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Research Article

Oxidative stress in childhood steroid sensitive nephrotic syndrome and its correlation with DNA damage

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ABSTRACT

Background: Imbalance in oxidative state has been hypothesized as causative factor in renal injury. Oxidant stress can result in DNA damage which can be measured by comet assay. The aim of the study was to assess the oxidative stress and DNA damage in children with NS.
Methods: Children of NS during first episode/relapse and remission were recruited. Blood malondialdehyde (MDA), reduced-glutathione (GSH) and superoxide dismutase (SOD) levels were done to assess oxidative state and compared with controls. DNA damage was assessed by comet assay. Percentage DNA in tail, tail length and olive tail moment (OTM) were calculated. Comparison of various groups was done and correlation determined by appropriate statistical tests.
Results: Study group consisted of 38 children in each group. MDA levels were high in NS group compared to remission and controls with statistical significance ($p<0.001$ and 0.005 respectively). GSH and SOD levels were lower in relapse and remission group compared to controls. p value was <0.001 when NS group was compared with control. When proteinuria group was compared with remission, p value was <0.001 for GSH and 0.045 for SOD. Mean percentage DNA in tail in children with proteinuria was significantly more compared to control ($p=0.052$) and it correlated best with MDA assay ($r=0.227$; $p=0.003$) suggesting a relation between oxidative stress and DNA damage.
Conclusions: Oxidative stress persisted in NS even after remission and DNA damage can occur as result of persistent oxidant stress. There was significant increase in antioxidants levels when children went into remission. The levels of oxidant MDA correlated with DNA damage.

Keywords: Comet assay, Glutathione, Malondialdehyde, Proteinuria

INTRODUCTION

Nephrotic syndrome (NS) is a common disorder in children characterized by alteration in permeability of the glomerular capillary wall resulting in its inability to restrict the urinary loss of protein. For decades, oxidative stress resulting from an imbalance between oxidants and antioxidants is implicated in the pathogenesis of several genetic or acquired diseases including NS.[1,2] Oxidative stress markers like malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) are easily measurable. MDA is a naturally occurring product of lipid peroxidation. The production of this aldehyde is used as a biomarker to measure oxidative stress. It combines with deoxyadenosine and guanosine to form large abnormal DNA molecules and is mutagenic.[3] GSH is a tripeptide containing sulfhydryl (thiol) group which acts as a proton donor and is responsible for its biological function. It is a co-factor for many detoxifying enzymes and scavenges hydroxyl radical and helps in regeneration of vitamins C and E to their active forms. Enzymatic antioxidant SOD by successive oxidative and reductive

processes neutralizes the superoxide ions and thus helps in protecting the cells from oxidant stress.[4]

Vascular, tubular, juxta glomerular and mesangial cells including podocytes and isolated glomeruli of the kidney produce oxygen free radicals which can have a negative influence in NS.[5] Reactive oxygen species (ROS) are suspected to be a common factor in the development of progressive renal injury. Chronic exposure to oxidative stress can result in permanent changes in the genome. In a recent study, investigators found that DNA damage and renal injury mediated by ROS was associated decline in podocyte number and glomerulosclerosis in animals.[6] The human body has many ingenious mechanisms of minimising oxidative damage. Increase in ROS levels beyond the capacity of its antioxidant defence results in accumulation of oxidative DNA damaged products including oxidative clustered DNA lesions and these can be efficiently repaired by base excision or nucleotide excision repairs.[7] If unrepaired, these lesions can lead to the formation of mutations, DNA double strand breaks and chromosome abnormalities. The various antioxidants either natural or synthetic when administered may have a beneficial role in prevention and attenuation of renal scarring in kidney diseases.[7,8]

The oxidative DNA damage has also been investigated using comet assay in chronic kidney disease.[9] Developed by Ostling and Johason, comet assay, also called as single cell gel electrophoresis assay is a technique by which DNA damage is measured. The highly ordered DNA when disrupted by any damage becomes disorganized and when electrophoresed migrates towards the cathode. DNA damage creates smaller fragments which travel farther whereas the large undamaged strands do not migrate far from the nucleus. The comet consists of a head, which represents intact DNA and a tail containing damaged and broken pieces. Comet assay measures and compares the overall fluorescent intensity of the DNA in the nucleus with DNA that has migrated out of the nucleus. Fluorescent signal from the migrated fragments is proportional to DNA damage.[10,11]

Several studies have established the oxidant stress in children and adults with NS during active phase continuing into remission. However there are no studies relating this to DNA damage. Thus this study was carried out with the objective of determining the presence of oxidative stress in children in various states of NS by measuring blood levels of MDA, GSH and SOD. In addition, DNA damage was assessed using comet assay and a correlation between the two if any was determined.

Subjects

The study was a case control study done after obtaining institutional ethical committee clearance and after taking informed consent from either parent with assent from children aged over seven years. Children diagnosed with first episode or relapse of NS, treated children in remission constituted the two study groups. Healthy children matched for age between one and 18 years constituted the controls. Subjects were diagnosed to have NS based on clinical presentation and relevant investigations. Children diagnosed with congenital or steroid resistant NS, previously or currently being treated with immunosuppressant, those with underlying systemic illnesses and chronic infections were excluded from the study. The duration of the study was two years.

METHODS

Biochemical assays

Blood was collected in plain vacutainer for MDA and in EDTA for GSH, SOD, and comet assays. MDA was assayed using 2-thiobarbituric acid (TBA). The colour of TBA chromogen was measured at 532 nm and values were expressed in micro moles per litre (µM/L).[12] GSH was assayed by spectrophotometer at 412 nm after oxidation with 5, 5'-dithiobis (2-nitro benzoic acid) and values were expressed as micro grams per ml (µg/ml).[13] Erythrocyte SOD was measured by Beauchamp and Fridovich method based on the inhibition of nitroblue tetrazolium (NBT).[14] Superoxide radicals inhibit NBT to form blue coloured compound, formazan. The absorbance was measured at 560 nm by UV absorption spectroscopy and expressed as units/gm Hb. One unit of SOD activity is defined as the amount of enzyme required to inhibit the reduction of NBT by 50%.

Comet assay

For alkaline comet assay sample was processed within three hours of collection. It was mixed with equal volumes of histopaque (polysucrose gradient) and was centrifuged. Buffy coat layer in the middle which contains large number of lymphocytes was removed with a micro-pipette and slides were prepared. After sample preparation, electrophoresis was carried at an alkaline pH of >13.[15] It was subjected to neutralizing solution and then stained with ethidium bromide and seen under fluorescent microscope with green filter. Pictures were taken with Q capture pro. Images were analysed using comet assay software. Values were scored based on percentage DNA in tail, tail length and olive tail moment (OTM) using comet assay software. Percentage DNA in tail is the difference between total percentage of DNA present in the comet and head. DNA tail is the distance of migration from the body of the nuclear core. OTM was calculated as measure of tail length × measure of DNA in tail.

Sample size calculation and statistical analysis

Sample size of 38 in each group was calculated for a power of 0.8 at α error of 0.05, 95% confidence interval (CI) and hypothesized difference of 5 between the means and population variance of 60 with 1:1 ratio of subjects and controls.

Data was analyzed using SPSS for windows software version (16.0). Comparison of various groups was done by independent sample t-test and ANOVA. Mean is expressed as ±standard error of mean (SEM). Pearson correlation was used to determine relation between comet assay scores and oxidative stress markers. A p-value ≤0.05 was considered significant.

RESULTS

Demography

Study group consisted of 114 children divided into three groups, 38 in each. Out of total 114 children in study group, 57 (50%) were between 1-5 years, 33 (29%) between 6-10 years and 24 (21%) were ≥11 years. Of this 64 (56.1%) were males. The first group of 38 cases included ten children with first episode of NS and 28 infrequent relapsers. All these children went into remission with steroids. Of this 34 cases were followed up and did not have relapse during the study period.

Oxidative stress

Oxidative stress was assessed across the groups of children and is shown in Table 1. The mean MDA levels in the three groups were 1.48±0.08, 1.21±0.06 and 1.01±0.06 µM/L respectively.

Table 1: Comparison of oxidative stress in children with various states of nephrotic syndrome (NS) and control as measured by malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) assays.

Subjects (n=38 in each)	MDA (µM/L)	GSH (µg/ml)	SOD (units/gm Hb)
	Mean±standard error of mean		
NS active	1.48±0.08	234.63±11.76	25.66±0.97
NS in remission	1.21±0.06	306.00±12.80	28.42±0.94
Controls	1.01±0.06	332.18±17.18	32.32±1.57
	95% confidence interval (p-value)		
Active vs. control	0.28 to 0.68 **(<0.001)**	-139.03 to-56.98 **(<0.001)**	-10.33 to-2.98 **(0.001)**
Active vs. remission	0.08 to 0.46 **(0.005)**	-106.01 to -36.73 **(<0.001)**	-5.46 to -0.06 **(0.045)**
Remission vs. control	0.04 to 0.37 **(0.017)**	-68.88 to 16.5 **(0.226)**	-7.54 to -0.25 **(0.037)**

There was statistically significant difference between children with proteinuria against remission (p=0.005) and controls (p <0.001). Mean GSH levels in µg/ml was 234.63±11.76 in children with proteinuria, lower compared to children in remission (306.00±12.80) and control group (332.18±17.18). The GSH was significantly lower with p-value of <0.001 when children with proteinuria were compared with remission and controls. Mean SOD levels in units/gm Hb were 25.66±0.97, 28.42±0.94 and 32.3±1.57 respectively.

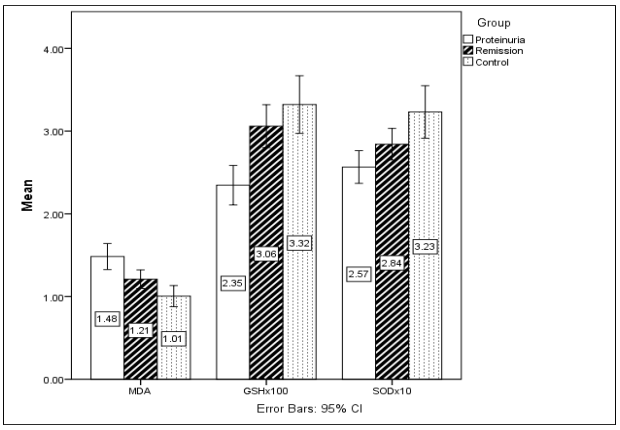

Figure 1: Mean levels of (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) with 95% confidence interval (CI) during the various states of NS in comparison with controls.

There was statistical significance when children with active disease were compared with remission and control groups (p=0.045 and 0.001 respectively) and also when children in remission and controls were compared (p=0.037). There was significant difference in MDA (p <0.001), GSH (p <0.001) and SOD (p <0.001) between the three groups. Figure 1 represents the ranges of MDA, GSH and SOD during the various states of NS in comparison with control.

DNA damage

Mean values of percentage DNA in tail, tail length and OTM were calculated. Mean percentage DNA in tail in children with proteinuria was 12.42±3.16, significantly more compared to control (5.26±1.74) with p value of 0.052. Mean tail length was comparable in children with active proteinuria and in remission and longer in comparison with controls though not statistically significant. Mean OTM was higher with active disease as compared to other groups but did not show statistical significance.

Table 2 gives the mean values of three indices calculated in comet assay with significance. Percentage DNA in tail correlated best with MDA assay (r=0.227; p=0.003) suggesting a relation between oxidative stress and DNA damage (Figure 2).

Table2. Comparison of DNA damage in children with various states of nephrotic syndrome (NS) and control.

Subjects (n=38 in each)	Percentage DNA in tail	Tail length	Olive tail moment
Mean ± standard error of mean			
NS active	12.42±3.16*	14.63±3.55	5.86±1.35
NS in remission	6.56±1.63	14.93±3.45	3.79±0.96
Controls	5.26±1.74*	9.96±2.69	3.53±1.25

*Relapse vs. Control: 95% Confidence interval -0.07 to 14.38; p=0.052

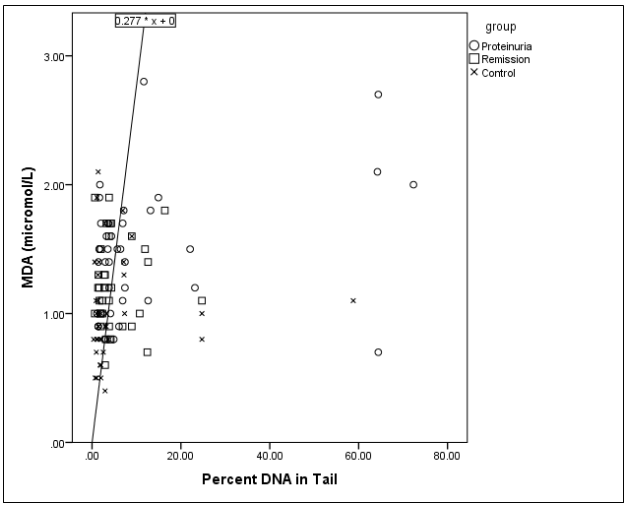

Figure 2: Linear correlation of serum malondialdehyde (MDA) with percentage DNA in tail depicting relation between oxidative stress and DNA damage.

DISCUSSION

The results of the present study suggest that oxidative stress especially MDA persisted in children with NS even after remission. There was a significant increase in the antioxidants levels that we measured when children went into remission. Also, the levels of oxidant MDA correlated with DNA damage. Various authors have used different markers to measure antioxidant levels in NS.[16] Fan et al, in a recent follow up study including 40 children with active proteinuria and remission, found significantly higher advance oxidation protein products as compared to 20 controls with predictive value for relapse. MDA levels were higher and SOD lower, but without statistical significance.[17] In similar studies done by Mishra et al and Bakr et al, comprising of three groups, there is recovery of antioxidant levels only with prolonged remission. Mao et al, in their meta-analysis of eight studies on NS, have concluded that MDA abnormalities persist even during remission.[18-20] Granquist et al, have shown decreased expression of antioxidant enzymes in the glomerular cells of nephrotic renal biopsy specimens by polymerase chain reaction.[21]

Alkaline comet assay maximizes sensitivity of detection of low levels of damage and is superior for evaluating broad spectrum of DNA lesions. Percentage DNA in tail is shown to be linearly related to damage. In our study percentage DNA in tail was statistically significant in children with active proteinuria and remission. Lopez et al. used alkaline comet assay to determine DNA damage in patients with chronic kidney disease.[22] They also measured markers of oxidative stress like MDA and advanced oxidation protein products. Their data showed significant increase of tail DNA intensity in patients with CKD ($p<0.001$) compared to control group. Ayakant et al also showed that DNA damage was significantly increased in children with CKD compared to healthy children ($p<0.001$).[9] Marshall et al in their *in vitro* study found that DNA damage was increased in cultured podocytes treated with puromomycin aminoglycoside (PA) induced ROS. They found that DNA repair enzymes were activated, providing evidence for attempted DNA repair. The PA-treated animals developed significant proteinuria and exhibited more DNA damage confirming that oxidative stress causes podocyte DNA damage.[6] In our study, the significant correlation between MDA levels and percentage DNA in tail suggests oxidant injury can result in DNA damage.

CONCLUSION

Oxidant stress exists in NS and may persist during remission. DNA damage can occur as a result of persistent oxidant stress. Antioxidants may have a role in NS to prevent injury from ROS and reduce the DNA damage.

A Long term follow up of children with NS is required to assess the persistent oxidative stress and relapse rate and the need for supplementing antioxidants to achieve prolonged remission.

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