of THP, ionic strength, pH and low levels of citrate tend to alter the kinetic behaviour of THP by promoting its aggregating properties. However, these explanations do not adequately explain the self-aggregating properties of THP which indicate the possibility of an alternative mechanism. Previous research from other laboratories has shown that *in vitro* oxidatively modified THP tends to lose its zeta potential. Free radicals that are released from electron leakage, metal ion-dependent reactions and auto-oxidation of lipids and sugars can cause damage to biomolecules.

Our findings suggest that free radicals such as superoxide, hydroxyl radical and hydrogen peroxide have profound effects on the kinetic behaviour of THP. Oxidative stress represents an imbalance between the levels of oxidant and antioxidant status in favour of the former. In our study, we found an extensive increase in the level of radicals such as superoxide, hydroxyl and hydrogen peroxide, as well as diminished antioxidant activity (SOD and catalase) and lowered antioxidant levels (plasma vitamin E). A decreased antioxidant status in hypertensive patients was reported by Koska *et al.* [22].

Table 2. Effect of vitamin E supplementation on CaOx* interaction, nucleation, nggregation, thiol content, sialic acid content and carbonyl content among the various groups

		CaOx interaction[1] pH 4.5	% Inhibition		Thiol content[3]	Sialic acid content[3]	Carbonyl content[3]
			Nucleation[2]	Aggregation[2]			
Control							
Group I ($n=100$)		$150.4 \pm 9.3^{b***c***}$	$13.5 \pm 1.3^{b***c***}$	$25.3 \pm 1.5^{b***c***}$	$57.8 \pm 6.5^{b***c***}$	$50.1 \pm 6.4^{b***c***}$	$56.6 \pm 11.5^{b***c***}$
Hypertensive subjects before treatment							
Group II ($n=200$)		$548 \pm 58^{a***}$	$-11.6 \pm 1.0^{a***}$	$-37.8 \pm 2.4^{a***}$	$35.0 \pm 3.6^{a***}$	$32.7 \pm 3.2^{a***}$	$146.9 \pm 17.4^{a***}$
Hypertensive subjects after treatment group III ($n=100$)							
3 months	Group IIIa	$250.3 \pm 28.7^{a***b***}$	$6.6 \pm 0.91^{a***b***}$	$15.7 \pm 1.5^{a***b***}$	$48.1 \pm 7.2^{a***b***}$	$42.5 \pm 2.8^{a***b***}$	$107.0 \pm 18.3^{a***b***}$
6 months	Group IIIb	$157.7 \pm 23.8^{a**b***}$	$12.8 \pm 2.4^{a**b***}$	$24.5 \pm 2.1^{a**b***}$	$54.2 \pm 9.5^{a**b***}$	$47.3 \pm 7.1^{a**b***}$	$61.2 \pm 12.4^{a**b***}$
9 months	Group IIIc	$541 \pm 58^{a***bNS}$	$-9.3 \pm 1.1^{a***b***}$	$-35.0 \pm 2.7^{a***b***}$	$38.9 \pm 6.3^{a***bNS}$	$35.5 \pm 2.0^{a***b***}$	$128.3 \pm 20.6^{a***b***}$
	GRP IV ($n=100$)	$152.7 \pm 23.7^{aNSb***}$	$13.2 \pm 1.0^{a*b***}$	$25.0 \pm 2.6^{aNSb***}$	$56.9 \pm 9.4^{aNSb***}$	$49.1 \pm 5.6^{aNSb***}$	$58.9 \pm 16.7^{aNSb***}$
Hyperoxaluric subjects before treatment							
Group V ($n=200$)		$575 \pm 43.1^{a***}$	$-12.3 \pm 1.3^{a***}$	$-43.5 \pm 3.1^{a***}$	$28.2 \pm 2.6^{a***}$	$28.1 \pm 3.3^{a***}$	$157.0 \pm 23.3^{a***}$
Hyperoxaluric subjects after treatment group V ($n=100$)							
3 months	Group VIa	$257 \pm 28.6^{a***c***}$	$5.50 \pm 0.71^{a***c***}$	$7.23 \pm 0.87^{a***c***}$	$45.2 \pm 3.3^{a***c***}$	$40.1 \pm 7.4^{a***c***}$	$115.0 \pm 13.8^{a***c***}$
6 months	Group VIb	$158 \pm 22.7^{a**c***}$	$8.7 \pm 0.72^{a***c***}$	$17.9 \pm 3.3^{a***c***}$	$50.0 \pm 9.3^{a**c***}$	$45.0 \pm 8.2^{a***c***}$	$73.4 \pm 17.9^{a***c***}$
9 months	Group VIc	$154.4 \pm 24.8^{a*c***}$	$12.8 \pm 2.4^{a**c***}$	$24.7 \pm 2.7^{a*c***}$	$55.0 \pm 8.0^{a*c***}$	$47.1 \pm 7.76^{a**c***}$	$60.2 \pm 17.5^{a**c***}$
	Group VII ($n=100$)	$543 \pm 58.8^{a***c***}$	$-10.0 \pm 1.1^{a***c***}$	$-35.8 \pm 1.9^{a***c***}$	$40.9 \pm 9.3^{a***c***}$	$37.2 \pm 3.8^{a***c***}$	$149.8 \pm 22.1^{a***c*}$

Values are means ± SD of six experiments; the comparisons include a = control *vs* various groups, b = hypertensives *vs* various hypertensive groups, c = hyperoxalurics *vs* various groups.
[1]pmol/mg protein; [2]percentage inhibition of promoting or inhibiting activity of 10 µg ofprotein; [3]µg/mg protein.
$^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

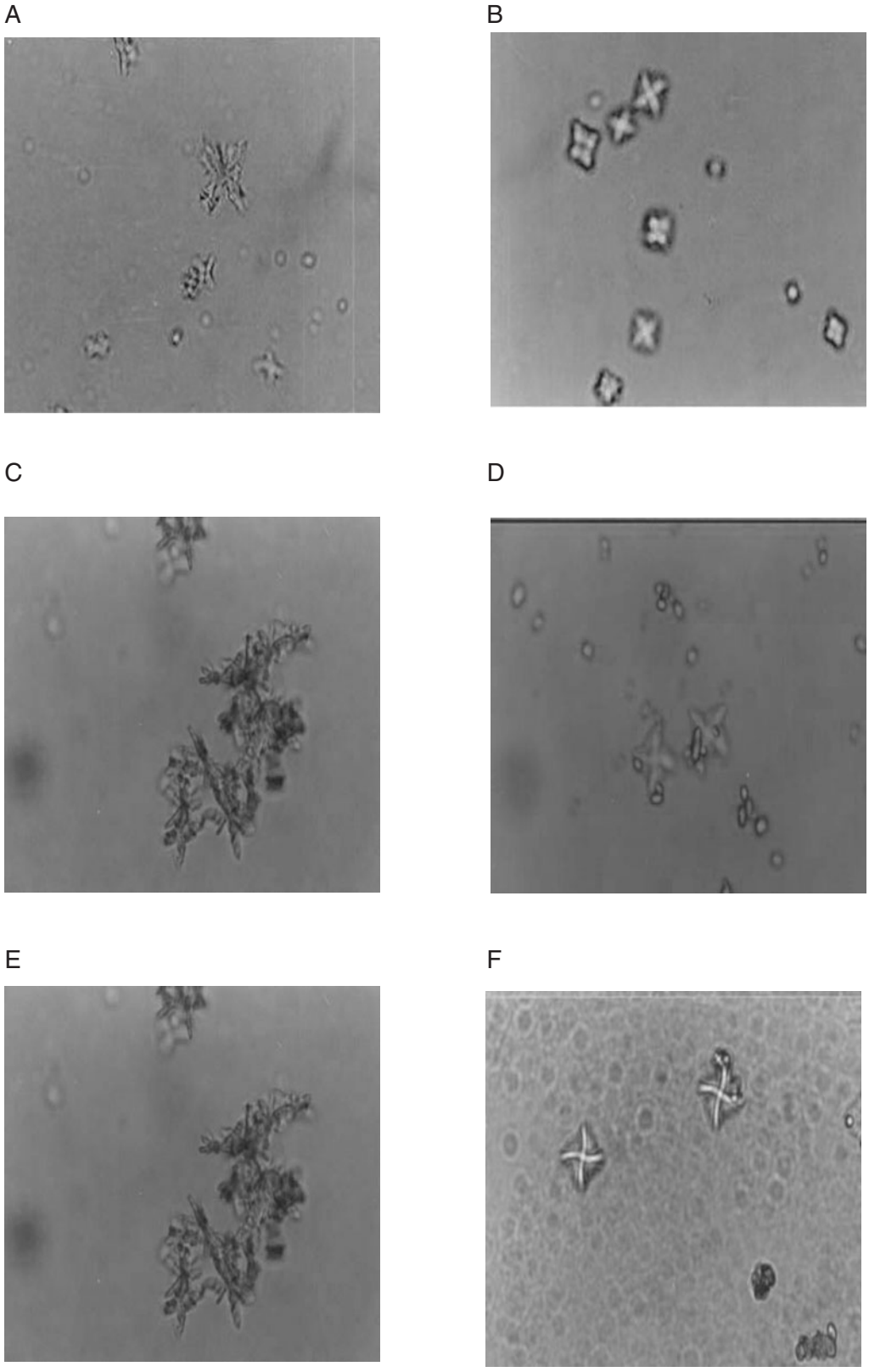

Fig. 2. Morphology of light microscopic crystals formed in the presence and absence of protein fractions. (A) Control CaOx monohydrate crystals. (B) Control THP showing CaOx dinohydrate crystals. (C) THP of hypertensive (placebo controls) patients showing highly aggregated CaOx monohydrate crystals. (D) THP isolated from hypertensive patients following 9 months of oral vitamin E supplementation showing CaOx dihydrate crystals. (E) THP of stone formers (placebo controls) showing highly aggregated CaOx monohydrate crystals. (F) THP isolated from stone formers following 9 months of oral vitamin E supplementation showing CaOx dihydrate crystals.

Our studies revealed that THP isolated from group II (hypertensive) and group V (hyperoxaluric) patients exhibited a profound degree of oxidative modification, as demonstrated by the increased level of carbonyl content that significantly normalized following vitamin E supplementation. We also found that deglycosylation of THP produced promoting effects in the crystallization medium and increased CaOx* (data not shown). Similar results were reported by Hallson *et al*. [23].

Although natural deglycosylation is not a common phenomenon *in vivo*, it may occur through oxidative stress. As a possible mechanism, the oxidized sugars may be defectively placed on the protein leading to abnormal sugar sequences on the protein [4] that may be trimmed off by an unknown mechanism. Indirect evidence for this hypothesis comes from the present studies, which showed that the sialic acid content of THP was normalized following oral vitamin E supplementation. Other factors that may influence the nucleation and aggregation property of the protein are the –SH groups that stabilize the major part of protein secondary structure though disulfide bridges. Group II, group V placebo controls, group IV and group VII showed significant depletion in thiol content compared with the control (group I) and vitamin E-treated groups (group III and group VI). THP possesses 49 cysteine residues that contribute to the –SH groups [4]. A loss of several S–S bridges by oxidation could cause alterations in the secondary structure of the protein. In our laboratory, we documented an *in vitro* oxidative modification of a 45 kDa protein that we had isolated [24]. This finding suggests that loss of –SH groups has a profound effect on inhibitory properties in a crystallizing medium [25]. This indicates that vitamin E mediates its action on protein kinetic behaviour primarily by curbing oxidative damage to the protein of the human subjects *in vivo*.

It may be possible to explain the increased CaOx* crystal interaction with THP from group II to group V patients. Oxidative damage to the protein, and especially to its sugar moieties and –SH groups, tends to expose the hydrophobic calcium-binding sites, as was observed by ligand–protein interaction studies (unpublished data). The role of oxidative damage in increasing the CaOx* crystal interaction was demonstrated by the finding that 9 months of oral vitamin E supplementation decreased the interaction between CaOx crystals while the levels of sialic acid and thiol became normalized.

Taken together, these findings indicate that THP under normal conditions may sequester calcium and thereby inhibit its interaction with oxalate. Further, from our results, we infer that the protein's sialic acid content and –SH content have a crucial role in this mechanism of sequestration. The THP's carbohydrate content tends to be 30%, of which sialic acid is the major contributor. The presence of a normal content of sialic acid on the protein's outer coat gives a high negative charge to the protein as evidenced by its low pI. This negativity effectively sequesters the nucleating CaOx crystals or oxalate from gaining access into the protein due to the negative nature of oxalate and the fact that like charges repel. However, a positively charged calcium ion gains entry to the calcium-binding hydrophobic domains embedded in the protein's interior structure stabilized by the S–S bridges evidenced by the –SH content of the protein.

In contrast, deglycosylation of the oxidized sugars weakens the protein's negativity, and the loss of S–S bridges, evidenced by the loss in the –SH content of the protein, disturbs the secondary structure, which evidently allows rapidly nucleating CaOx crystals to interact with the protein and oxalate to interact with already embedded calcium crystals within the protein. These processes convert THP to a glue-type protein, which can harbour CaOx crystals and enhance its growth.

In conclusion, THP tended to act as a heterogeneous nucleator in our patient groups, thus acting as a bridge in predisposing essential hypertensive patients to renal lithiasis and increasing the incidence of recurrence in hyperoxaluric patients even while on standard drug supplementation. The loss of inhibitory activity was mediated by various factors, and especially by free radicals. Thus, the occurrence and recurrence of renal lithiasis in essential hypertensive and hyperoxaluric patients may be satisfactorily explained by non-quenched free radical-mediated damage. The normalization of the kinetic properties of THP following vitamin E supplementation indicates that renal injury is an important prerequisite for retention of renal calculi. Altogether, these findings suggest that a supplementary antioxidant combination along with the standard drug supplementation may be highly beneficial for the treatment of renal lithiasis.

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Conflict of interest statement. None declared.

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