

Journal of Nephrothology

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Platelet glycogen synthase kinase 3 β regulates plasma β amyloid and phosphorylated tau levels in chronic kidney disease patients with cognitive dysfunction; therapeutic role of erythropoietin

Vinothkumar Ganesan¹, Krishnakumar Sethuraman^{2,3}, Sureshkumar⁴, Venkataraman Prabhu^{1*}¹Department of Medical Research, SRM Medical college Hospital, SRM Institute of Science and Technology, Chennai, India²Department of Nephrology, SRM Medical college Hospital, SRM Institute of Science and Technology, Chennai, India³Diaverum Dialysis Center, Al Hassa, Saudi Arabia⁴Department of Neurology, Balaji Medical College Hospital, Chrompet, Chennai, India

ARTICLE INFO

Article type:
Original Article

Article history:
Received: 5 June 2021
Accepted: 3 November 2021
Published online: 5 December 2021

Keywords:
Chronic kidney disease
Cognitive impairment
 β
Tau protein
GSK3 β
Recombinant human erythropoietin

ABSTRACT

Introduction: Patients with chronic kidney disease (CKD) have increasingly been diagnosed with cognitive impairment. Glycogen synthase kinase 3 β (GSK3 β) is directly causing both phosphorylated tau (pTau) and amyloid β (A β) accumulation in Alzheimer's disease (AD). GSK3 β expression is more abundant in human platelets than in other blood cells. Recombinant human erythropoietin (rHuEPO) is a common medicine for treating anemia in patients with CKD, as well as a neuroprotective agent.

Objectives: The goal of this research is to find out how platelet GSK3 β regulates plasma A β , total Tau and tau phosphorylated at threonine 181 (p-tau181) levels in CKD patients with cognitive dysfunction and also the efficacy of rHuEPO treatment.

Patients and Methods: The study included 60 participants, which consist of 30 CKD without cognitive dysfunction and 30 CKD with cognitive dysfunction based on the neuropsychological examination. The expression of GSK3 β in platelets was evaluated using a western blot and plasma A β , total Tau, pTau 181 levels were quantified by ELISA. The data were compared statistically ($P < 0.05$) to AD, normocytic normochromic anemic and healthy patients.

Results: In CKD with cognitive dysfunction subjects, platelet GSK3 β expression and plasma A β , total Tau and pTau181 levels were significantly ($P < 0.05$) altered like AD when compared to normocytic normochromic anemic, healthy and CKD without cognitive dysfunction subjects. In post rHuEPO (100 IU/kg, weekly twice, six months) treatment, the altered protein abnormalities were retrieved significantly ($P < 0.05$) compared to pre-treatment.

Conclusion: This study established that platelet GSK3 β expression and plasma A β , total Tau, pTau181 are the candidate biomarkers for cognitive dysfunction in CKD patients. The clinical utility of rHuEPO as a GSK3 β inhibitor and therapeutic agent for cognitive dysfunction in CKD has been determined.

Implication for health policy/practice/research/medical education:

Glycogen synthase kinase 3 β (GSK3 β) is directly causing phosphorylated tau (pTau) and amyloid precursor protein (APP) isoforms accumulation and increased secretion of A β in cognitive dysfunction. Platelet GSK3 β has been a focus of the growing literature on blood-based biomarkers for cognitive dysfunction. Recombinant human erythropoietin (rHuEPO) is a standard therapy for management of anemia in CKD and also acts as a neuroprotective agent. rHuEpo acts as neuroprotective through inhibition of GSK3 β .

Please cite this paper as: Ganesan V, Sethuraman K, Sureshkumar, Prabhu V. Platelet glycogen synthase kinase 3 β regulates plasma β amyloid and phosphorylated tau levels in chronic kidney disease patients with cognitive dysfunction; therapeutic role of erythropoietin. J Nephrothol. 2022;11(x):exx. DOI: 10.34172/jnp.2022.xx.

Introduction

Patients with chronic kidney disease (CKD) have twice the chance of developing dementia and cognitive dysfunction. The presence of hyperphosphorylated tau protein clusters and also neurofibrillary tangles (NFT) and extracellular amyloid (A β) protein aggregates (senile plaques), are clinically indicative of cognitive dysfunction and Alzheimer's disease (AD). A β is a heterogeneous mixture of 39-43 amino acids of small peptide with a molecular weight of around 4 kDa. The action of BACE1 and secretases results in proteolytic cleavage of an integral membrane protein known as amyloid precursor protein (APP).

In the pathogenesis of dementia and AD, oxidative stress has been implicated. Excess reactive oxygen species (ROS) formation is manifested in CKD patients by high serum lipid peroxidation (LPO) indices and inadequate antioxidant levels. Oxidative stress is thought to be a critical element in the development of AD and mild cognitive impairment (MCI) (1). Studies have shown that oxidative stress and β amyloid development are proportionally connected because β amyloid induces oxidative stress in vivo and in vitro and oxidative stress increased the production of β amyloid by proteolytic cleavage of APP (2).

Tau is a major protein associated with microtubules (~55 kDa) that plays an important role in axonal stabilization, neuronal growth, and neuronal polarity and among its post-translational modifications; Tau's phosphorylation has been studied most widely (3). A characteristic of many neurodegenerative diseases, collectively known as tauopathies, is NFT composed of hyperphosphorylated tau protein. Studies have shown that elevated levels of plasma A β , total tau, and tau phosphorylated at threonine 181 (p-tau181) in patients with AD and moderate cognitive impairment compare to healthy subjects (4) and p-Tau181 in plasma is more common to AD.

Platelet serves as a cellular model and systemic marker in various neurodegenerative diseases. Studies also have shown that platelets and neuron are strikingly close, pointing to a possible cross communication pathway between the systemic environment and the brain. Neuropeptides, neurohormones and neurotransmitters are stored in intercellular storage compartments in neurons that are close to granules of platelets, with the use of similar vesicle trafficking mechanisms. Normal neuronal biochemical processes include blood platelet aggregation and neurotransmitter release, sensitivity to calcium concentration fluctuations and membrane formation of other substances. Platelets have molecular mechanisms of neuronal exocytosis in common with neurons, and they include crucial proteins and polypeptides that cause

neuronal depolarization.

Glycogen synthase kinase-3 (GSK-3) is a serine-threonine protein kinase that is strongly expressed in the central nervous system (CNS) and platelets. Among mammals two GSK-3 isoforms are encoded by separate genes (GSK3 α and GSK3 β). GSK3 β is involved in neuronal cell death, which is a common pathogenic mechanism in neurodegenerative illnesses. It causes oxidative damage. GSK3 β has been implicated in the phosphorylation of tau in the normal and AD brain. GSK3 β activity and expression were found to be higher in AD brains, which could be owing to hypomethylation of its promoter region (5). In the affected brain regions of patients with AD, expression of neuronal protein phosphatase 2A is down-regulated, allowing both phosphorylated tau and APP isoforms to accumulate and increased secretion of A β peptides (6). Studies have shown that oxidative damage causes over activation of GSK3 β in neuronal cells (7). GSK3 β is also expressed in larger quantities in human platelets than in other blood cells. Recent studies indicate platelet GSK3 β plays a biomarker for MCI and AD (8). Till now, GSK3 β expression in platelets and its relation with plasma levels of A β , total Tau and pTau-181 in CKD patients with cognitive dysfunction has so far been not studied.

Typically, anemia in CKD is normocytic, normochromic, and hypoproliferative. Recombinant human erythropoietin (rHuEPO) is routinely administered in the treatment of anemia in CKD and studies have shown that cognitive function also improves along with hemoglobin levels (9) after EPO therapy. Over the past few years, we have acquired experience on studies in handling of human platelets and plasma in CKD patients with cognitive dysfunction, healthy, normocytic normochromic anemic, AD subjects (9). In this study, we determined the impact of rHuEPO therapy on GSK3 β expression in platelet by western blot method and A β , total Tau and pTau-181 levels in plasma by enzyme linked immunosorbent assay (ELISA) in CKD with cognitive dysfunction and compare the parameters with other groups such as CKD patients without cognitive dysfunction, healthy, normocytic normochromic anaemic and AD subjects.

Objectives

The objective of this study is to determine the regulative role of platelet GSK3 β on plasma A β , total Tau, p-tau181 levels in CKD with cognitive dysfunction and also determine the impact of rHuEPO treatment.

In the current study, we also discussed the feasibility and possible benefits of using human platelets and plasma to assess protein levels for clinical laboratory explorations and therapeutic outcomes monitoring.

Patients and Methods

Chemicals and drug

For western blot analysis, Rabbit Anti GSK3 β polyclonal (bs-0028R), rabbit Anti-beta-actin Polyclonal (bs-0061R) and appropriate secondary antibody, Goat against Rabbit IgG Antibody (H+L). The HRP conjugates were purchased from Bioss in the United States, to detect GSK3 β and β actin expressions in platelets. The levels of A β , total tau and pTau181 in plasma were detected by highly sensitive sandwich ELISA. E1230Hu kit for A β (1-40), E1264Hu kit for A β (1-42) and E1333Hu kit for total tau were purchased from Bioassay Technology Laboratory, China. KHO0631 kit for pTau181 was purchased from Invitrogen, USA.

Recombinant human erythropoietin administration

The dose and duration of rHuEPO therapy were chosen based on previous publications (9,10) for CKD patients with cognitive impairment.

Participants

A total of 130 subjects were studied including healthy (n=30), normocytic normochromic anemic (n=30), CKD without cognitive dysfunction (n=30), CKD with cognitive dysfunction (n=30) and to compare the parameters, we also observed in AD (n=10).

Inclusion criteria

A total of 60 CKD patients (20 and 50 years) of both genders were included in the study subjects as 30 CKD with cognitive dysfunction and 30 CKD without cognitive dysfunction based on neuropsychological questionnaires.

Exclusion criteria

Uremic encephalopathy, type 1 DM, malnutrition, thrombocytopenia, CVA and critically ill patients were excluded from this study.

Methods

For screening of cognitive status, CKD patients were administered with MMSE, WMS- I and TOL test scoring followed by standard methods (9,10).

Sample collection and preparation for platelet

Blood was drawn into tubes coated with 0.1M sodium citrate. The blood samples were homogenized in 300 μ L of 0.09M EDTA and centrifuged for 10 minutes at 1300 rpm. Platelet rich plasma was isolated from blood cells, and platelets were extracted at room temperature using centrifugation at 2400 rpm for 10 minutes. Pellets were re-suspended in lysis buffer containing 10mM Tris-HCl, pH-7.4, 1mM EGTA, 100mM PMSE, and protease inhibitors after being rinsed with 5 mL of 10mM Tris,

pH-7.4. The quantities of platelet protein in each sample were measured using the Lowry technique.

Estimation of platelet lipid peroxidation

Platelet LPO level was calculated using Devasagayam and Tarachand's technique. The data was calculated as nmol MDA produced per mg protein. Following rHuEPO (100 IU/ kg, twice weekly, six months) treatment, the parameters were examined again in CKD patients with cognitive dysfunction.

Determination of platelet GSK3 β expression

The expression of GSK3 β in platelets was evaluated using the western blot technique according to Forlenza et al (8).

Quantification of A β , total Tau and phospho Tau 181 protein levels

After overnight fasting, blood sample from all the experimental community was obtained in standard vacuum potassium EDTA tubes and plasma was removed for 10 min after centrifugation at 3000 rpm. The plasma aliquoted a protease inhibitor into polypropylene tubes that held 40 μ L. We placed the samples at -20° C. In accordance with the manufacturer's instructions, plasma A β , total Tau and p-tau181 protein levels (expressed in ng/ L) were quantified using a highly sensitive sandwich ELISA.

Statistical analysis

The results were analysed statistically by SPSS software version 21.0 using analysis of variance (ANOVA) and Turkey's multiple comparison tests. The level of probability <0.05 was considered to be statistically significant. The correlation coefficient of Pearson's was used to assess the possible association between expression of the platelet GSK3 β and plasma A β , total Tau, p-tau181 protein abnormalities in the respective experimental groups.

Results

Effect of rHuEPO on platelet LPO and plasma AD markers level in CKD with cognitive dysfunction

Increased AD markers in plasma could be attributable to elevated levels of LPO in platelets of CKD individuals with cognitive dysfunction, compared to healthy, normocytic normochromic anemia, and CKD without cognitive dysfunction subjects. In post-rHuEPO treatment, the altered protein abnormalities and LPO were retrieved significantly compared to pre-treatment (Table 1).

Effect of rHuEPO on platelet GSK3 β expression in CKD with cognitive dysfunction

Increased level of platelet GSK3 β expression was observed in CKD with cognitive dysfunction, like AD patients

Table 1. Clinical feature of study subjects

Parameters	Healthy group (n=30)	Normocytic normochromic (n=30)	CKD without cognitive dysfunction (n=30)	AD (n=10)	CKD with cognitive dysfunction (n=30)	
					Pre - rHuEPO therapy	Post - six months rHuEPO therapy
Platelet LPO (nmol/mg protein)	4.48 ± 0.1	5.39 ± 0.2	6.47 ± 0.3	9.46 ± 0.1	8.4 ± 0.3	5.5 ± 0.2***
Aβ40 (ng/L)	13 ± 1.8	16.7 ± 1.5	22.4 ± 2.2	50.7 ± 2.3	48 ± 3.6	27.5 ± 2.5***
Aβ42 (ng/L)	20 ± 1.9	21.7 ± 2.1	29.2 ± 2.5	61.8 ± 3.9	53.8 ± 1.9	28.9 ± 2.3***
Total Tau (ng/L)	24.9 ± 2.1	25.7 ± 1.5	35.1 ± 1.5	63.9 ± 3.6	56.3 ± 3.1	33.4 ± 1.6***
p-Tau 181 (ng/L)	22 ± 1.4	22.7 ± 1.1	32.4 ± 1.4	55.1 ± 2.3	41 ± 1.7	31 ± 1.9***
Aβ 42/Aβ 40 (ng/L)	1.5 ± 1	1.2 ± 1.4	1.3 ± 1.1	1.2 ± 1.6	1.1 ± 0.5	1 ± 0.9*
Total Tau/p-Tau 181 (ng/L)	1.1 ± 1.5	1.1 ± 1.3	1 ± 0.1	1.1 ± 1.5	1.3 ± 1.8	1 ± 0.8*

The data is presented as the mean ± SD comparison between the respective patients. Statistical significance was defined as a P value of < 0.05. ***P<0.0001, *P<0.5.

compared to healthy, normocytic normochromic anemia, and CKD individuals without cognitive dysfunction subjects. In post-rHuEPO treatment, the altered platelet GSK3β expression was retrieved significantly compared to pre-treatment (Figures 1 and 2).

Correlation between platelet GSK3β expression and LPO level in respective patients

In this study, like AD, we observed increased levels of platelet LPO in CKD patients with cognitive dysfunction when compared to other groups. This may be due to increased platelet GSK3β expression. The altered platelet LPO and GSK3β recovered substantially in post-rHuEPO treatment compared with pre-treatment (Figure 3).

Correlation between platelet GSK3β expression and plasma AD markers in respective patients

In this study, like AD, we observed increased levels of plasma Aβ, total Tau and p-tau181 in CKD patients with cognitive dysfunction when compared to other groups. This may be due to increased platelet GSK3β expression. The altered protein levels recovered substantially in post-

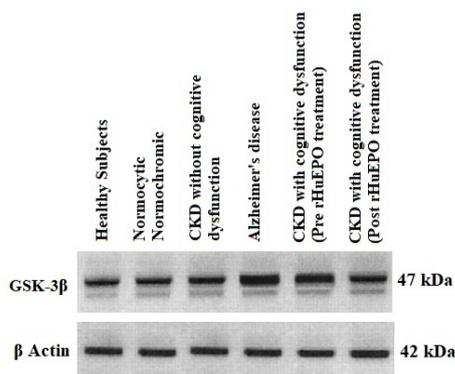


Figure 1. Representative WB image of GSK3β in platelets from respective groups. The internal/loading control was β actin.

rHuEPO treatment compared with pre-treatment (Figures 4 to7).

Discussion

The risk of cognitive dysfunction in CKD patients with AD is significantly greater than in patients without CKD, not only in aged patients with CKD, but also in young patients with CKD (20-49 years) (9,10). It has been believed for a long-time that kidney function is associated with brain activity. According to clinical research, CKD patients are more vulnerable to cognitive impairment and AD, and the severity of cognitive impairment is closely linked to the development of CKD and renal failure. EPO is produced primarily by the kidney. EPO stimulates the production of RBC cells and maintains normal levels of

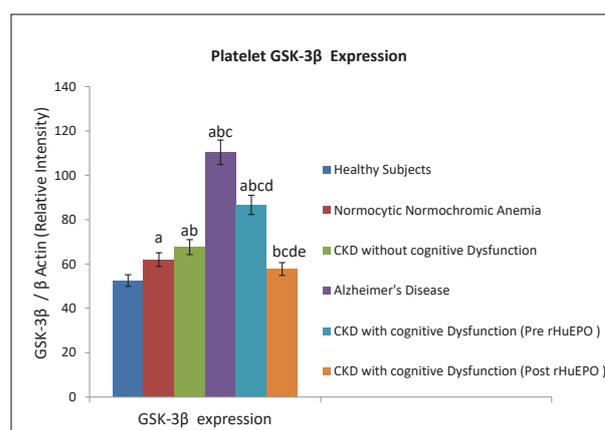


Figure 2. The histogram represent mean ± SEM from three independent experiments. Letters a, b, c, d and e denote the statistical significance of the data at the level of P < 0.05. 'a' denotes healthy subjects versus. Other groups; 'b' denotes normocytic normochromic anemia versus. CKD without cognitive dysfunction, AD and pre - post rHuEPO treatment of CKD with cognitive dysfunction; 'c' denotes CKD without cognitive dysfunction versus. AD and pre - post rHuEPO treatment of CKD with cognitive dysfunction; 'd' denotes AD versus. Pre - post rHuEPO treatment of CKD with cognitive dysfunction; 'e' denotes pre versus. Post - rHuEPO treatment of CKD with cognitive dysfunction.

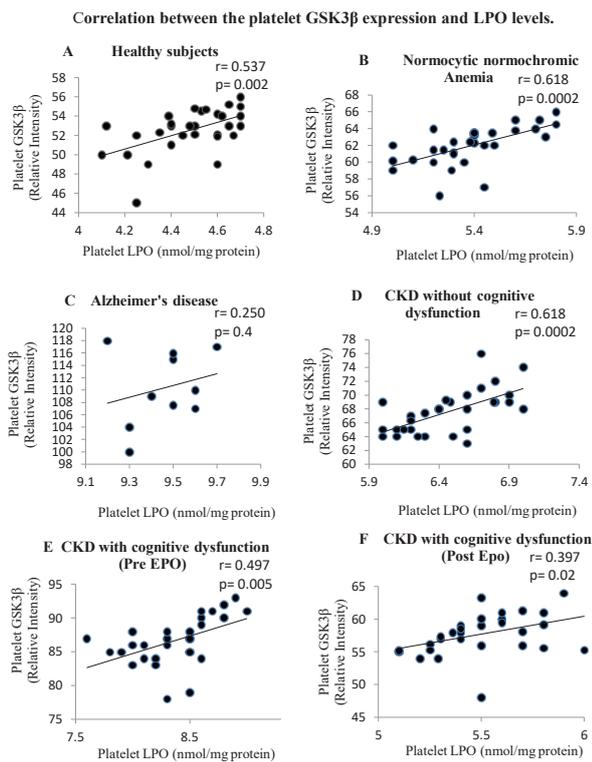


Figure 3. Correlation between platelets GSK3β expression and LPO levels in respective subjects. Each point represents a separate subject. Correlation coefficients and P values were determined using Pearson correlation.

hemoglobin concentrations. Hemoglobin and EPO serum concentrations in patients with CKD are slightly lower and patients with CKD are more vulnerable to anemia and anaemic hypoxia (11). A large cohort study of 3591 participants showed that patients with CKD were more likely to show cognitive impairment (12), this suggests that the strong association was primarily due to the change in the concentration of hemoglobin in patients with CKD. The concentration of hemoglobin was significantly correlated with cognitive impairment. In addition, a study has shown that EPO can have neuroprotective effects (13), since the production of EPO can be stimulated by anemic hypoxia in the brain, and anemia is an independent risk factor of cognitive decline (14). Moreover, the fact that cognitive function is substantially improved in CKD patients after treatment with rHuEPO suggests a correlation between CKD and AD. The mechanisms behind this linkage, however, remain unclear.

Oxidative stress is a major factor in the onset and progression of CKD. Overproduction of ROS combined with a lack of antioxidant mechanisms leads to increased oxidative stress. Platelets have a high number of mitochondria and most mammalian cells and platelets are an essential cause of ROS. Over 90% of mitochondrial ROS output can result in oxidative stress to mitochondrial

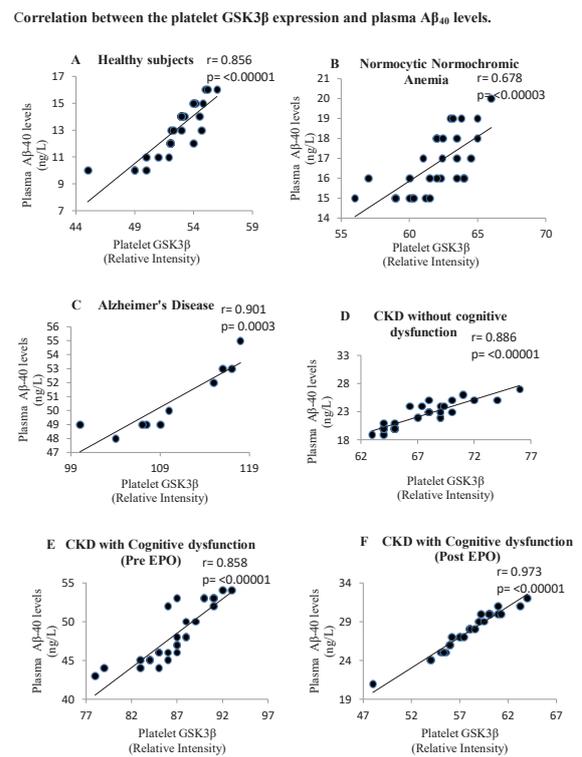


Figure 4. Correlation between platelets GSK3β expression and plasma Aβ₄₀ levels in respective subjects. Each point represents a separate subject. Correlation coefficients and P values were determined using Pearson's correlation.

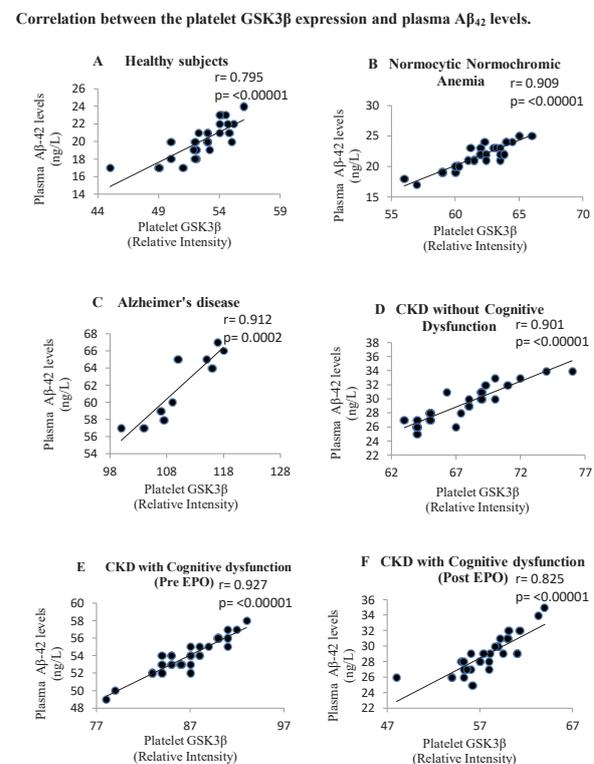


Figure 5. Correlation between platelets GSK3β expression and plasma Aβ₄₂ levels in respective subjects. Each point represents a separate subject. Correlation coefficients and P values were determined using Pearson's correlation.

Correlation between the platelet GSK3β expression and plasma total Tau levels.

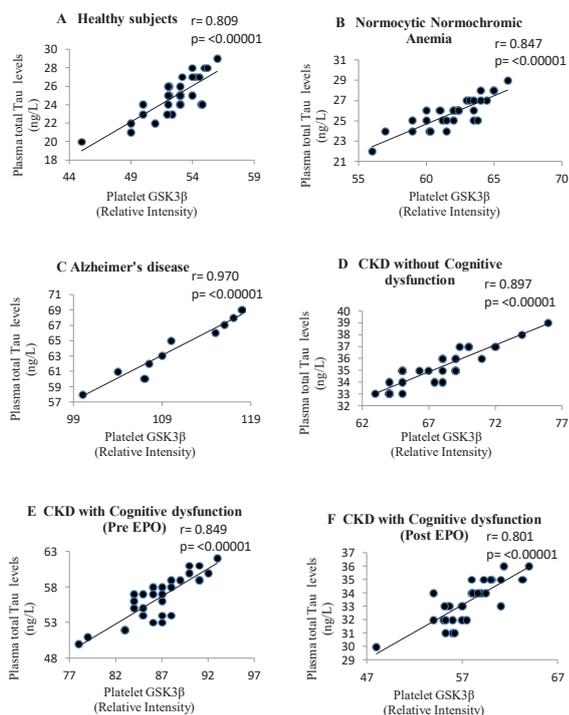


Figure 6. Correlation between platelets GSK3β expression and plasma total Tau levels in respective subjects. Each point represents a separate subject. Correlation coefficients and P-values were determined using Pearson's correlation

proteins, membranes and DNA, impairing mitochondrial capacity to synthesize adenosine triphosphate (ATP) (15). Several studies have shown that GSK3β is found in the mitochondria and is highly activated (16). Mitochondrial GSK3β activity controlled the activity of the mitochondrial complex I, production of ROS and morphology of the mitochondria. Conversely, under oxidative stress, GSK3β activity is upregulated. Grimes et al (17) propose that oxidative stress causes GSK3β activation in neuronal cells, while GSK3β inhibition is involved in oxidative stress regulation in neuronal hippocampal cell lines (7). This research proves a link between GSK3β and oxidative stress. Increased GSK3β expression in platelets were also identified in this investigation, which could be attributable to higher levels of platelets LPO in CKD with cognitive dysfunction, similar to AD patients compared to healthy, normocytic normochromic anemia, and CKD without cognitive dysfunction (Figures 1 and 3 and Table 1).

In neurons, GSK3β is the most significant Tau kinase. It helps to build NFT and senile plaques. GSK3β has been found to hyperphosphorylate Tau in AD models, causing microtubule disintegration and feature loss (18). In AD patients and animal models, increased GSK3β activity has been associated to elevated levels of Aβ formation and deposits, tau hyperphosphorylation, and synaptic impairment in several studies (19). Inhibition of GSK3β

Correlation between the platelet GSK3β expression and plasma p-Tau 181 levels.

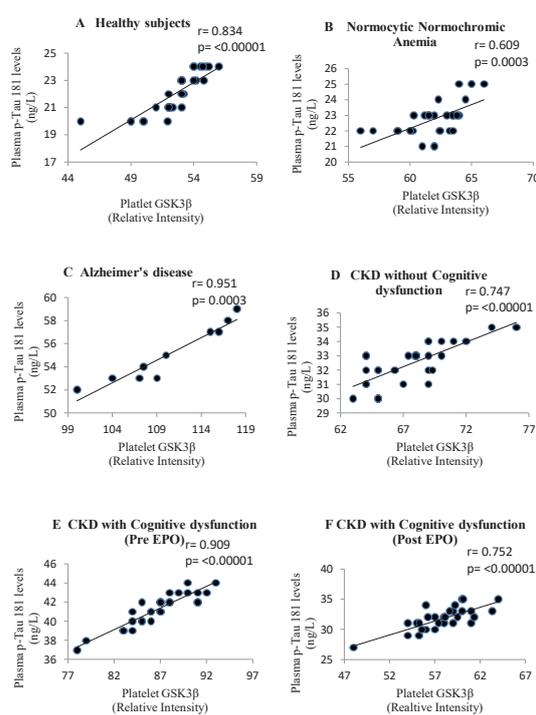


Figure 7. Correlation between platelets GSK3β expression and plasma p-Tau 181 levels in respective subjects. Each point represents a separate subject. Correlation coefficients and P-values were determined using Pearson's correlation.

has been demonstrated to decrease Aβ generation in AD patients as well as memory impairment in a rat model (20) and to decrease Aβ induced neurotoxicity in cultured neurons (21). In this study, like AD, we observed greater levels of Aβ, total Tau and p-tau181 in CKD patients with cognitive impairments when compared to other groups. This may be due to increased platelet GSK3β expression (Figures 4 to 7).

The effects of rHuEPO treatment on platelet GSK3β expression, plasma Aβ, total Tau and p-tau181 levels in CKD patients with cognitive dysfunction were also investigated in this study. rHuEPO was commonly used in clinical practice especially CKD associated anaemia (Figure 8). In the present study, the expression of platelet GSK3β and plasma Aβ and total Tau and p-tau181 levels, were reviewed in CKD patients with cognitive dysfunction after treatment with rHuEPO. Compared to pre-rHuEPO treatment (Figures 1 and 2 and Table 1). All protein defects were substantially recovered along with increased scoring of neuropsychological tests which were published in our previous study (9). Wang et al (22) recently discovered that EPO is a key neuroprotective agent in the context of cognitive dysfunction associated with diabetes. EPO ameliorates diabetes induced oxidative stress in vitro and in vivo. EPO suppresses the synthesis of LPO and protects against oxidative stress by

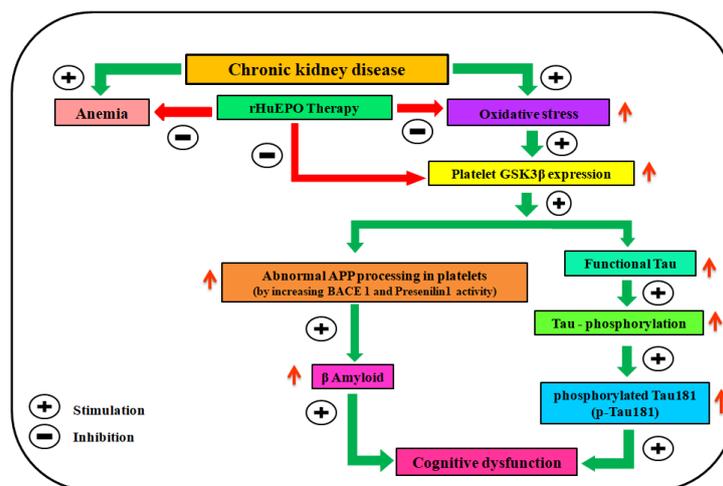


Figure 8. Recombinant human erythropoietin (rHuEPO) therapy inhibits the production of GSK3β, resulting in the deactivation of APP processing and prevents the formation of Amyloid β (Aβ) and hyperphosphorylated tau (pTau) levels in anemic CKD patients with cognitive dysfunction.

boosting the enzymatic antioxidant system, according to a recent research (23). Katavetin et al (24) showed that EPO promotes cytoprotection by reducing oxidative stress and directly exerting antioxidant effects by regulating antioxidant intracellular systems such heme oxygenase-1 and glutathione peroxidase. According to the study by Ma et al (25), EPO is neuroprotective through a PI3K/Akt-dependent mechanism including GSK3β inhibition, which results in reduced Aβ 25–35 apoptosis in PC 12 cells. In addition, accumulating studies have shown that the EPO has an inhibitory effect on GSK3β. In this experiment, based on the above data, we indicated that the recovered expression of platelet GSK3β and plasma Aβ, total Tau and pTau181 levels in post-rHuEPO therapy in CKD patients with cognitive dysfunction may be due to a substantial decrease in platelet LPO levels (Table 1) and the same trend was also observed in previous studies (9).

Conclusion

In conclusion, when compared to healthy, normocytic normochromic anemia and CKD without cognitive dysfunction, CKD with cognitive dysfunction participants have dramatically changed platelet GSK3β expression, plasma Aβ, total Tau and p-tau181 levels, similar to AD participants. The altered protein defects recovered substantially in post-rHuEPO treatment compared with pre-treatment. Platelet GSK3β expression and plasma Aβ, total Tau, p-tau181 are prospective biomarkers for cognitive dysfunction in CKD patients, according to this study. The clinical utility of rHuEPO as a GSK3β inhibitor and therapeutic treatment for cognitive dysfunction in patients with CKD has been established.

Authors' contribution

GV conducted all the experiments with the guidance of

PV. SK conducted the assessment and selection of CKD patients. SK conducted the assessment and selection of AD and cognitive impairment patients. All authors read and approved the final version of manuscript.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The study protocol was authorized by the Institutional Ethical Committee (Clearance No. 58/ IEC/2010) of SRM Medical College Hospital and Research Centre, SRM Institute of Science and Technology, India. The informed consent was collected from all the participants. This research was based on the Ph.D thesis of Vinothkumar Ganesan (Thesis#T0629) at SRM IST.

Conflict of interests

The authors report no biomedical financial interests or potential conflicts of interest.

Funding/Support

This research was funded by the Indian Council of Medical Research – ICMR (Grant# GIA/ 77/ 2014-DHR), New Delhi, India.

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