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# Estimation of DNA damage through Comet assay in children with Congenital Heart Disease - Case-control study.

**Vidya G, Suma H Y, Vishnu Bhat B*, Parkash Chand, Ramachandra Rao K, Harichandrakumar K T****

Department of Anatomy, Pediatrics* and Medical Biometrics and Informatics**, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.

## Abstract

**The present case-control study was undertaken to assess the level of DNA damage in children with congenital heart disease and compare the same with that of age and sex matched controls using *submarine gel electrophoresis (Comet Assay)*.The clinical diagnosis of CHD was confirmed with echocardiography. Subsequently the cases and the controls were subjected for alkaline version of *comet assay* – Single cell gel electrophoresis using lymphocytes isolated from the peripheral blood. Scoring of comets and recording of the data of comet metrics was done after capturing the images of various sizes of comets under bright field microscopy using the Olypmus BX 51trinocular research microscope (Japan) and an automated Comet Scoring Software TriTek V 1.5. Comet parameters were compared between the cases and controls using Unpaired 't' test using the statistical software SPSS version 19. The mean *comet tail length* in cases and control was 19.90µm and 0.89 µm respectively which was statistically significant (p<0.001). Other comet parameters like *total comet length* and *%DNA in head* also showed a statistically significant difference (p < 0.05) between cases and controls. The current investigation unraveled increased levels of DNA damage in children with congenital heart defect as evidenced by statistically significant levels of the comet metrics in *cases* when compared to the *controls*.**

**Keywords:** Congenital heart disease, Comet assay, Total comet length, Comet tail length, DNA damage.

## Introduction

Congenital Heart Disease (CHD) is linked to chronic hypoxia usually due to mixing of blood as a result of shunt either from structural defects or increased pulmonary blood flow as a result of non-obliteration of embryonic vessel/s leading to stages of either gradual or sudden onset of cyanosis and ultimately to hypoxia [1]. In chronic hypoxia, there is liberation of reactive oxygen species (ROS) due to tissue injury as a result of ischemia. It also leads to induction of *hypoxia inducible factor – 1*(HIF-1) and *p53* which in turn activate pro-apoptotic factors leading to alteration in the regulation of pro-apoptotic gene Blc-2 which is involved in causing the DNA damage [2,3].

The data available in literature with regards to oxidative stress and DNA damage in CHD is scanty. Earlier investigators focused only on the comet tail length under the comet metrics and documented DNA damage exclusively in cases with tetralogy of Fallot (TOF) [2]. Hence the complete comet scores in various types of CHD were undertaken in the current study.

## Methods

The present ***case-control*** study on children with congenital heart diseases was conducted in the Cytogenetic laboratory attached to Department of Anatomy, JIPMER in collaboration with the Department of Pediatrics after obtaining the approval from ***Institute Research Council and Human Ethical Committee. Written Informed consent*** was also obtained from the concerned parents. Forty children of various congenital heart diseases diagnosed clinically and confirmed by echocardiography - ***Philips iE 33*** were included as ***cases*** and an identical age and gender matched healthy children were selected as the ***control*** group. About one ml of whole blood was drawn under aseptic condition and lymphocytes were isolated using ***histopaque- SIGMA 1077.*** Isolated lymphocytes were

layered over agarose for performing conventional comet assay [4, 5]. Fifty comets of various dimensions were selected randomly from each slide and observed at 20X. The captured images (Figure 1 and 2) were analyzed using comet software - Tritek cometscore™ version 1.5. The data of the comet configurations that were measured and analyzed in the current study were *-Total comet length (TCL), Head diameter (HD), % of DNA in head (% DH), Comet tail length (TL) and % of DNA in tail (% DT).*

The parametric 'Unpaired 't' test' was used to estimate differences among the comet metrics in cases and control group at a significance threshold of $p<0.05$. All statistical tests were performed using SPSS-version 19 statistical software.

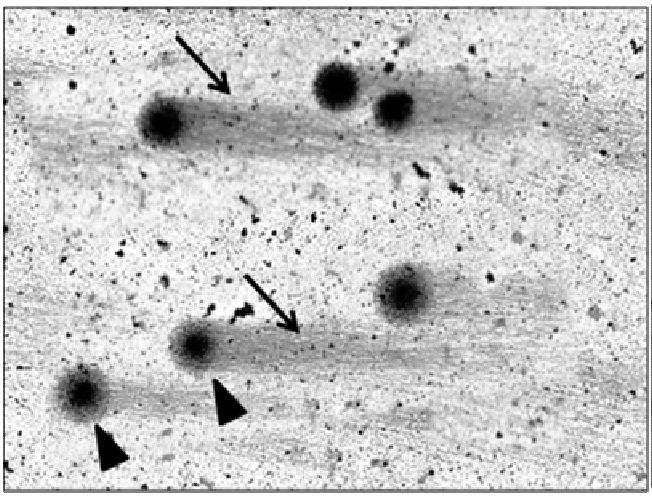

***Figure 1.*** *Comet –Cases*

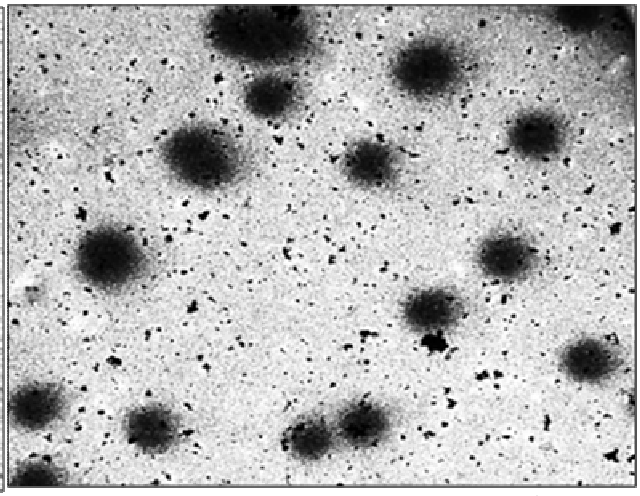

***Figure 2.*** *Comet –Controls*

*(Black arrows- comet tail; Arrow head – comet head)*

## Results

Among the cases investigated, majority were infants contributing to 67.5% of the total while the remaining were confined to >24 < 60 months of age. The cases were classified on the basis of clinical examination into **a*cyanotic and cyanotic groups*** (Table 1). Out of forty cases, 24 comprised of acyanotic group while the remaining cyanotic. In the ***acyanotic group***; septal defects were observed in the form of ventricular septal defect (VSD) or atrial septal defect (ASD). In addition, structural anomalies and non-obliteration of the great vessels were also observed in the form of patent ductus ateriosus (PDA) and coarctation of aorta (COA). In the ***cyanotic group***, all cases were of TOF.

The *mean age* of the *cases and controls* investigated was 16 months. Regarding the details of DNA damage, the *total comet length* among the cases (68.13±14.68) was observed to be significantly higher *(p<0.001) than controls (*30.33±5.19). Similarly *% DNA in head (*88.01±10.47 in cases and 89.02±7.61 in controls) *and comet tail length* (9.90±11.29 in cases and 0.89±1.55 in controls) *also found to be significantly higher (p<0.001) in cases* when compared to the controls. However the *% DNA in tail* (13.32±5.01in cases and10.98±7.61 in controls) and *head diameter* (48.28±9.36 in cases and 29.5±5.35 in controls) were found to be higher in cases than control, but the difference was not statistically significant (Table 2).

***Table 1.*** *Baseline characteristics of the study group*

| Variables | | **Cases** | | **Controls** | |
|---|---|---|---|---|---|
| | | Number | Percentage | Number | Percentage |
| Age in months | ≤6 | 11 | 27.5 | 11 | 27.5 |
| | 6-12 | 16 | 40.0 | 16 | 40.0 |
| | ≥12-24 | 06 | 15.0 | 06 | 15.0 |
| | >24 | 07 | 17.5 | 07 | 17.5 |
| Gender | Male | 19 | 52.5 | 19 | 52.5 |
| | Female | 21 | 47.5 | 21 | 47.5 |
| Type of CHD | Acyanotic | 24 | 60 | NA | NA |
| | Cyanotic | 16 | 40 | NA | NA |

*NA – Not Applicable*

***Table 2.*** *Comparison of Comet Metrics between cases and control*

| Study group | Total Comet Length (µm) | Head Diameter (µm) | Comet Tail Length (µm) | % DNA in Head | % DNA in Tail |
|---|---|---|---|---|---|
| **Cases** (n=40) | 68.13±14.68 | 48.28±9.36 | 19.90±11.29 | 88.01±10.47 | 13.32±5.01 |
| **Controls** (n=40) | 30.33±5.19 | 29.50±5.35 | 0.89±1.55 | 89.02±7.61 | 10.98±7.61 |
| **P value** | P<0.001 | P=0.602 | P<0.001 | P<0.001 | P=0.108 |

*All comet metrics are expressed as mean ±SD*

## Discussion

The present *case-control* study was carried out to evaluate the level of DNA damage in the peripheral lymphocytes of the children with CHD and compare the same with age and sex matched healthy children. The study showed increased DNA damage in children with CHD when compared to controls. Individuals of cyanotic and acyanotic CHD, either due to septal defect or great vessel anomaly are more prone for hypoxia. As cyanosis and hypoxia are interrelated, oxidative stress phenomenon is said to be playing a major role in the said states and the tissue damage due to ***oxidative stress reaction*** [6]. Oxidants and anti-oxidants have deleterious effects on the cellular macromolecules like proteins, lipids and DNA [7]. This is evidenced in the current study as migration of deranged and detached particulate matter of DNA as a trail of the comet.

There is a single study regarding DNA damage through comet assay on individuals with CHD that too being confined to individuals of cyanotic state with tetralogy of Fallot with statistically significant increase in comet tail length in males and females [2]. In the current study, the cases of both cyanotic and acyanotic children of less than 5 years of age were compared with an identical age and sex matched controls. The comet metrics did not show any significant difference between the males and females of the cases investigated. Regarding the extra comet parameters that were studied exhaustively in cases and controls which being TCL, HD, % DH and % DT, the data of TCL, % DH and TL between cases and controls were significantly different indicating enormous degree of DNA damage in children with CHD. The current data agree with the biochemical findings of Ercan et al. [6] with reference to elevated oxidative stress in children with CHD; where the oxidative tools like Malonaldialdehyde (MDA), plasma catalase, Total Antioxidant Status (TAS), Total Oxidant Status (TOS) and Oxidant Stress Index (OSI) were used. Although there is a strong evidence of oxidative stress in the condition investigated, the approach and the methodology used in the current investigation and the one just mentioned were entirely different; one being serology and the other cyto-morphological i.e. plasma chemistry in the former and comet later. The results of the present study corroborates with the findings of Rokicki et al. [8], wherein an imbalance between the oxidant and anti-oxidant in children with CHD had been established.

In the current series, sample selection was exclusively carried with reference to individuals with ***non-syndromic*** congenital heart defects. Oxidative stress has also been estimated in aneuploidy condition such as 21 trisomy-Down syndrome by Sulthana et al.[9,10] due to the presence of selective locus for superoxide dismutase (enzymatic anti-oxidant) gene in the long arm of 21$^{st}$ chromosome to rule out the possibility of the role of ***gene-dosage*** effect by estimating the oxidants like MDA and protein carbonylation [9] and anti-oxidants such as superoxide dismutase, glutathione peroxidase and plasma catalase [10] which were found to be marginally elevated except lastly mentioned anti-oxidant i.e. plasma catalase; where the levels were below to that of the controls. Regarding the results of comet assay in cases of Down syndrome [11], the comet length in cases was 45.38 ±12.5 compared to controls 24.48 ± 3.2. While comparing the total comet length in children of CHD with that of Down syndrome individual, data of the DNA damage seems to be more in non-syndromic children with heart defects.

As mentioned earlier, ROS which is formed as a result of oxidative stress due to hypoxia seems to be playing a major role in individuals with CHD which has the potential to cause DNA damage by attacking either the sugar or the nucleotide moiety of DNA thereby leading to the *formation of damaged DNA* which is estimated / quantified through comet assay. Chronic hypoxia in CHD also leads to induction of hypoxia inducible factor – 1and p53 which in turn activates pro-apoptotic factors leading to alteration in the regulation of pro-apoptotic gene Blc-2 to be involved in causing the DNA damage. It probably indicates that the role of dual mechanisms contributing for the structural defect and alteration leading to fragmentation/ dissociation of DNA as evidenced by the comet.

## Conclusion

Children of CHD of non-syndromic entity irrespective of whether being cyanotic or acyanotic showed significantly elevated levels of DNA damage when compared with

healthy controls as evidenced by the data of comet configurations. There is a positive indication of the phenomena of oxidative stress in the present series playing a major role in the release of ROS and occurrence of ***oxidative DNA damage***. Under these circumstances estimation of oxidant and antioxidant levels is very much warranted in establishing the phenomenon of oxidative stress and the role played by it being the prime for the liberation of damaged DNA in CHD.

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**Correspondence to:**

Suma H.Y.
Department of Anatomy
JIPMER, Puducherry 605006
India