DOES VITAMIN E INFLUENCE OXIDATIVE STRESS AND RENAL ANAEMIA IN HAEMODIALYSED PATIENTS?

BY

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ABSTRACT

There is increasing evidence that oxygen radicals are involved in the progression of renal damage and of uraemic symptoms. Haemodialysis (HD) is known to be one major cause of oxidative stress. Renal anaemia is one of the main complications seen in HD patients and resulting from many pathogenic factors. In this study we aimed to evaluate the role of oxidative stress as a contributing factor in renal anaemia through studying the effect of vit E supplementation as an antioxident on the markers of oxidative stress as well as on haemoglobin in HD patients. Subjects and methods: Erythrocyte activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), plasma malondialdehyde (MDA), selenium (Se), total protein levels, haemoglobin (Hb) concentration and haematocrit (Hct %) were measured in 43 haemodialysed pateints and compared with 20 healthy, age matched controls. After 12 weeks of vitamin E supplementation in a dosage of 400 mg/day, the baseline parameters were reassessed in the same patients group. Results: Mean plasma level of MDA was significantly higher in HD patients ($p<0.0001$) than in healthy controls. The erythrocyte activity of SOD, GSH-Px, plasma level of selenium and haemoglobin concentration were significantly lower ($p<0.0001$). Furthermore, there was an inverse correlation between plasma levels of MDA and haemoglobin concentration ($r=-0.62$, $p=0.002$). After vit E supplementation there were statistically significant increase in erythrocyte activity of GSH-Px and SOD ($p<0.0001$), decrease in MDA concentrations ($p<0.0001$) and non significant increase in selenium level, haemoglobin concentration and haematocrit. Conclusions: Redox imbalance in HD pateints might be a contributing factor in renal anaemia. Treatment with vit E might decrease radical generation effectively in HD patients with insignificant effect on renal anaemia.

Key words: *Haemodialysis; Oxidative stress; Antioxidant enzymes; Lipid peroxidation; Vitamin E; renal anaemia.*

INTRODUCTION

Oxidative stress defines an imbalance between generation of reactive oxygen species (ROS) and antioxidative defence mechanisms (Ichikawa et al., 1994). Protection from oxidant injury involves complex pathways of antioxidant defence mecha-

nisms at both the blood and intracellular levels. There are at least three groups of antioxidant enzymes: superoxide dismutases (SOD), catalases (CAT) and glutathione peroxidases (GSH-Px) which neutralize ROS. The trace elements; selenium, copper and zinc are bound to the active sites of these enzymes and play an important role in the antioxidant defence system (Bronislaw et al., 2006).

Haemodialysis is known to be one major cause of oxidative stress due to the activation of polymorphonuclear neutrophil leukocytes (PMNL) through contact of the blood with the dialysis membranes. Activated PMNL are able to mediate lipid peroxidation (LPO) in red blood cells (RBC), which results in anaemia in uremic patients (ward and McLeish 2003). Anaemia is a common and disabling feature of chronic renal failure (CRF) resulting from many pathogenic factors, mainly erythropoietin deficiency (Sarnak etal., 2002). It seems to be a main cause for redox imbalance in uraemic patients (Klahr, 1997). Correction of renal anemia by erytheropoietin therapy decrease radical generation effectively in haemodyalized patients due to increase in the number of red blood cells and blood haemoglobin concentration (Vaziri, 2004).

Vitamin E (Vit E), particularly in the form of α-tocopherol has been proposed for the prevention or treatment of numerous health conditions, often based on its antioxidant and anti-inflammatory properties. There is a growing interest in the possible benefits of Vit E in kidney diseases with high oxidative stress (Thabet and James, 2006). So, in this study we were interested to investigate the relationships between the markers of oxidative stress and haemoglobin levels and if there is a possible role of redox imbalance in the development of renal anaemia through studying the effect of vit E supplementation as an antioxidant on the markers of oxidative stress and haemoglobin levels in HD patients

SUBJECTS AND METHODS

Fourty three patients (25 males and 18 females), mean age (47±3.5) years with CRF on maintenance HD referred from the Dialysis Unit and Internal Medicine Department, Mansoura University Hospital were enrolled in the study. The underlying renal pathology caused by chronic glomerulonephritis (n=12), pyelonephritis (n=10), diabetes (n=4), arterial hypertension (n=8), nephrolithiasis (n=3) and unknown (n=6).

All patients had been on regular haemodialysis for at least 6 months and the dialysis sessions were performed three times a week and each session lasted 4 hours using Fresenious dialysers with polysulfone dialyzing membranes and bicar-

bonate buffer with glucose. Twinty three patients were receiving angiotensin converting enzymes (ACE) inhibitors, 16 receiving beta blockers, 10 receiving calcium antagonists and 6 patients were receiving digitoxin. The exclusion criteria were patients with acute infections, acute phase of rheumatological disorders and previous blood transfusions or iron therapy two months before the study.

Twenty healthy subjects (14 males and 6 females), mean age (42±3.2) were chosen as control. They did not receive any supplements that could increase selenium concentration within the previous two months. An informed consent was obtained from all participants.

All participants underwent thorough history taking and general examination. The blood samples were taken from the patients before the dialysis session and repeated at the end of three months of vitamin E supplementation.

Assay; Six ml fasting blood samples were collected into polypropylene tubes containing EDTA (1.5 mg/mL). 1 mL was used for measurement of haemoglobin (Hb) concentration and hematoctit while, the rest of sample was separated into plasma and erythrocytes fraction by centrifugation at 3000 g for 10 minutes at 4°C. The separated plasma was used for assay of selenium (Se), malondialdehyde (MDA), creatinine, urea nitrogen and total protein levels.

Preparation of haemolysates: After separation of plasma, the packed erythrocytes were washed 3 times with an isotonic saline and the washed erythrocytes were hemolyzed with osmotic shock technique through three freeze-thaw cycles and by addition of ice-cold water 1:5 (v/v). The erythrocyte membranes were removed by centrifugation (Cruz-Pastor et al., 1998). Erythrocyte activity of SOD and GSH-Px was determined immediately in the haemolysate.

Erythrocyte glutathione peroxidase (GSH-Px) activity: It was measured by an enzymatic method according to (Plagia and Valentine, 1967) using tert-butyl hydroperoxide as the substrate. The kit was supplied by Randox lab. Ltd, UK. Enzyme activity was expressed in units per gram hemoglobin (U/g Hb). One unit of GSH-Px was defined as 1 μmol NADPH oxidized per minute per gram hemoglobin. The absorbance was measured by JENWAY, 6105 UV-VIS Spectrophotometer.

Erythrocyte superoxide dismutase (SOD) activity: It was measured according to (McCord and Fridovich, 1969), which is based on the capacity of SOD to inhibit the reduction of ferricytochrome C by the xanthine/xanthine oxidase system. One SOD unit was defined as the amount

of enzyme that inhibited the rate of cytochrome C reduction by 50%. The result was expressed as U/g Hb. The kit was supplied by Randox lab. Ltd, UK.

Plasma Malondialdehyde (MDA) level: Plasma MDA level was measured spectrophotometrically at 532 nm using thiobarbituric acid reagents under acidic conditions to generate a pink colored product and recorded as nmol/L (Gavino et al., 1981).

Haemoglobin concentration: Was measured by (Quanti Chrom haemoglobin assay kit) according to (Green and Teal, 1959) based on an improved cyanohaemoglobin method, in which the haemoglobin is converted into a uniform colored end product. The intensity of color was measured at 400 nm. Micro-Hematocrit method (Dacie and Lewis, 2001) was used to measure the hematocrit (Hct %) using micro-Hematocrit centrifuge (10000 g for 5 minutes) and the standard capillary tubes.

Determination of plasma creatinine, urea nitrogen and total protein levels: Plasma creatinine and total protein levels were measured by spectrophotometric method according to (Jaffe et at., 1986 and Layne, 1957) respectively, while urea nitrogen was measured by an enzymatic method according to (Talke and Schubert, 1965).

Plasma selenium level (Jacobson and Lockitch, 1988): Plasma selenium was measured using Atomic Absorption Spectrometer (ASS 2380 Perkin Elmer). The optimum parameters were: lamp current; 4mA; air flow, 211 min^{-1}, slit width, 2 nm and wave length of 196.0 nm. The plasma samples were prepared by nitric acid ultrapure grade (65%, Merck, Germany) and hydrochloric acid ultrapure grade (30%, Merck, Germany). Then selenium in sample was reduced with 1 ml of 25 mmol/L ascorbic acid. High purity selenium stock solution (1000 mg Se/L as SeO_2 in 0.5 mol/L HNO3, Merck, Germany) was used for preparation of working standard (40 µg Se/L).

Statistical analysis:

Statistical analysis was done by using SPSS Software version 10.0 (SPSS, Chicago, IL, U.S.A.). The data were expressed as mean ± standard deviation for patients and control separately. Differences were analyzed using t-student test for comparison between two groups and ANOVA test to detect differences among groups. Pearson's correlation coefficient was done to study the relation between variables. P value was considered significant if less than 0.05.

RESULTS

Tables (1, 2) showed that there was statisticaly significant decrease in the

erythrocyte activity of GSH-Px and SOD enzymes and Se level compared to the control values ($p<0.0001$) however, MDA level in HD patients were increased significantly ($p<0.0001$). Also there was a significant decrease in Hb, hematocrit and plasma protein concentration in the patients when compared to control ($p<0.0001$). After vit E supplementation there was a significant decrease in MDA level ($p<0.0001$) also, there was a significant increase in erythrocyte activity of GSH-Px and SOD as well as plasma protein concentration ($p<0.0001$ and $p=0.010$) respectively. While, there was insignificant increase in Se level, Hb concentration and hematocrit ($p= 0.072$, $p=0.081$ and 0.62) respectivly.

Table (3) showed that there was significant decrease in creatinine and urea level after vit E supplementation ($p<0.0001$).

Table (4) showed that there was a statisticaly significant negative correlation between Hb and MDA levels (fig.1) as well as between Hct and MDA. While, there was a significant positive correlation between Hb and each of SOD (fig.2), GSH-Px (fig.3) and Se. Also, there was significant positive correlation between Se and each of GSH-Px and plasma protein. Furthermore, there was a significant positive correlation between MDA and creatinine, however SOD correlate negatively with urea.

DISCUSSION

This study demonstrated a relationship between markers of oxidative stress and haemoglobin levels in HD patients and investigated the effect of vit E supplementation on both oxidative stress markers and renal anaemia.

MDA is an end product of lipid peroxidation and as an index of oxidative damage, in the current study was significantly increased in group of HD patients before vit E supplementation than in control. These findings are in agreement of Ozden et al. (2002) and Esma et al. (2006). They reported that increased MDA level might be a concequence of uremia per se which could prime phagocyte oxidative burst. HD, far from improving the uraemic status, results in an enhancement of ROS owing to bioincompatibility of the dialyser membrane. Since a dialysis membrane is an artificial biomaterial, the leukocytes and complements are activated to produce a variety of ROS, including superoxide, hydrogen peroxide and hypochlorous acid.

Furthermore, interleukins and anaphylatoxins produced during HD sessions are potent activators for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme that is responsible for overproduction of ROS (Morena et al., 2005).

Also, HD could mediate the platelets activation which interact with neutrophils through P-selectin and increasing their production of ROS (Clermont et al., 2001).

Moreover, high levels of plasma homocysteine, which accumulates at the early stages of CRF, could promote prooxidant state by interacting with H_2O_2 (Marion et al., 2001).

Protection from such oxidant injury involves numerous enzymatic and non-enzymatic pathways. GSH-Px is one of the main antioxidants in the intra- and extracellular compartments. It catalyzes the inactivation of hydrogen peroxide and lipid hydroperoxides to neutral substance and water in the presence of reduced glutathione (GSH). High levels of GSH are maintained in the red blood cells by the NADPH dependent enzyme glutathione-reductase. Hexose monophosphate (HMP) pathway in erythrocytes is the principal source of the reductive equivalents NADPH (Kose et al., 1997).

However, the selenium (Se) status is important to the antioxidant defense because Se is incorporated into the red blood cell GSH-Px during erythropoiesis (Ozden et al., 2002).

Therefore, lowered erythrocyte activity of GSH-Px in this study could be attributed to Se deficiency. This explanation might be supported by significant correlation between erythrocyte GSH-Px activity and plasma Se level in this study as reported by Bogye et al. (2000).

However, metabolic blockage of the HMP shunt as well as low GSH in uremic patients could be involved in lower GSH-Px activity (Stepniewska et al., 2006).

In the present study the decreased Se level in HD patients group before vit E supplementation could be a concequence of selenium loss through the pores of polysulfone membranes during haemodialysis (Bogye et al., 2000).

However, restricted protein intake by uremic patients has been suggested as an important cause of Se deficiency because the protein foodstuffs contain the largest amount of Se (Kaminska-Galwa et al., 1994).

On the other hand, it might be due to impaired intestinal absorption, abnormal binding to Se transport proteins and protein loss with urine (Story et al., 1999).

The correlation between Se and plasma protein concentration in the current study could support the dependency of selenium level on protein status.

However, a strong inflammatory response with redistribution of trace

elements from plasma to other tissues as well as dilution of the circulating compartment by resuscitation fluids could be also explain Se deficiency (Berger, et al., 2004).

In the present study there was a significant reduction of erythrocyte SOD activity which in accordance to Chugh et al. (2000), who stated that SOD is zinc and copper dependent, and therefore decreased these serum ions levels in HD patients, could contribute to its low activity.

However, an increased H2o2 as a consequence of reduced GSH-Px activity in this study could be involved in lower erythrocyte SOD activity (Stepniewska et al., 2006).

Thus, GSH-Px could be considered as a complementary to SOD which represent an appropriate response for continuing erythrocyte integrity (Kose et al., 1997).

On the other hand Weinstein et al. (2000) suggested that uraemia itself could alter the erythrocyte antioxidant system and an additional insult to the system is the presence of free iron in the circulation, which aggravates the toxicity of ROS.

The increased oxidative stress in the present study assessed by MDA level and erythrocyte activity of SOD and GSH-Px was found to be correlated with the degree of renal dysfunction before vit E as measured by plasma creatinine and urea levels as reported by Giray et al. (2003).

In the current study there was decrease in Hb concentration and hematocrit % in HD patients group before vit E supplementation which could be a consequence of oxidative stress in those patients as reported by Stepniewska et al. (2006). They stated that the enhanced ROS generation together with reduced erythrocyte GSH-Px activity reactions in uremia would contribute to the lipid peroxidation within the RBCs membrane resulting in reduction of the lifespan of the erythrocytes and hemolysis.

Also, we found correlation between Hb concentration and each of MDA, GSH-Px, and SOD in those patients which could support the involvement of oxidative stress in the pathogenesis of renal anemia.

Furthermore, aminophospholipid phosphatidylserine (PS) of erythrocyte is located normally in the membrane's inner leaflet and ureamia is associated with retention of compound(s) which increase the exposure of PS on the outer leaflet of the erythrocyte membrane. This surface-exposed PS would enhance the susceptibility of RBCs to phagocytosis and shortened erythrocyte survival (Bonomini et al., 2006)

On the other hand, increased activity of pro-inflammatory cytokines, tumor necrosis factor-alpha, interleukins-1(IL-1) and IL-6 in maintenance hemodialysis patients have an inhibitory effect on erythroid precursors in the bone marrow, leading to development of erythropoietin resistance (Stepniewska et al., 2006).

Moreover, anaemia per se is connected with progression in nephron loss due to its mitogenic and fibrogenic stimuli by causing local tissue hypoxia and alterations of renal sympathetic nerve activity (Deicher and Horl, 2003).

However, since vitamin E deficiencies have also been reported in uroemic patients undergoing maintenance HD, beneficial effects of vitamin E, administered orally or bound to dialysis membranes have been suggested in these patients (Galli et al., 2001).

In this study re-evaluating indices of oxidative stress of the same patients after supplementation of the vitamin E in a dosage of 400 mg/daily for 12 weeks revealed a decrease in MDA concentrations, an increase in erythrocyte activity of GSH-Px and SOD as well as slight increase in selenium level. These results could be attributed to its capacity to scavenge ROS and decrease superoxide production by activated phagocyte (Morimoto et al., 2005).

On the other hand the improvement in indicies of oxidative stress after vit E supplementation was not associated by correction of renal anaemia.

Although, haemoglobin levels did not increased significantly after the vit E supplementation, Morimoto et al. (2005) have reported that vitamin E might improve the rheology of circulating red blood cells and reduce the requirement of erythropoietin doses in HD patients.

However, Taccone-Galluci et al. (1999) have suggested that renal anaemia is caused probably by some high molecular weight or protein-bound toxins that have a property to inactivate the antioxidant defense system.

Recently Luchi et al. (2007) have reported that oxidative stress lead to increased erythrocyte vulnerability with continuous destruction of oxidized erythrocytes. This induces the formation of autoantibodies against certain erythrocyte components resulting in anaemia through autoimmune process.

The present findings allow us to conclude that: Redox imbalance in HD pateints might be a contributing factor in renal anaemia. Treatment with vit E might decrease radical generation effectively in HD patients with insignificant effect on renal anaemia.

Table (1): The studied parameters in HD patients before vit E compared to control.

Parameters	Control	HD patients before vit E	p-value
Se (µmol/l)	1.09±0.225	0.582± 0.07	p<0.0001
MDA (nmol/l)	1.75±0.71	8.18± 1.99	p<0.0001
GSH-Px (U/g Hb)	32.76± 6.31	19.06± 1.99	p<0.0001
SOD (U/g Hb)	895.63± 8.98	451.26± 40.25	p<0.0001
Plasm protein (g/l)	7.48±1.02	6.23±0.73	p<0.0001
Hb (g/dl)	11.31±8.55	7.14±.95	p<0.0001
Haematocrit (%)	34±0.51	23 ±0.38	p<0.0001

p significance between control and HD before vit E

Table (2): The studied parameters in HD patients before and after vit E supplementation.

Parameters	HD patients		p-value
	before vit E	after vit E	
Se (µmol/l)	0.582±0.07	0.612± 0.04	p=0.072
MDA (nmol/l)	8.18±1.99	6.08± 1.79	p<0.0001
GSH-Px (U/g Hb)	19.06±1.99	22.46± 3.14	p<0.0001
SOD (U/g Hb)	451.26± 40.25	557.46± 74.34	p<0.0001
Plasm protein (g/l)	6.23± 0.73	6.82± 0.95	p=0.010
Hb (g/dl)	7.14± .95	7.94± 1.35	p=0.081
Haematocrite %	23 ±0.38	23.5 ±0.12	p=0.062

p: significance between HD before and after vit E

Table (3): Plasma creatinine and urea in HD patients before and after vit E supplementation.

Parameters	Control	HD patients		p-value
		before vit E	after vit E	
Creatinine (mg/dl)	0.96±0.16	10.98±2.23	5.71±1.5	p_1<0.0001 p_3<0.0001
Urea (mg/dl)	27.7± 6.58	210.33± 26.11	84.40± 15.9	p_1<0.0001 p_3<0.0001

p_1: significance between control and HD before vit E
p_3: significance between HD before and after vit E

Table (4): Correlation between the studied variables .

Parameters		HD patients	
		before vit E	after vit E
Hb and MDA	r	-0.62	-0.60
	p	0.002	0.013
Hb and GSH-Px	r	0.368	0.43
	p	0.043	0.025
Hb and SOD	r	0.42	0.54
	p	0.02	0.016
Hb and Se	r	0.420	0.53
	p	0.021	0.022
Hct and MDA	r	-0.207	-0.127
	p	0.045	0.003
Se and GSH-Px	r	0.403	0.55
	p	0.027	0.014
Se and plasma protein	r	0.303	0.402
	p	0.017	0.028
MDA and creatinine	r	0.480	0.510
	p	0.007	0.006
SOD and urea	r	-0.032	-0.0397
	p	0.040	0.030

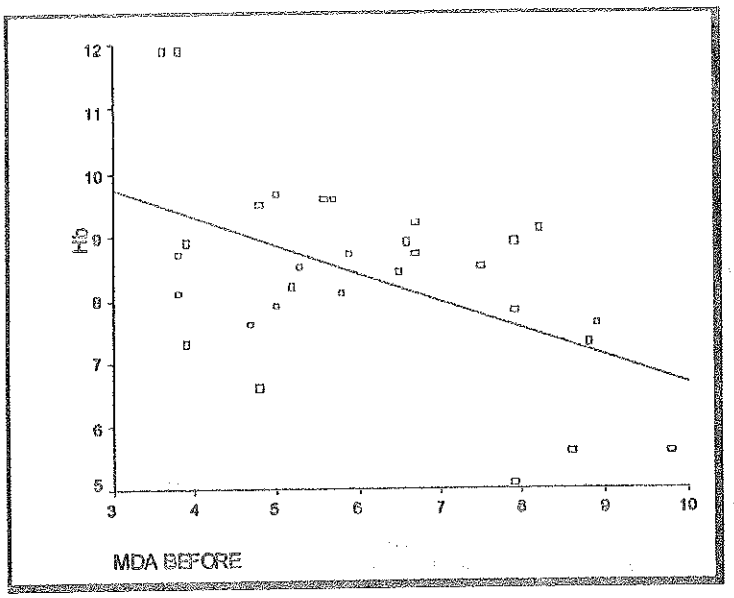

Fig. (1) : Correlation between MDA and Hb

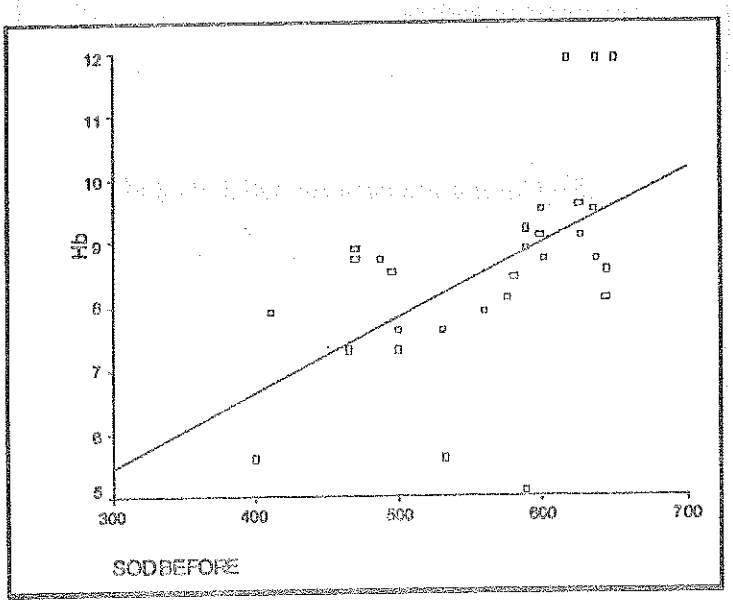

Fig. (2) : Correlation between SOD and Hb

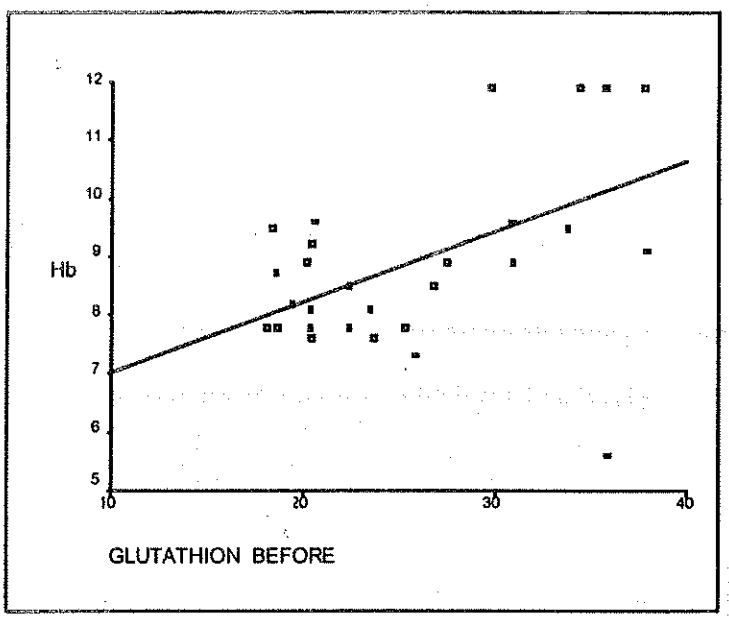

Fig. (3): Correlation between GSH-Px and Hb.

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هل يؤثر فيتامين هـ على الإجهاد التأكسدى والأنيميا الكلوية فى مرضى الغسيل الكلوى ؟

المشتركون فى البحث

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مرضى الغسيل الكلوى معرضون للإجهاد التأكسدى نتيجة لعوامل متعددة، الأنيميا الكلوية من أهم مضاعفات الغسيل الكلوى والتى تنتج عن عوامل مختلفة متعلقة بنشوء المرض.

تهدف الدراسة إلى تقييم دور الإجهاد التأكسدى كعنصر يسهم فى الأنيميا الكلوية وذلك من خلال دراسة تأثير إضافة ڤيتامين هـ (بوصفه من مضادات الأكسدة) على دلالات الإجهاد التأكسدى وكذلك الأنيميا فى مرضى الغسيل الكلوى، تم قياس نشاط إنزيم سوبر أوكسيد دسميوتيز وإنزيم جلوتاثيون بيروكسيداز فى كرات الدم الحمراء والمالونالدهيد والسيلنيوم والبروتين فى البلازما كذلك تركيز الهيموجلوبين والهيماتوكريت فى ٤٣ من مرضى الغسيل الكلوى بالمقارنة مع ٢٠ من الأصحاء من نفس العمر كمجموعة ضابطة.

أوضحت النتائج :

- زيادة ذات دلالة إحصائية فى مستوى المالونالدهيد فى المرضى مما كان عليه فى المجموعة الضابطة.

- إنخفاض ذو دلالة إحصائية لنشاط إنزيم سوبر أوكسيد دسميوتيز وانزيم جلوتاثيون بيروكسيداز ومستوى كل من السيلنيوم والبروتين كذلك تركيز الهيموجلوبين والهيماتوكريت فى المرضى عن المجموعة الضابطة.

- علاقة إرتباط عكسية بين مستوى المالونالدهيد وتركيز الهيموجلوبين.

- بمعاودة قياس مستوى دلالات الإجهاد التأكسدى، والأنيميا بعد ١٢ إسبوعاً من العلاج بجرعة (٤٠٠ ملجرام) ڤيتامين هـ يومياً لوحظ.

- زيادة ذات دلالة إحصائية فى نشاط إنزيم سوبر أوكسيد دسميوتيز والجلوتاثيون بيروكسيداز.

- إنخفاض ذو دلالة إحصائية فى مستوى المالونالدهيد.

- بينما الزيادة فى مستوى كل من السيلنيوم وتركيز الهيموجلوبين والهيماتوكريت لم تكن ذات دلالة إحصائية.

نستنتج من البحث أن الإجهاد التأكسدى فى مرضى الغسيل الكلوى قد يكون أحد أسباب الأنيميا الكلوية.

وأن العلاج بفيتامين هـ يقلل توليد الشوارد الحرة وأن الأثر الإيجابى لفيتامين هـ لم يقترن بتحسن الأنيميا الكلوية فى مرضى الغسيل الكلوى.

الكلمات الرئيسية : الغسيل الكلوى؛ الإجهاد التأكسدى ، الإنزيمات المضادة للأكسدة، فيتامين هـ؛ والأنيميا الكلوية.