Is the Oxidative DNA Damage Level of Human Lens Epithelial Cells Correlated with the Total Antioxidant Capacity (TAC) in Aqueous Extract of HLECs in Senile Cataract Patients?

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Abstract

Background: Various experimental evidence suggests that in cataract pathogenesis oxidative stress plays an important role in causing DNA damage, but there is still a lack of data on in vivo assessment of DNA damage and the role of antioxidants.

Objectives: To measure the percentage of DNA damage and the total antioxidant capacity (TAC) of patients with cataracts directly in human lens epithelial cells and compare these with a control group followed by whether there is any statistical correlation between oxidative DNA damage and total antioxidant status in senile cataract patients.

Patients and methods: A total number of capsulorhexis from thirty senile cataract cases aged 50-80 years, who were admitted to the ophthalmology ward of AVBRH for cataract surgery, were used for in vitro assessment of DNA damage in human lens epithelial cells by Comet assay and TAC assays. 12 controls were collected from healthy cadavers who or their relatives donated their eyes for TAC assays.

Results: Oxidative DNA damage level of human lens epithelial cells is not correlated with the TAC in aqueous extract of HLECs in senile cataract patients**.**

Conclusion: The conclusion in the present study should not be taken that supplementing antioxidants through diet has no role in the prevention of oxidative DNA damage against oxidants. There may be another mechanism may be responsible for oxidative DNA damage.

Keywords: Cataract; antioxidants; DNA damage.

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Introduction

A cataract is gradual progressive age-related opacification of a human lens that affects nearly 37 million people and is the leading cause of reversible blindness worldwide (**World Health Organization, 2020).** Ultraviolet radiation, light exposure, cigarette smoking, and endogenous oxidants have been proposed as etiologic factors in the pathophysiology of age-related cataracts **(Klaus, 1985).** A higher incidence of cataracts is observed in patients with diabetes mellitus, advancing age is considered a risk factor for cataracts **(Boyle, 2010)** Various mechanisms have been proposed in the development of cataracts such as excessive tissue sorbitol production in type 2 Diabetes, abnormal glycation of lens proteins, and increased free-radical generation **(Andreas Pollreisz and Ursula Schmidt-Erfurth, 2010)**

The formation of multiple reactive oxygen species like superoxide ($O2^{-}$), hydrogen peroxide (H2O2), and hydroxyl radicals (OH) can initiate and propagate free radicals (FR), caused by exposure to light through photosensitizing mechanisms **(Kohen and Nyska, 2002).** Oxidative stress can arise from the overproduction of reactive oxygen species by various metabolic reactions, where oxygen is utilized. So there is an alteration in the balance between oxidant and antioxidant statuses, and the balance shifts towards oxidants **(Birben et al., 2012).** ROS are produced by cellular metabolic activities and environmental factors, such as air pollutants or bidi/cigarette smoke. Reported targets of reactive oxygen species in the lens are proteins, membranes, and deoxyribonucleic acid (DNA) **(Nita & Grzybowski, 2016).** A. Different types of DNA damage may be associated with different types of insults, and some contribution of the reactive oxygen species to DNA damage in human cataractous lens epithelium cells **(Sedgwick et al., 2007)**.

The purpose of this study was to measure the percentage of DNA damage and the total antioxidant capacity of patients with cataracts directly in human lens epithelial cells and compare these with a control group followed by whether there is any statistical correlation between oxidative DNA damage and total antioxidant status in senile cataract patients

Patients and methods

Informed consent was obtained from the subjects after a detailed explanation of the nature and possible consequences of the study. The "Institutional Ethics Committee" sanctioned letter vide letter No. DMIMSU/IEC/2008-09/151 dt 30.05.2008".

Selection of Subjects

A total number of capsulorhexis from Thirty Senile cataract cases aged between 50-80 years who were admitted to the ophthalmology ward of AVBRH for cataract surgery, were used for in vitro assessment of DNA Damage in human lens epithelial cells by Comet assay and TAC assays. 12 lenses were collected from healthy cadavers who or their relatives donated their eyes for TAC assays.

Selection Criteria: The patients who were not having Type II Diabetes mellitus, Hypertensives, trauma, steroid administration, and any other systemic illness, were included in this study. Patients with traumatic or congenital cataracts were excluded. None of the subjects were chronic smokers and were

taking multivitamin supplements such as vitamins A, C & E.

Detailed examination of senile cataract patients: Detailed ocular history was obtained from the participants. Preoperative confirmation of cataracts and their type was recorded and confirmed by Professors of the Ophthalmology Department of Our Institute.

The procedure followed for removing the lens anterior capsule from senile cataract patients. Cataract patients were operated on under local anesthesia using an injection of 2ml 2% lignocaine, through a clear corneal incision (2.75 mm in length was made using a 2.2-mm double-blade corneal knife), 5.5 mm continuous curvilinear capsulorhexis was done by with the help of capsulorhexis forceps and 25-gauge needle.

The anterior capsule was extracted in all cases with Visco expression through a clear corneal incision and the anterior capsule was collected using forceps by an experienced surgeon. No further handling or irrigation was done to avoid any direct harm to the HLEC. Once the removal anterior capsule is removed, the sample was instantly kept in an Essential Medium (containing 10% fetal bovine serum) and immediately transferred to the Research laboratory. A single rhexis for preservation was kept in a Minimal Essential Medium (MEM), containing 10% fetal bovine serum, and incubated in an incubator containing five percent carbon dioxide at 37 °C **(Gajjar et al., 2008).** The maximum time-lapse from the collection of the sample to the process was 15-20 min. HLEC viability testing: Before starting the comet assay, the trypan blue exclusion test was used to check if the human lens epithelial cells were viable or not **(Nanavaty et al.,2006).** All collected samples were viable.

Sample preparation: Mechanical shaking technique was used for the preparation of cell suspension of the human lens epithelial cells using the capsule (in fifty µl of Dulbecco's phosphate-buffered saline with pH 7.2), by hand, for a duration of up to 15 min at 4 °C; human lens epithelial cells were shaded from the lens capsule. To study the degree of DNA damage in human lens epithelial cells of senile cataract patients, cell suspension of the lens epithelial cells was used for Comet assay. For conducting the Comet assay, we followed the steps which were designed by **(Singh et al., 1988),** with some variations:1) Precoating of glass microscopic slides: 75 µl of 1 % of HMPA at 65o C was dropped onto the slide, covered it with 18 mm x 18 mm coverslip and was kept at 4o C for 10 minutes. Then the slide was kept outside to attain room temperature and carefully removed coverslip so that the gel is not damaged. 2) Embedding of human lens epithelial cells: Mix 50 µl of cellular suspension in 50 µl with 2% low melting point agarose (LMPA) at 37oC, then 75 µl of this mixture was dropped onto the slide, covered with a cover slip, and kept at 4oC for 10 minutes. Then, the slide was kept outside to attain room temperature, and carefully removed coverslip. Then, the last layer of 1% LMPA was applied on the slide in the same manner; and 3) Lysis of lens epithelial cells was done by dipping the slide in the ice lysis solution at 4 °C for 8 hours instead of 2 hours. Slides were kept in a tank containing a denaturing buffer (NaOH 0.3 M, EDTA, Na2 1.0 mM) for 30 minutes. The level of the solution is to be kept 3-4 mm above the gel & the process of electrophoresis was

carried out for 30 minutes, by applying an electric current of 25 volts. Slides were washed with a neutralizing solution for 5 minutes and were kept at room temperature for drying. It takes 30 minutes. For visualization, drop 100 microliters of ethidium bromide solution onto the slide and it is covered by a coverslip. Incubated for 30-40 minutes in the dark and then rinsed with water. After drying, it is observed under a fluorescent microscope for one hour. No less than fifty cells from each sample were counted and the amounts of DNA in the main body and tail were measured by fluorescence intensity. The amount of damage is represented by an increased fragment of DNA in the nucleus similar to the tail of a comet. The DNA fragments are generated by the break of DNA double-strand and single strand; the length and fragment content of the tail is directly proportional to the extent of DNA damage measured by fluorescence intensity.

Comet assay photos under a fluorescent microscope for case and control photographs were taken for analysis shown in (**Fig. 1A and 1B**).

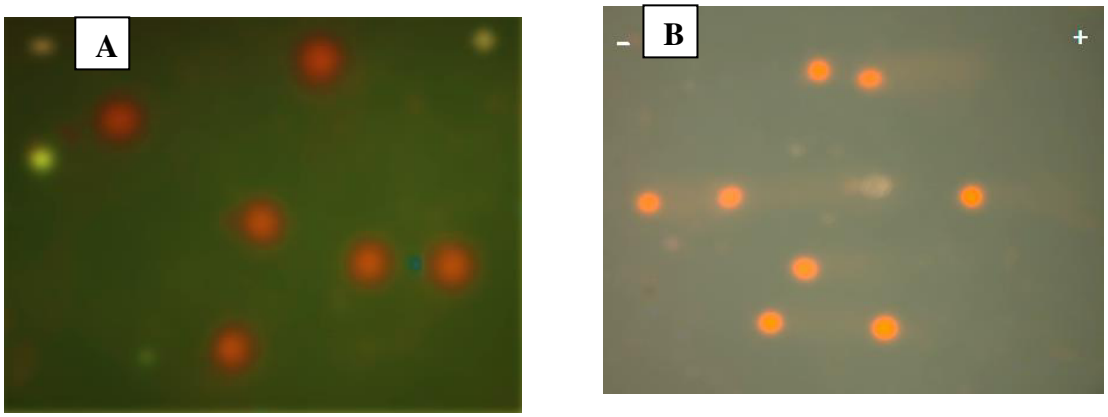

Fig. 1A. Comet assay in HLECs of a Control . Fig. 1B. Comet assay in HLECs of a Cataract patient

The ratio of DNA content in the head & tail (normal DNA versus damaged DNA) was estimated by Comet Score™ software called Comet score.

Estimation of Total Antioxidant Capacity (TAC) in Aqueous Extract of HLECs

Principle: At low pH, the reduction of ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex to ferrous form gives intense blue color which is directly proportional to the total reducing power of electron-donating antioxidant present in the reaction mixture. The intensity of this blue color was measured at λ_{593}.

FRAP: Ferric reducing assay power

Reagents:

I. FRAP Reagents:

a. Acetate buffer: 300 mM, pH 3.6
   - Sodium acetate: 2460 mg
   - Glacial acetic acid: 1.7 ml

   (in 100 ml water)

b. TPTZ: 10mM in 40 mM HCl
   - TPTZ: 3.2mg
   - HCl: 35 µL

   (in 100 ml water)

c. $FeCl_3$, 20 mM: 34.4 mg in 10 ml water.

Mix the above three reagents in a ratio of 10:1:1 to make the FRAP reagent.

II. Standard

Solution of Trolox 1000 µM: Dissolve 5mg of Trolox in 50 µL of NaOH of 5M solution and make a volume of up to 10 ml with water.

Protocol:

FRAP reagent: 1000µL.

Standard/Sample: 75µL.

Mix, wait for 5 mins at 37ºC & take A593

Calculation:

TAC (µmol of Trolox/ml) of sample = A_{593} of sample x Factor

Standardization of Total Antioxidant Capacity (TAC) assay

Fig.2 depicts the standard graph of TAC using standards of 2, 1, 0.5, 0.25, 12.5, and 6.25µmol/ml of Trolox, the straight graph shows the linearity of the total antioxidant capacity of Trolox.

$$\textbf{Factor: } \frac{\textbf{conc of trolox}(1.0\mu\textbf{mol/ml})}{\textbf{A593}(0.9747)} = 1.0257$$

Statistical analysis

GraphPad Prism (United States) was used to analyze the statistical data. To compare the means of the two groups, an unpaired sample t-test was used. For two groups simple linear regression A *p*-value of 0.05 or less was considered statistically significant.

Results

A total of 30 rhexis samples were collected from senile cataract patients, having mean ages of 68.6 ± 8.24 (Mean ±SD) years for males (n=15) and 71.6 ± 10.58 (Mean ± SD) years for females (n=15). 12 samples from cadavers having mean ages of 45.87 ± 4.7 (Mean ±SD) years for males (n=8) and 39.75± 4.02 (Mean ± SD) years for females (n=4) shown in **(Table1)**

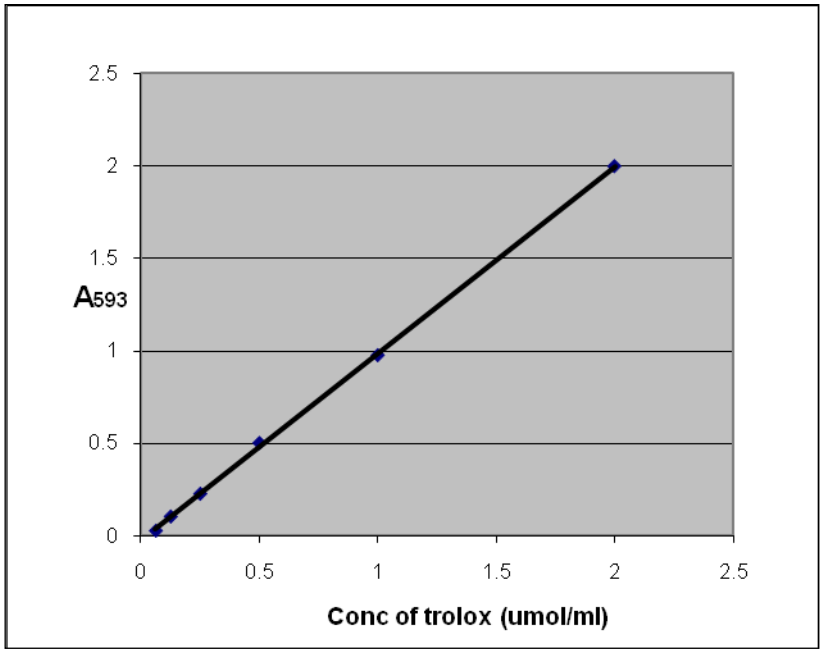

Fig.2 .Standard graph of Total Antioxidant Capacity (TAC)

Table 1. Demographic data

Senile Cataract Cases(n=30)		**Control (Healthy Cadaver) (n=12)**	
Males (n-15)	68.6 ± 8.24 (Mean ±SD) years	**Males(n=8)**	45.87 ± 4.7 (Mean ±SD) years
Females (n=15)	71.6 ± 10.58 (Mean ± SD) years	**Females(n=4)**	39.75± 4.02 (Mean ± SD) years

The percentage of DNA damage in the tail of the comets was: 17.77 ±6.14 for the control subjects **(Table 3)** and 46.12 ±13.85 for the cataract patients **(Table 2).** The difference was significant ($p<0.001$).

Table 2 . Results of Comet assay of HLEC in Senile Cataract

S.N.	Senile Cataract	
	Head %DNA	Tail %DNA
1	70.63	29.37
2	79.22	20.78
3	64.45	35.55
4	68.53	31.47
5	64.97	35.03
6	56.57	43.43
7	62.40	37.60
8	18.79	81.21
9	52.88	47.12
10	17.53	22.47
11	75.91	24.09
12	37.56	62.44
13	36.55	63.45
14	74.14	25.86
15	68.45	31.55
16	35.05	64.95
17	32.90	67.10
18	45.01	54.99
19	32.33	67.67
20	81.66	18.34
21	39.44	60.56
22	54.90	45.10
23	68.20	31.80
24	11.20	88.80
25	68.88	31.12
26	68.90	31.10
27	54.20	45.80
28	15.04	84.06
29	61.34	38.66
30	37.96	62.04
Mean + Standard Deviation		46.12 ±13.85

Table 3. Results of Comet assay of HLEC in Healthy Control

	Control	
S.N.	Head %DNA	Tail % DNA
1	88.26	11.74
2	75.56	24.44
3	85.04	14.36
4	86.04	13.34
5	75.75	24.25
6	75.19	24.81
7	81.78	18.22
8	80.2	19.8
9	88.26	11.74
10	75.56	24.44
11	85.04	14.36
12	88.26	11.74
Mean + Standard Deviation		17.77 ±6.14

DNA damage to the Tail of %DNA in Cataract and control individuals is shown in **Fig.3.** The value of total antioxidant capacity in control TAC (µmol of Trolox/ml) was: 1.846 ± 0.396 for the control subjects **(Table 5)** and 1.098 ± 0.524 for the cataract patients **(Table 4).** The difference was significant ($p<0.001$).

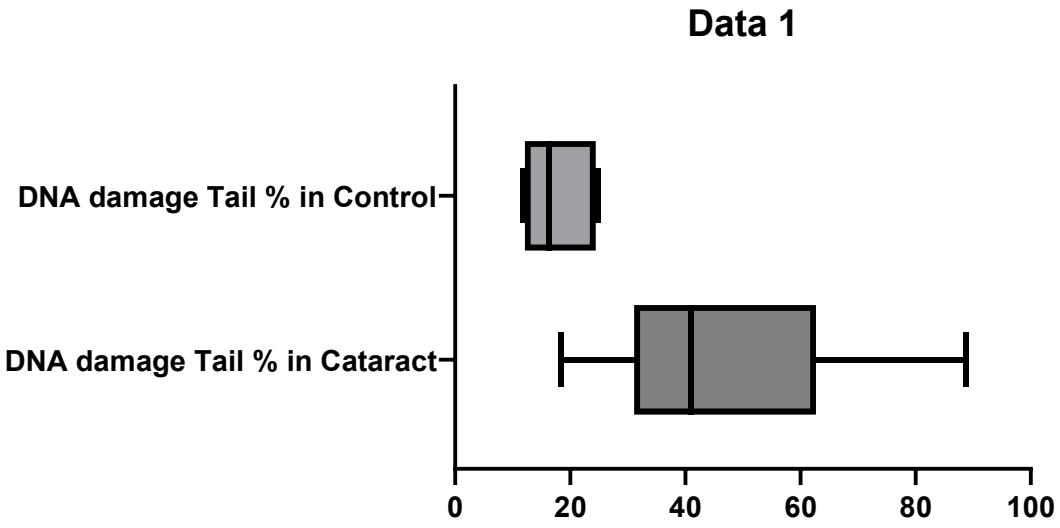

Fig.3. Percentage of DNA damage in cases and controls. The Maximum and minimum damage to the Tail of %DNA in Cataract and control individuals is shown**.**

Table 4.Results of Total Antioxidant Capacity in Cataract Patients

S.N.	Total Antioxidant Capacity in Cataract Patients TAC (µmol of Trolox/ml)
1	0.737
2	0.479
3	1.301
4	2.052
5	1.538
6	1.395

7	0.741
8	1.092
9	0.699
10	0.476
11	0.804
12	1.472
13	1.261
14	1.93
15	1.756
16	0.855
17	0.995
18	0.825
19	1.284
20	0.672
21	1.123
22	0.596
23	0.357
24	0.395
25	1.33
26	1.106
27	1.151
28	2.118
29	1.355
30	1.059
Mean	1.098
Standard Deviation	0.524

Table 5. Results of Total Antioxidant Capacity in Control Patients

S.N.	Total Antioxidant Capacity in Control Patients TAC (µmol of Trolox/ml)
1	2.418
2	1.819
3	1.251
4	1.521
5	2.553
6	1.736
7	1.831
8	2.31
9	1.48
10	1.837
11	1.57
12	1.82
Mean	1.846
Standard Deviation	0.396

There is no significant correlation between oxidative DNA damage and total antioxidant capacity in senile cataract patients Shown in **Figure 4.**

There is no significant correlation between oxidative DNA damage and total antioxidant capacity in controls shown in **Figure 5.**

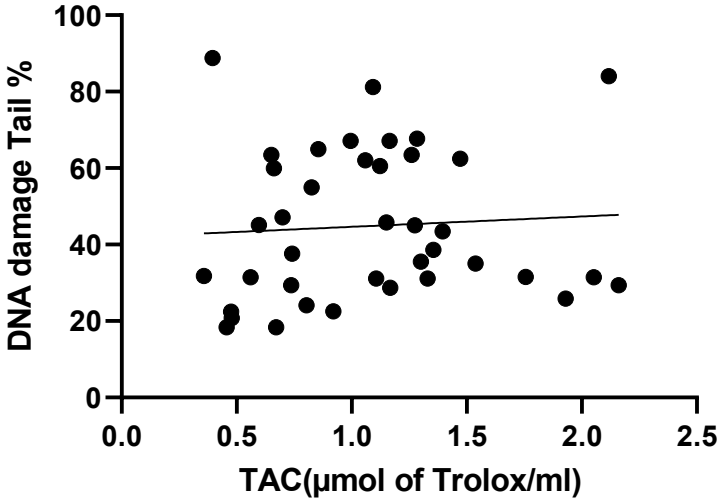

Fig 4. Correlation between Oxidative DNA damage and total antioxidant capacity in Senile Cataract Patients

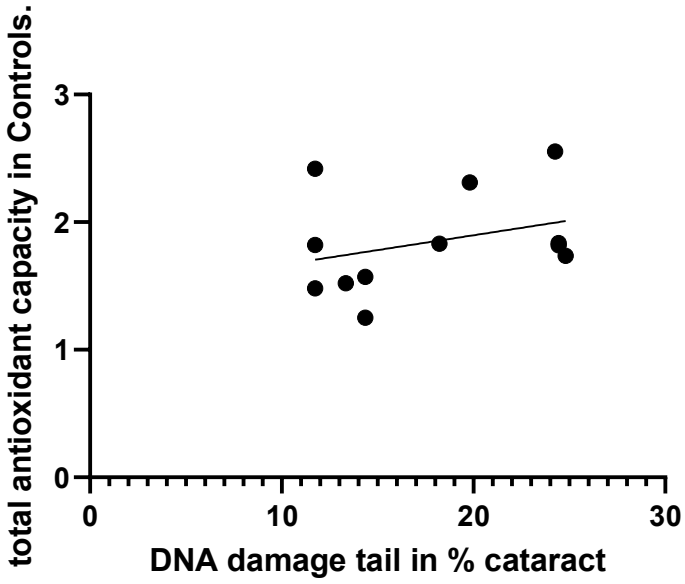

Fig.5. Correlation between Oxidative DNA damage and total antioxidant capacity in Controls

Discussion

Total antioxidant capacity (TAC) in senile cataract patients has been studied in serum **(Gul et al., 2009)** and it was significantly reduced as compared to the control. Most of the experimenters had studied the anti-oxidative enzymes e.g. Superoxide Dismutase, Catalase, Glutathione peroxidase, reductase, etc discretely or in different combinations and non-enzymatic anti-oxidants e.g. glutathione reductase, Vitamin C, Retinoic acid and α-tocopherol, etc separately or in different combinations in senile cataract patients plasma **(Zoric,2003; Krepler and Schmid,2005)** in animal LECs **(Shang et al., 2003)** or few instances, in HLECs (**Xing and Lou, 2002**). This is the first study where TAC

had been assayed directly in HLECs. In our study, we found decreased TAC levels in senile cataract patients and this decrease was highly significant as compared to the control.

During oxidative stress, consumption of non-enzymatic antioxidants to scrap the damaging properties of ROS. So, TAC level declines significantly in HLECs on oxidative stress.

Oxidative stress produces ROS which leads to DNA damage. DNA damage was found by comet assay encouraged by microwave radiation **(Yao et al., 2008)** and soft x-ray radiation **(Norman et al., 2008)** in mice LECs. In our study, DNA damage in cataract patients was found to be highly significant.

The main aim of our study was whether there is any statistical correlation between oxidative DNA damage and total antioxidant status in senile cataract patients.

Oxidative DNA damage level of human lens epithelial cells is not correlated with the TAC in aqueous extract of HLECs in senile cataract patients**.** We first examined individual oxidative DNA damage levels in human lens epithelial cells by comet assay and compared them with AC in an aqueous extract of HLECs.
The antioxidant capacity in our study is the total antioxidant capacity (expressed in TAC) contributed by aqueous humor extract. The aqueous humor contains enzymatic and non-enzymatic antioxidant defenses. Antioxidants, such as reduced glutathione, Vitamin C, and amino acids like cysteine, and tyrosine, have been recognized in aqueous humor **(Richer et al., 1998)**. High concentration of ascorbic acids by their scavenging action against superoxide anion, hydroxyl radical, and singlet oxygen **(Garland 1991; Rose and Bode 1991; Tso 1987). (Duthie et al., 1996)** found that there is an insignificant correlation observed between oxidative DNA damage and plasma concentrations of various antioxidants. This finding is in agreement—with our conclusion: no correlation between oxidative DNA damage of human lens epithelial cells and TAC levels in aqueous extract of HLECs in senile cataracts. In other studies, **(Collins et al., 1998)** have found that oxidative DNA damage in lymphocytes is not prevented after 12 weeks of supplementation of carotenoids.

Conclusion

The findings of the current study should not be interpreted to mean that adding antioxidants to one's diet has no part in preventing oxidative DNA damage. If antioxidants are unable to stop oxidative DNA damage, there may be another mechanism at play.

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