Original Article

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# DNA Damage Analysis in Children with Non-syndromic Developmental Delay by Comet Assay

Anatomy Section

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## ABSTRACT

**Introduction:** Majority of the developmental delays in children are non-syndromic and they are believed to have an underlying DNA damage, though not well substantiated. Hence the present study was carried out to find out if there is any increased DNA damage in children with non-syndromic developmental delay by using the comet assay.

**Aim:** The present case-control study was undertaken to assess the level of DNA damage in children with non syndromic developmental delay and compare the same with that of age and sex matched controls using submarine gel electrophoresis (Comet Assay).

**Materials and Methods:** The blood from clinically diagnosed children with non syndromic developmental delay and controls were subjected for alkaline version of comet assay – Single cell gel electrophoresis using lymphocytes isolated from the peripheral blood. The comets were observed under a bright field microscope; photocaptured and scored using the Image J image quantification software. Comet parameters were compared between the cases and controls and statistical analysis and interpretation of results was done using the statistical software SPSS version 20.

**Results:** The mean comet tail length in cases and control was 20.77±7.659μm and 08.97±4.398μm respectively which was statistically significant ($p<0.001$). Other comet parameters like total comet length and % DNA in tail also showed a statistically significant difference ($p < 0.001$) between cases and controls.

**Conclusion:** The current investigation unraveled increased levels of DNA damage in children with non syndromic developmental delay when compared to the controls.

**Keywords:** Damage to Deoxy-ribonucleic acid, Delayed development, Gel electrophrosis, Malonyldialdehyde

## INTRODUCTION

Developmental delay occurs when a child is unable to attain the desired milestones for that age and it is a common problem among children. Around 2 to 3% of the children are affected with ongoing severity of developmental delay. Among the various factors that contribute to developmental delay, chromosomal anomalies contribute to only a minor extent, whereas majority of the causes for developmental delay are non-syndromic in origin [1-3]. Asphyxia during the perinatal period, damages the DNA by oxidative stress, thereby harming the neurons by causing their death either in a programmed manner or by self-destruction [3,4]. It is not fully known if this DNA damage still persists in those babies who do not improve neurologically. Hence, this present study was undertaken. Though there are a lot of techniques to assess the damage inflicted upon the DNA like FISH, TUNEL, PCR, etc, the comet assay still stands unique in its role in assessing DNA damage, because it is quicker, economical and sensitive [5]. Hence, the comet assay has been used to detect DNA damage in this study.

## MATERIALS AND METHODS

The study was carried out in the Cytogenetics Laboratory of the Department of Anatomy, JIPMER in collaboration with the Department of Paediatrics and Biochemistry, JIPMER, from December 2013 to March 2015, after getting approval from the Institute Research Council and Human Ethics Committee. Clearance from the Institute Human Ethics Committee was obtained on the 2nd of February 2014. The study was carried out only after a proper written, informed consent was obtained from the parents or guardians of the children participating in the study, as per the guidelines given by the Institute Ethics Committee. The subjects for the study were selected from the out-patient department of Paediatrics, JIPMER. The hospital case sheets of the children with developmental delay were viewed for evidence of chromosomal anomalies or other features suggestive of syndromes. They were also examined for dysmorphic features. Children under five years, without any evidence of syndromic features or who were clinically diagnosed of having non-syndromic developmental delay, attending the Paediatric out-patient department of JIPMER, were included as cases. Age and sex matched children, without developmental delay, who attended the hospital for some other minor ailments, were included as controls. Children with chromosomal anomalies and sick children were excluded from this study. The developmental ages of the children who were included as cases, were assessed using the Revised Trivandrum Developmental Screening Chart for children aged 0 to 6 years [6].

Heparinized blood samples (2ml) from both cases and controls were collected under strict aseptic precautions after a written, informed consent from the parents or guardians of the children. A standard operative procedure was also prepared and approved by the Paediatrics department for this study, as per the advice given by the ethics committee. Lymphocytes were separated by centrifugation method using Histopaque (SIGMA). Buffy coats containing the lymphocytes were removed. They were then sandwiched between two layers of agarose gel and subjected to lysis buffer (NaCl, Triton X, EDTA,TRIS). Electrophoresis was carried out in an alkaline medium followed by neutralization of slides. The slides were then stained with silver nitrate and observed under a light microscope. The images were captured by a digital camera and the comet parameters were scored and analysed using the image quantification software. The plasma that was obtained from the samples after centrifugation was separated out using a micropipette and stored in separate vials after labelling them, in a deep freezer at -80ºC for estimation of MDA levels using the TBARS kit method.

The sample size calculated was 60, that is 30 cases and 30 controls. The sample size was calculated using the N-masters software. The primary variable for assessing the outcome was DNA damage. The standard deviation of DNA damage in case

and control groups was 5 and 2.6 micrometers respectively in a previous study on Down's syndrome [7]. We estimated that 28 patients in each group would be required to detect a mean difference of 3 micrometre in DNA damage between the 2 groups. Calculations were based on 80% power; $\alpha$=0.05 and 2 sided 95% confidence interval.

## STATISTICAL ANALYSIS

Descriptive and inferential statistics were used to analyse the data. The baseline characteristics were analysed by descriptive statistics. The data on the level of comet parameters and levels of MDA were expressed as mean with standard deviation or median with range whichever was appropriate. The students t-test was used to compare the continuous variables between the groups; while the Mann-Whitney U test was used in case of a non-parametric distribution. The comet parameters were correlated with the plasma levels of MDA by applying the Spearman's rho correlation. All statistical analyses were carried out at 5% level of significance and the p-value <0.05 was considered significant. The SPSS software, version 20, was used for statistical analysis. The graphs were plotted using Microsoft office excel work sheet.

## RESULTS

There were 30 cases and 30 controls in this study. The controls were age and sex matched as shown in [Table/Fig-1].

| Age in months | Cases ( N=30) | | Controls ( N=30) | |
|---|---|---|---|---|
| | n | % | n | % |
| 0 - 12 | 14 | 46.7 | 14 | 46.7 |
| 13 - 60 | 16 | 53.3 | 16 | 53.3 |
| **Gender** | | | | |
| Males | 17 | 56.7 | 17 | 56.7 |
| Females | 13 | 43.3 | 13 | 43.3 |

**[Table/Fig-1]:** Age and gender distribution among cases and controls.

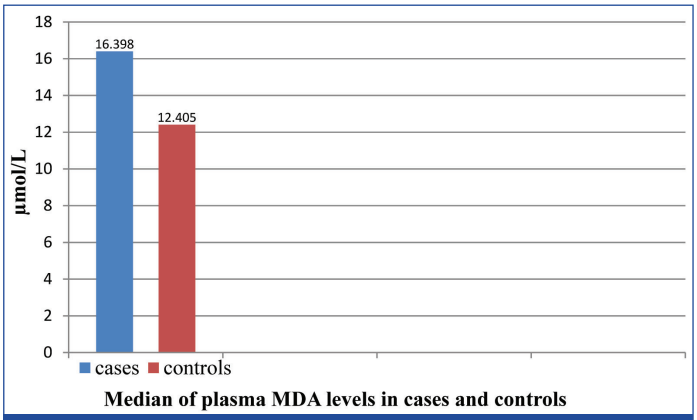
From the table it is evident that majority of the developmentally delayed children fell into the age group of 13 to 60 months, while the remainder of them were less than or equal to 12 months of age at the time of presentation. The gender distribution shows that there was a slight tendency of a male dominant distribution among children with non-syndromic developmental delay.

The images of the comets that were obtained in cases and controls are shown in [Table/Fig-2]. From the figure it is evident that controls had a negligible amount of DNA damage as compared to cases, as the comet tail in controls was hardly ever seen. The assessment and comparison of the magnitude of DNA damage between cases and controls is shown in [Table/Fig-3].

From the table it is deciphered that, the total comet length, head diameter, tail length and percentage of DNA in tail were significantly higher in cases as compared to controls. However, the percentage of DNA in head was significantly less in cases when compared to controls.

| Parameter | Mean with standard deviation | | p-value |
|---|---|---|---|
| | Cases (n=30) | Controls (n=30) | |
| Total comet length (µm) | 50.97 ± 7.332 | 35.40 ± 5.069 | < 0.001 |
| Head diameter (µm) | 30.20 ± 3,662 | 26.43 ± 3.775 | |
| Comet tail length (µm) | 20.77 ± 7.659 | 08.97 ± 4.398 | |
| % of DNA in tail | 71.50 ± 7.001 | 18.63 ± 9.242 | |
| % of DNA in head | 34.20 ± 13.905 | ± 8.011 | |

**[Table/Fig-3]:** Comet parameters among cases and controls




**[Table/Fig-4]:** Comparison between plasma MDA levels of cases and controls.

| Correlation of Plasma MDA levels with | Spearmann's rho correlation coefficient (r) |
|---|---|
| Total comet length | 0.148 |
| Head diameter | 0.228 |
| Comet tail length | -0.032 |
| % of DNA in tail | 0.231 |
| % of DNA in head | 0.115 |

**[Table/Fig-5]:** Correlation between MDA and comet parameters.

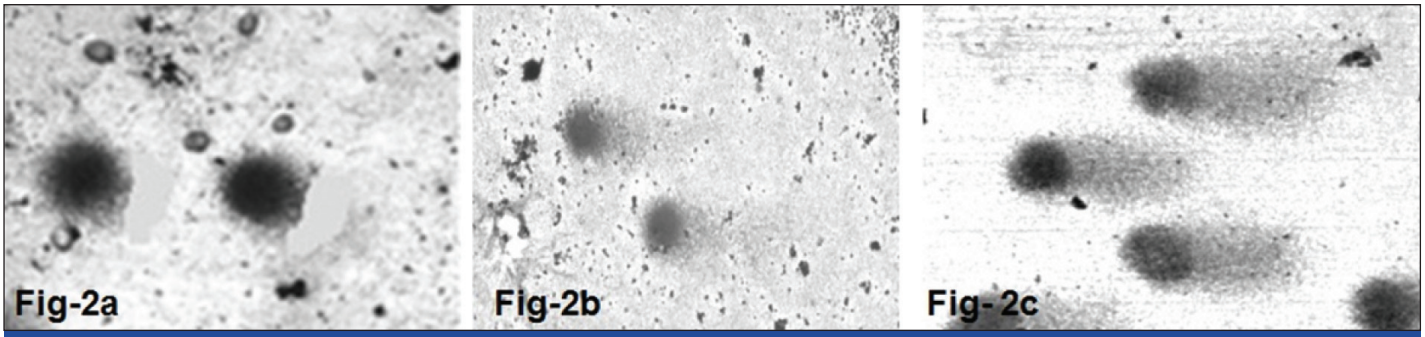
### Levels of Malondialdehyde

The levels of oxidative stress marker malondialdehyde (MDA), that were obtained from the plasma samples of 30 cases and 30 controls, were estimated by the calorimetric method using the TBARS assay kit (96 wells) [8]. The median values of plasma MDA levels for cases were more when compared to controls. However, it was found that the difference in plasma MDA levels between the two groups was not statistically significant [Table/Fig-4].

**Correlation of DNA damage with MDA levels among cases:** Significant correlations were not obtained between plasma MDA levels and comet parameters [Table/Fig-5].

## DISCUSSION

DNA damage among children with non-syndromic developmental delay occurs because of oxidative stress which produces breaks in its strands leading to failure of inherent correcting capabilities within the cell to repair those breaks [9-12]. Sometimes this DNA




**[Table/Fig-2]:** The comet images of cases and controls are shown. (a) No DNA Damage, (b) Mild DNA Damage, (c) Severe DNA Damage.

damage can arise due to freshly occurring genomic imbalances. Wu et al., have observed deletions and rearrangements occuring within the chromosomes of children at the microscopic level just below their telomeric ends [9]. New mutations have also been reported in non-syndromic developmental delay [13,14].

The results in this study show that the comet tail length, percentage of DNA in tail and total comet length were significantly elevated in cases when compared to controls. Previous studies that have been done using the comet assay for various other conditions, have shown that the comet tail length and the percentage of DNA in the comet tail are the two best parameters that indicate the extent of DNA damage, because they basically reflect upon the migration of fragmented DNA particles as a result of breaks towards the anode due to the liberation of supercoils [15-18]. Hence the findings in this study are supportive of those of the other studies, with regards to the comet parameters, thereby proving that the severity of DNA damage is more in children with non-syndromic developmental delay when compared to controls.

Oxidative stress which is a cause for DNA damage releases malondialdehyde (MDA) as a by-product due to peroxidation of lipids [8,19]. Perinatal asphyxia is a well known risk factor for developmental delay [3]. Manoj et al., had shown that in children with asphyxia, the MDA levels in their serum increased. In their study, it was shown that there was a strong correlation between the percentage of DNA in tail and the MDA levels in serum [20]. In this study, it was found that the comet parameters were poorly correlating plasma MDA levels. The possible reason behind the poor correlation of DNA damage with MDA in our study could be that, apart from oxidative stress, there could be other environmental factors that would have acted as stressors and induced DNA damage in children with non-syndromic developmental delay, or this DNA damage could have arisen spontaneously. Moreover oxidative stress could be an acute and static event that has already damaged the DNA either in utero or during the perinatal period, hence the time gap between the occurrence of oxidative stress and evaluation of MDA could be a factor. This is further explained by the fact that in this study, though the plasma MDA levels in cases were more when compared to controls, still the increase was not statistically significant. The other possible reason could be the small sample size in this study.

## CONCLUSION

This study shows that children with non-syndromic developmental delay have increased levels of DNA damage when compared to normal children. But, the increase in plasma MDA levels in cases, is not significant and the MDA levels are not strongly correlating with DNA damage. This could possibly be due to the small sample size in this study, which is a limitation of this study. The other possible reason being the time lag between the estimation of MDA and occurrence of oxidative stress that could have occurred before birth, which is another limitation in this study. However, this study suggests that DNA damage due to oxidative stress has already occurred and there is no further deterioration of DNA damage. This also shows that non-syndromic developmental delay due to perinatal asphyxia and other stress factors, is a non progressive, static event.

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