Pediatr Nephrol (2006) 21: 771–777
DOI 10.1007/s00467-006-0061-2

ORIGINAL ARTICLE

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Tissue factor pathway inhibitor in childhood nephrotic syndrome

Received: 4 July 2005 / Revised: 6 December 2005 / Accepted: 9 December 2005 / Published online: 31 March 2006

Abstract It is now recognised that the extrinsic tissue factor pathway is the main trigger to the coagulation system in vivo. Its main inhibitor, tissue factor pathway inhibitor (TFPI), has never been studied in childhood nephrotic syndrome. The aim of the study was to monitor the level of TFPI in childhood nephrotic syndrome. One hundred and thirty-nine nephrotic children were classified into the following groups: group 1 (n=25), in relapse and receiving no treatment; group 2 (n=37), in relapse but receiving steroid treatment; group 3 (n= 45), in early remission and on steroids; group 4 (n=24), in established remission and receiving no steroids; group 5 (n=8), steroid-resistant. The controls (n=84) were healthy and age-matched. There was significant elevation of total TFPI levels in groups 1 and 2 and 3; levels were comparable to those of the healthy controls in group 4. The highest levels of total TFPI were recorded in group 5. Like total TFPI, the levels of the free form of TFPI showed a statistically significant increase in groups 1, 2, 3 and 4, when compared with levels in healthy controls. The highest levels of free TFPI were recorded group 5. We concluded that the elevated levels of both the total and free TFPI in various phases of nephrotic syndrome add another natural anticoagulant mechanism, which will attenuate the hypercoagulability of childhood nephrotic syndrome.

Keywords Nephrotic syndrome · Tissue factor pathway inhibitor · TFPI · Haemostasis

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Introduction

Haemostasis is delicately balanced between pro- and anticoagulant mechanisms, and an imbalance of this equilibrium may result in thrombotic disease. Nephrotic syndrome is one example of a hypercoagulable state, with high risk for the development of thromboembolism [1–6]. However, the thromboembolic complications associated with nephrotic syndrome occur with much lower frequency in children than in adults, despite the more pronounced prothrombotic coagulation abnormalities in the former [2].

The prothrombotic changes in childhood nephrotic syndrome include an increase in the activity of clotting factors and platelet number and function and a diminution in fibrinolytic activity and coagulation inhibitor antithrombin III [1–10]. Recently, some emphasis was focused on increased red blood cell (RBC) aggregation and plasma viscosity as haemorheological risk factors for thromboembolic disease that compound further the prothrombotic haemostatic changes, particularly during the relapse of nephrosis [11–13]. These alterations occur simultaneously and are enhanced by concurrent elevation in the plasma levels of fibrinogen and IgM [11] and hypercholesterolaemia [12, 14].

The possible role played by the natural anticoagulants as protective against thromboembolism in childhood nephrosis has also received particular attention in many recent studies. Despite some disagreements, the weight of evidence was in favour of the elevation of the natural anticoagulants, proteins C and S, in childhood nephrotic syndrome [5–7, 15–18].

Recently, it has been increasingly realised that the extrinsic (tissue factor) pathway of activation of the coagulation system is the main trigger to the coagulation system in vivo [19], with the intrinsic system playing an amplification role. Tissue factor pathway inhibitor (TFPI) is the major physiological inhibitor to the tissue factor-mediated extrinsic pathway of blood coagulation. TFPI binds to factor Xa and, in this combination, binds to and inhibits tissue factor/factor VIIa complex [19, 20]. TFPI originates from vascular endothelium. Of the total body

TFPI, 85% is bound to the vascular endothelium, leaving 15% to be blood-born, and this exists in two forms: 80% is present in bound form (total to lipoproteins). The rest (15%), which is free in plasma (free form), is the physiologically active anticoagulant part of TFPI [19].

In vitro studies have shown that direct activation of factor X by VIIa-TF complex is quickly suppressed by TFPI [21]. Also the depletion of TFPI sensitises rabbits to disseminated intravascular coagulation (DIC) induced experimentally by tissue factor [22] or endotoxin [23], while the infusion of TFPI ameliorates the DIC induced by tissue factor [24] or endotoxin [25]. Further support for the pivotal role of TFPI in the prevention of thrombosis comes from the observation that high concentrations of TFPI can prevent venous thrombosis in rabbits [26]. These and other pieces of recent evidence in human and animal studies [19] indicated that the deficiency of this inhibitor can lead to a thrombotic tendency; in this sense TFPI can be included as a natural coagulation inhibitor. This encouraged us to assess the fluctuations of TFPI in childhood nephrotic syndrome, an area that has not been studied so far as we know.

Aim of the study

To monitor the level of TFPI, total and free, in various phases of childhood nephrotic syndrome.

Subjects and methods

Patients A total of 139 children with nephrotic syndrome was recruited; they were from the Paediatric Nephrology Clinic or were in-patients in the Nephrology Ward, King Khalid University Hospital, Riyadh; 100 were males and 39 were females. Their ages ranged from 2.5 years to 14 years. Their physical characteristics are summarised in Table 1. This study was approved by the ethics committee, which is an offshoot of the College of Medicine Research Centre (CMRC). Informed consent was obtained from the parents of the children who participated in the study.

The nephrosis was considered to be in relapse if the following criteria were fulfilled: (1) serum albumin below 25 g/l in association with (2) heavy proteinuria by dipstick (>2+) or 24-h urine showing more than 40 mg/h per m^2 surface area. The duration of the relapse varied between 1 week and 2 weeks. Remission was defined as protein-free urine for 3 successive days. Relapse was treated in each case with prednisolone (2 mg/kg per day) and increased to a maximum of 60 mg, given in divided doses, two to three doses/day, until remission was induced, or until 28 days of treatment had elapsed. The dose was then changed to 2 mg/kg every other day for 4 weeks. Thereafter, it was tapered slowly over the next 2 to 3 months, provided the patients responded to therapy. If there was no response the patients were considered to be steroid resistant and were put on cyclophosphamide.

Table 1 Physical characteristics of children with nephrotic syndrome (n=139) and healthy controls (n=84). Results are expressed as means ± standard deviations

Characteristic	Group 1 (relapse, no steroids), n=25	Group 2 (relapse, on steroids), n=37	Group 3 (remission, on steroids), n=45	Group 4 (remission, no steroids), n=24	Group 5 (steroid resistant), n=8	Controls, n=84
Age (years)	7.0±4.1	7.2±4.2	7.4±2.7	6.5±3.5	6.3±2.94	7.3±3.1
Weight (Kg)	31.1±22.5	28.1±11.4	25.6±8.7	23.7±13.2	25.9±11.4	23.3±10.4
Height (cm)	109.6±32.1	115.1±19.4	116.6±17.7	115.2±24.2	106.8±16.4	117.2±19.5
Creatinine (μmol/l)	41.9±19.1	39.1±11.3	39.8±15.9	44.7±18.4	37.9±19.6	62–115[a]
Urea (μmol/l)	4.33±2.5	3.7±1.1	3.9±1.2	3.5±1.7	4.5±2.0	2.5–6.4[a]

[a]Laboratory reference range

The patients were investigated during different phases of the disease with and without steroid therapy.

In order to study the relationship between the clinical profile and haemostatic laboratory findings, we divided the steroid responding patients into four groups (retrospectively), as in a previous report [9]:

Group 1, (n=25):
Nephrotic patients in relapse before receiving any treatment.
Group 2, (n=37):
Patients in relapse but receiving steroid treatment.
Group 3, (n=45):
Patients in early remission (serum albumin between 25–30 g/l) and still on steroids.
Group 4, (n=24):
Patients in established remission receiving no steroids and with serum albumin levels >30 g/l.
Group 5, (n=8):
Patients who were steroid resistant.

Control subjects The control subjects (n=84) were either healthy siblings of the patients or children who attended the Outpatient Department for routine health checks; 45 were males and 39 were females, and their ages ranged from 1 year to 14 years (mean ± SD 7.3±3.1 years) (Table 1).

Blood samples

Blood (10 ml) was collected by venepuncture from an easily accessible vein in the antecubital fossa using the minimum of stasis, directly into a Vacutainer tube (Teromu, Japan) containing sodium citrate (0.11 M), to give a blood: citrate ratio of 9:1. The contents of the tube were mixed gently by inversion, and the tube was placed into crushed ice and transported without delay to the Coagulation Laboratory. The tube was then centrifuged at 3,000 rpm for 15 min, and the platelet-rich plasma was separated using a plastic pipette and put in aliquots into plastic tubes, which were stored at −80°C until assays were undertaken in batches at a later date.

Laboratory methods

Coagulation inhibitors

All the following factors were assayed with commercial kits and according to the manufacturers' instructions.

Tissue factor pathway inhibitor: total and free [enzyme-linked immunosorbent assay (ELISA), Stago Diagnostica, France].

Protein-C assay: the Asserachrom protein-C test kit (Stago Diagnostica), using the ELISA.

Protein-S assay (total and free): the one-step sandwich technique of enzyme immunoassay (EIA), using

Table 2 Summary of the results of the haemostatic variables in children in different phases of nephrotic syndrome compared with healthy controls. Results are expressed as mean ± standard deviation

Haemostatic parameter	Relapse, no steroids *P*	Relapse, on steroids *P*	Remission, on steroids *P*	Remission, no steroids *P*	Steroid resistant *P*	Controls
Fibrinogen (mg %)	555±310*, *P*=0.003	555±305.8*, *P*=0.001	351.2±140.9, *P*=0.059	374±219.6, *P*=0.289	510±224.8*, *P*=0.023	299±106.7
Protein C (%)	124.2±42.0*, *P*=0.001	149.3±47.5*, *P*=0.001	105.3±27.14*, *P*=0.001	94.8±33.28, *P*=0.68	120.6±30.0*, *P*=0.011	83.5±15.2
Total protein S (%)	89.4±18.3*, *P*=0.001	97.5±15.3*, *P*=0.001	97.5±17.3*, *P*=0.001	96.6±13.3*, *P*=0.001	103.1±13.3*, *P*=0.001	74.1±18.8
Free protein S (%)	66.9±23.6, *P*=0.51	70.4±16.7, *P*=0.286	66.0±19.2, *P*=0.661	64.0±13.1, *P*=0.028	88.8±23.8*, *P*=0.015	68.7±19.0
TFPI (total) (ng/ml)	100.8±45.6*, *P*=0.001	101.2±36.1*, *P*=0.001	75.6±33.6*, *P*=0.011	62.9±13.8, *P*=0.971	118.6±56.1*, *P*=0.021	60.0±13.9
TFPI (free) (ng/ml)	14.0±6.7*, *P*=0.005	12.5±4.4*, *P*=0.001	10.3±3.2*, *P*=0.001	10.6±6.7*, *P*=0.002	18.6±11.3*, *P*=0.011	7.6±2.3
Thrombin–antithrombin complexes (ug/l)	1.8±1.1, *P*=0.429	3.6±6.8, *P*=0.286	1.9±1.8, *P*=0.438	2.6±1.8, *P*=0.57	1.4±0.8, *P*=0.371	2.4±1.9
Prothrombin fraction 1+2 (nmol/l)	0.8±0.3, *P*=0.482	1.0±1.3, *P*=0.507	0.7±0.3, *P*=0.346	0.8±0.4, *P*=0.389	1.2±0.4, *P*=0.649	1.4±0.8
Plasminogen activator inhibitor (PAI) (nmol/l)	23.6±10.1*, *P*=0.011	29.4±15.8*, *P*=0.012	33.6±25.5*, *P*=0.009	23.7±17.4*, *P*=0.15	59.3±22.8*, *P*=0.012	20.7±13.3
Tissue plasminogen activator (tPA)-(nmol/l)	2.2±2.2, *P*=0.869	2.9±2.9, *P*=0.843	1.9±1.8*, *P*=0.011	1.3±1.4*, *P*=0.014	6.5±4.3*, *P*=0.012	3.5±1.7

Level of significance of the P values (*)≤0.05

the Asserachrom total and free protein-S kits (Stago Diagnostica).

Tests of thrombin generation Thrombin–antithrombin (TAT) complex and prothrombin fragment 1+2 by the sandwich enzyme immunoassay (Enzygnost Micro, Behring/Dade Mannheim, Germany).

Tests of fibrinolysis Tissue plasminogen activator (tPA) and plasminogen activator inhibitor-I (PAI-I): were both assayed by the enzyme immunoassay (EIA; Asserachrom, Stago Diagnostica).

Plasma fibrinogen: the turbidometric method of Ellis and Stransky [27].

Activated protein C resistance: the functional clotting assay (Stago Diagnostica, France).

Statistical methods

The data analysis was performed with the SPSS Statistical Package (Version 10.01). The results were expressed as the calculated mean and standard deviation, and the two-tailed Student's *t*-test for independent groups was employed to compare different variables in various groups of patients with healthy controls. We assumed there was significant difference if P was <0.05.

Results

Tissue factor pathway inhibitor

Total TFPI There was significant elevation of total TFPI mean level (100.8 ng/ml) in the relapse of nephrotic syndrome when patients were not on steroids (group 1), and the level remained elevated (101.2 ng/ml) when the relapsing patients were put on steroids (group 2); mean level dropped slightly but remained significantly elevated (75.6 ng/ml) in patients in the remission phase while still on steroid therapy (group 3) but dropped in the remission stage (group 4) to levels (62.9 ng/ml) comparable to healthy control mean levels (60.0 ng/ml). The highest levels of total TFPI (118.6 ng/ml) were recorded in the steroid-resistant nephrotic patients (group 5) (Table 2).

Free Tissue factor pathway inhibitor Like the total form of TFPI, the mean levels of the free form of TFPI showed a statistically significant increase (14.0 ng/ml) in group 1, and the levels remained elevated (12.5 ng/ml) in group 2, and dropped slightly to 10.3 ng/ml and 10.6 ng/ml, in groups 3 and 4, respectively, when compared with mean level in healthy controls (7.6 ng/ml). The highest levels of free TFPI (18.6 ng/ml), approaching three times the control levels, were noted in group 5, (Table 2).

Protein C mean levels were significantly elevated in the relapse of nephrotic syndrome (124.2% and 149.3 mg% in group 1 and group 2, respectively), remained elevated in group 3 (105.3%) and normalised (94.8%) in group 4, when compared with mean concentration in healthy controls (83.5%). Protein C levels increased significantly (120.6%) in steroid-resistant patients (group 5), (Table 2).

Total Protein S mean concentrations showed statistically significant elevation in the five groups of nephrotic patients (89.4%, 97.5%, 97.5%, 96.6% and 103.1% in groups 1, 2, 3, 4 and 5, respectively), when compared with healthy controls (74.1%) (Table 2).

Free protein S levels did not show any significant fluctuations in groups 1 to 4, but the mean level was significantly higher in the steroid-resistant (88.8%) patients (group 5) than in controls (68.7%) (Table 2).

Plasma fibrinogen mean concentrations were significantly elevated in the relapse of nephrotic syndrome patients when compared with controls. Hyperfibrinogenaemia (510 mg%) was notable in group 5 (steroid-resistant) patients (Table 2).

Tests of fibrinolysis There was significant elevation of the mean plasminogen activator inhibitor (PAI) levels in all the steroid-responsive nephrotic patients in both the relapse and remission phases of nephrosis, with and without steroid therapy (23.6 nmol/l, 29.4 nmol/l, 33.6 nmol/l and 23.7 nmol/l). However, the highest mean levels of PAI were recorded in the steroid- resistant nephrotic patients (59.3 nmol/l) when compared with the healthy control mean level (20.7 nmol/ml) (Table 2). The changes in tPA were not remarkable in the four groups of steroid-responsive nephrotic patients, but elevated levels were recorded in the steroid-resistant group (Table 2).

Tests of thrombin generation Thrombin–antithrombin complex, prothrombin fraction 1+2 levels did not display any significant differences between the various groups of patients and healthy controls (Table 2).

Activated protein C resistance Three patients (out of 139) in the steroid-responsive patients and one (out of 84) in the healthy control subjects gave positive results for activated protein C resistance (APCR).

Correlation between the levels of TFPI and parameters of kidney function The correlation between total and free TFPI and serum albumin and creatinine did not attain statistical significance: *Total TFPI* and albumin $r=-0.118$; creatinine $r=-0.02$; urea $r=-0.05$. *Free TFPI* and albumin $r=-0.101$; creatinine $r=-0.02$; urea $r=-0.065$).

Discussion

Nephrotic syndrome continues to present an ideal model to characterise the way the interaction of multiple thrombogenic anomalies will eventually end in frank thrombosis. Schlegel [6] described the following thromboembolic risks in childhood nephrosis: albuminuria, hyperfibrinogenaemia, low ATIII, elevated levels of D-dimer and molecular markers of coagulation activation, in addition to a genetic

predisposition for thromboembolism, in the form activated protein C resistance due to V Leiden, in individual patients. He stated further "The responsibility of each anomaly per se in triggering thrombotic complications is not yet known and today it is understood that the coexistence of several factors is necessary to induce these complications".

The possible role played by the natural anticoagulants as protective against thromboembolism in childhood nephrosis has received extensive attention in many recent studies. Previous reports from our institution [9, 15] as well as those of others agree on the elevation of levels of the natural anticoagulants, proteins C and S, in childhood nephrotic syndrome, particularly during the relapse phase of the disease, when the prothrombotic haemostatic changes are at their highest [1–7, 16–18].

The distinctive finding of the current study relates to the fluctuations of TFPI in different phases of the disease. A careful search in the literature succeeded in identifying only two studies of adult nephrotic syndrome (NS) [28, 29].

The first study [28] was undertaken in one group of nephrotic adults and with no staging of the disease process; their mean age was 47 years (SD 19 years). In this study, the levels of both total and free TFPI were found to be higher than in controls, and this led the authors to conclude that the hypercoagulable state of adult NS could not be attributed to TFPI deficiency [28]. The second study [29] was also on nephrotic adults whose age range was 29 years to 74 years. In this study, the levels of both TF and TFPI antigens were found to be higher in nephrotic patients than in healthy controls, while the TF and TFPI activity levels were of comparable magnitude.

The current study on childhood NS followed a different design, related to the staging of the nephrotic process into relapse and remission with and without CS therapy. Employing the current widely used ELISA for total and free TFPI, we found that the plasma levels of *total* TFPI were significantly elevated above normal control values in the relapse of NS before and when patients were put on CS therapy, dropped in early remission, while the patient was under CS therapy, and normalised during established remission, when CS therapy was discontinued. On the other hand, the *free* form of TFPI maintained an elevated level during the relapse and remission of NS. It is worthwhile noting that the fluctuations in TFPI levels corresponded closely with those of the natural coagulation inhibitors, proteins C and S.

The mechanism responsible for the elevated TFPI levels in childhood NS is open to speculation and is probably multi-factorial. It is known that the circulating level of a haemostatic factor is a balance between production and consumption, degradation or clearance. The source of TFPI is vascular endothelium, and, therefore, the observed elevated TFPI blood levels could be accounted for by excessive endothelial release of this inhibitor. It is also possible that clearance and catabolism of TFPI may also play a role in the fluctuations of TFPI in childhood NS. TFPI is cleared from the circulation primarily by the liver and kidneys [30, 31]. Two receptors, LDL receptor-related protein (LRP) and heparin sulphate proteoglycan (HSPGs) are believed to bind TFPI, resulting in its degradation and eventual clearance in vivo [32, 33]. It has also been shown that a 39-kDa protein inhibits the interaction of TFPI with LRP, in both the kidney and the liver, resulting in prolongation of the half-life of infused ^{125}I-TFPI [32]. The precise role both LRP and HSPGs play in determining the circulating level of TFPI in childhood NS has not been studied. However, by speculation, it is possible that the function of either of these receptors is down-regulated in childhood NS, resulting in reduced plasma clearance of TPFI, prolonging its half-life in plasma and accounting for the elevated plasma levels of this inhibitor.

Urine loss of TFPI, which was not measured in our patients, was reported in children with meningococcal meningitis [34]. Therefore, the elevated levels of TFPI, especially in the relapse of nephrosis, could be a compensation to loss in urine. Once the kidney inflammatory process is brought under control with CS therapy, proteinuria and urinary loss of TFPI would presumably cease, and the release of this inhibitor from vascular endothelium would drop, as seen in the established remission of NS.

Albuminuria has also been invoked as a causal factor in the release of TFPI from vascular endothelium. In a recent study [35] Leurs et al. reported higher levels of both basal and post-heparin TFPI activity (assayed by a chromogenic technique) in type I diabetes complicated with albuminuria, than in patients with uncomplicated diabetes or those with retinopathy without albuminuria. This led the authors to conclude that the mechanism involved in the release of TFPI from vascular endothelium could be related to altered endothelial glycosaminoglycan characteristics. In the current study the levels of both total and free TFPI were highest during the relapse (albuminuric phase) of nephrosis than during remission. Although this finding lends some support to the proposition of Leurs et al., albuminuria cannot be a major factor in the release of TFPI from vascular endothelium, as its levels remained well above control levels in the remission of childhood nephrosis, when the albuminuria had ceased. Also, heparin is known to stimulate the synthesis and release of TFPI from vascular endothelium [36–38]; however, none of our patients was on heparin at the time of the collection of blood sample for this study.

Hypercholesterolaemia has also been mentioned as a causative factor for elevated TFPI levels [39, 40]. Elevated cholesterol levels are one of the features that define NS [41] and are highest during the active (relapse) phase of the disease and disappear with the resolution of the proteinuria [42]. However, in our institution, cholesterol measurements are usually done only in newly diagnosed cases and not during routine follow-up visits. Nonetheless, high cholesterol levels could be an additional factor accounting for the elevated levels of TFPI during the relapse phase of nephrosis with or without CS therapy, when it is assumed that cholesterol levels are highest [42].

Our findings relating to TFPI in childhood NS add yet one further physiologically active coagulation inhibitory mechanism to the already well-studied proteins C and S

that are present in elevated concentrations during the relapse of nephrosis, when the hypercoagulable (prothrombotic) changes are at their maximum and the risk of thromboembolism is paramount. The elevated levels of these coagulation inhibitors (proteins C and S as well as TFPI) would serve a vital physiological function attenuating the hypercoagulability that prevails in NS.

The results of the current study did not show any significant fluctuations in the levels of the markers of thrombin generation (F1+2 and TAT) in all phases of nephrotic syndrome. Two small recent studies found the levels of F1+2 and TAT elevated significantly above control levels in steroid-responsive nephrotic children, before and 2 weeks after the commencement of steroid therapy [43, 44]. This finding was taken to confirm the existence of a prothrombotic state, particularly in the relapse of nephrosis.

The fibrinolytic system has not been studied extensively in childhood nephrotic syndrome, and the evidence from the current as well as previous studies [45, 46] supports the existence of a hypofibrinolytic state in nephrotic syndrome, which contributes further to the hypercoagulability, elevated concentrations of PAI being a prime feature [45, 46]. In the current study, in which we resorted to more detailed categorisation of nephrotic patient groups, there was a clear marked elevation of PAI concentrations when patients were on steroid therapy (both in relapse and in remission of nephrosis). Maximum PAI levels were recorded in the steroid-resistant patients. Indeed, in the current study, the steroid-resistant nephrotic patients showed the most remarkable haemostatic perturbations. They displayed markedly inhibited fibrinolytic activity (the highest levels of PAI), and hyperfibrinogenaemia, when compared to other categories of steroid-sensitive patients. But they also seemed to be protected by the simultaneous marked elevation in the levels of the natural anticoagulants, particularly TFPI.

In the functional assay for APCR, only one child out of 84 in the control group, and three out of 138 patients, gave positive results for APCR, indicating that this known risk factor of arterial and venous thrombosis [47] need not be sought as an additional prothrombotic marker of the hypercoagulability of childhood nephrotic syndrome. A careful search in the literature for relevant references in this area uncovered one case report of a young man who suffered deep vein thrombosis, and the hypercoagulable screen showed that he had APCR [48].

In the current study attempts were made to find clinical evidence of thrombosis and thromboembolism, as invasive methods are not ethically applicable. No evidence was found, and, therefore, we assumed that our patients were free from thrombotic complications.

In conclusion, the current study uncovered yet one additional natural anticoagulant system (viz. TFPI) that is highly mobilised in childhood nephrotic syndrome, particularly in the relapse of the steroid-responsive form of the disease, but is remarkably more elevated in the steroid-resistant form of nephrosis. This finding may help clarify further the physiological role of TFPI in patients at risk of developing thrombotic disease.

Acknowledgements We are grateful to Mr. M.A. Hamid, Mr. Lugman A.G. El-Sid and Mrs. M.C. Aradillous for their technical assistance, to Mr. Amir Abdul Aziz for statistical help, and to Mrs. F. Chatila for her secretarial work. This work was supported by a grant (no. AT 18–73) from King Abdul Aziz City for Science and Technology (KACST), Riyadh, Saudi Arabia.

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