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Detection the Effect of Smoking and Age on Total Anti- Oxidant Capacity Level and DNA Damage of Individuals Exposure to Low Dose Ionizing Radiation

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Abstract: The aim of this study to detect the total anti- oxidant capacity (TAC) level and DNA damage by use alkaline comet assay in different measurements as biomarker of double and single strand breaks that caused by smoking and relation with age of individuals exposed to ionizing radiation, this study including 100 male blood samples (30-65) years, TAC level were measured as biomarker for detecting the effect of smoking and age of exposed individuals, in this research recorded non- significant increase in total TAC level in blood serum for the non-smoker individuals as compared with smoker, in other hand also we recorded non -significant result of TAC in three age groups (Less than 40, 40-50 and more than 50) years, but the single cell gel electrophoresis (comet assay) appear highly significant increase ** ($P<0.01$) with Mean ±SE (18.63± 0.72) and LSD –value (3.386 **) of DNA damage(high damage measurement HD) in smoker individuals when compared with nonsmoking, the present study shows significant difference in comet high damage cells (* $P<0.05$) for some age groups that be less than 40 and more than 50years is 12.67 and 17.37 respectively, the study results show that the difference increased of comet assay measurements may be caused by smoking , aging and also effect of works environment. The level of TAC was non more difference in age groups and in smoker individuals or non- smokers . In conclusion, found that an negative correlation between TAC level and oxidised bases that measured by comet assay (single and double strands breaks), and age. This study , indicated that can use the different measurement of comet assay as biomarkers to detect influence of age and smoking in peripheral blood lymphocytes DNA. Also, the results confirmed usefuless of the TAC as biomarker of smoking and aging effect in exposed individuals.

Keywords: DNA damage , TAC, comet assay , lymphocyte , smoking effect, free radical.

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Introduction:

The level of DNA damage was increased in human individuals exposed to pollution factors such as radiation , pesticides and other reagent , some of studies of pollution was focus on polycyclic aromatic hydrocarbons (PAH), X-ray, heavy metals and addicted smoking (1,2). Even before the extensive studies of DNA structure, it was become apparent that chemical, physical and biological agents could interact with the genetic material and lead to mutations (3,4), which are associated to genomic instability and cancer (5). Cancer is one of the important health problems of the current era and also a leading cause of death among populations. Cancer can simply be defined as a malignant tumor ormalignant neoplasm. It is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Direct growth in the neighboring tissue occurs through invasion. Implantation at distant sites follows metastasis. Cancer may affect people at all ages, but the risk tends to increase with age (6,7). Smoking is a

leads to and cause the changes in the genetic materials of the human cells, then leads to many of diseases as lung diseases heart failure and may be causes stroke, and cancer (particularly lung cancer) (8, 9). Other factors such as age, drug can increased DNA damaging. The agencies in some of countries require genotoxicity assays as part of drug effectiveness, the European Medicines Agency (EMA) is one from the regulatory agencies. (10,11). There are many of tests was used involved in *vivo* and in *vitro* assays to show the drug possibility To causes genetic mutation or any change in chromosomal structure (12,13). suggests two options of battery tests such as the comet assay (14) The comet assay is a precious test for quantify of damaging DNA in individuals exposed to different types and doses of genotoxic agents as chemical and physical reagent, it can employ to study the 'biological effect dosing' of occupational and environmental exposures. The plurality of the environmental exposure research in which the comet assay is used as test with inhalation toxicity and air pollution exposure. The test show to be useful, along with other biomarkers of oxidative stress, to study the oxidative possibility of particulate matter (PM) exposure reviewed in (15), and the other option was used total anti-oxidant TAO or spectrophotometric assays for total antioxidant capacity (TAC) in human serum as biomarker for oxidative stress (16), A free radical is atom or any molecular capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain, common properties that are shared by most radicals. Most of radicals are unstable and reactive. They can either, donate an electron to or accept an electron from other molecules, therefore behaving, as oxidants (16). The most important oxygen free radicals in many disease states are hydroxyl radical, superoxide, anion radical, hydrogen, peroxide, oxygen singlit and peroxy nitrite radical. These are highly reactive species capable in the nucleus, and in the membrines of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids (17). Free radicals attack important, macromolecules leading to cell damage. and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. To protect the cells and organ systems of the body against, reactive oxygen species (ROS), humans have evolved a highly sophisticated, and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals(18) these components include endogenous antioxidants such as NADPH and NADH, Thiols, glutathione, lipoic acid, N and Ubiquinone. (coenzyme Q10). The Enzymes are antioxidant involved, selenium-dependent. glutathione peroxidase and iron-dependent catalase vitamin C, and vitamin E ,are represent dietary antioxidants(17)

Dusinska *et al*. (19), studying some of workers (exposed LDIR), found an an negative correlation between activity of enzyme glutathione S transferase (GST) and oxidized DNA measured as EndoIII- and FPG, and also relation with base exession reaper capacity. Staruchova *et al*. (20) found same result as inverse correlations between repairing DNA and both (GPx) and catalase. The correlation between

signaling of damaging DNA pathways, biotransformation of enzymes, and the DNA regulation repair is a topic attracting increase the interest (19). It is generally presumed that damaging of DNA increased with age, therefore the result of comet assay should appear higher levels of single strand breaks and/or oxidized of bases in Peripheral blood mononuclear cells (PBMC)that isolated from older age-groups. In fact, the results are not harmonious , and not all result from studies giving an age-related increasing damaged of DNA(20,21,22).Furthermore, currently studies pointed out that the association of TAC and comet assay is the best battery experiment to estimate the potential mutagenic, since comet assays are very sensitive, not complex and we can to detect breaks at single and double strand break levels, TAC not more effected by smoking or aging but highly effected by environmental stress and may be fund some of relation between TAC and other measurements showing in discussion. The aim of study focused on a possibility of using the change in comet damage and TAC in lymphocyte of smoker individual exposed (LDIR).

Materials and methods:

a-Blood sample collection:

Blood samples occupationally were obtained from 100 male individuals from individuals exposed to low dose of IR (mineral plant and welding test workers), Distribution according to age in to three groups, Less than 40 was 39 ranged from 30 to 39 represent by 37.50%, This was followed by 44 (42.31%) individuals in the age group of 40-50 years, More than 50 were 21 (20.19 %) individuals in age group of ranged from 50- 65 years (table 1). Blood samples were drawn in two tubes the firs tube containing EDTA as anticoagulant for the comet assay to determined DNA breaks according to ref (23) and other tub content gel to isolation serum for TAC test. Individuals study included 64 samples of smoker affected by environmental factors. Their ages ranged from 37-65 and 36 non-smokers also effected by environmental factors from the same place.

Table (1): Distribution of sample study according to age groups.

Age group (years)	No	%
⩽40	39	37.50
40-50	40	42.31
⩾50	21	20.19
P-value	----	0.0083 **
**** (P<0.01).**		

b- Lymphocyte separation:

Lymphocytes were isolation from peripheral blood samples using the process described by Boyum,(1968) (24) and DNA breaks were measured by using comet assay technique under alkaline conditions , three mL of blood centrifuged at 1000 rpm for 15 min. collected the buffy coat and diluted with 5mL PBS or RPMI 1640 (cell suspension) in a 10 mL centrifuge tubes. Five mL of the diluted cell suspension was layered on 3 mL of

ficoll-isopaque separation fluid (lymphoprep; specific gravity = 1.077). Centrifuged the tubes at 2000 rpm for 30 min at 4ºC. After centrifugation visible cloudy band between the PBS and lymphoprep layers, this mononuclear cells. The band between the PBS and lymphoprep layers was collected in a 10 mL test tube and the cells were suspended in 5 mL RPMI 1640, the tube centrifuged at 2000 rpm for 5 min (first wash), discarded the supernatant and the cells were resuspended in 5 mL RPMI 1640 (This step was repeated twice). The suspension was centrifuged at 1000 rpm for 10 min, the supernatant was discarded,the precipitated cells were resuspended in 1mL RPMI solution and transferred to cryovials that were then submerged into a pre cooled 4ºC then immediately placed in a -80 ºC freezer to used in the planned experiments.

c- Determination breaks of DNA strand by the(SCGE) comet assay:

The comet assay relatively simple method for measuring DNA damage at the level of individual cells. Originally devised in the 1980s to measure DNA strand breaks (SB) (25-26), it was soon modified to detect a variety of other lesions, such as oxidised bases(27-28). Because of its sensitivity and speed, it has been widely adopted as a biomarker assay in human studies, for example monitoring occupational exposure to mutagens, testing dietary supplementation with antioxidants, or checking levels of oxidative stress in relation to diverse diseases (21). Comet assay in order to determine DNA damage. Oxiselect comet assay kit was used to perform the test (29-30) detect double-stranded DNA breaks by neutral comet assay, whereas in this study detect single and double-stranded DNA breaks by Alkaline Comet Assay. Briefly prepared lyses solution and cooled at 4°C for at least 20 minutes before use. LMA garose were melted placed in a beaker in water at 100ºC for 5 minutes, with the cap loosened. The bottle was placed in 37°C water bath for 20 minutes at least for cooling, then100 µl of lymphocytes cells were combined at 1 x 10^5/mL with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipetted 50 µl onto comet slide, slides flat should be placed in the dark at 4°C (*e.g.* place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of comet slide area. improves adherence of samples in high humidity environments was increasing gelling time to 30 minutes. then transferred the slides to tank of electrophoresis with alkaline electrophoresis buffer (pH13)for 20 minutes at 25ºC (room temperature) or 1 hour at 4°C, in the dark, slides were labeled adjacent to black cathode and the comet assay electrophoresis(ES-unit), ~850 mL 4°C alkaline electrophoresis solution was added, then placed slides in electrophoresis slide tray and covered with slide tray overlay. Setted power supply to 21volts and voltage was applied for 30 minutes. slides were dried and stained with 50 µl SYBR ®Green solution(1:5000) then selected randomly scored was calculated from the ratio of (L/W) comet to determine the Comet Index (CI). Scored range from 1.2 to2 considered low DNA damage (LD), from 2.1 to 3 medium DNA damage (MD), and up to 3 high DNA damage (HD) (31) , as well as no damage (ND) the analysis software was calculate many parameters for each comet, three parameters were estimated to indicate DNA migration, mean tail moment

(product of tail DNA/total DNA by the tail center of gravity) tail length (distance from the head center to the end of the tail), and tail DNA%=100X tail DNA intensity/cell DNA intensity, but the results of automated method neglected because of interferences of some cells.

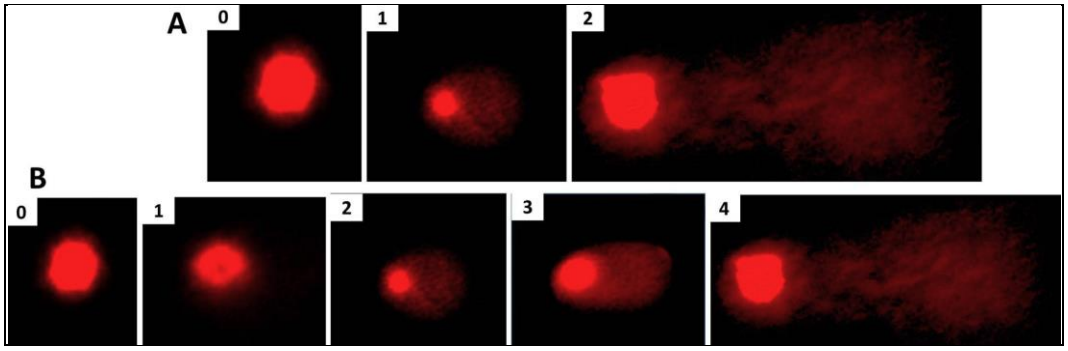

Figure (1): Classification of nucleoids according two systems: (A) 0–2, in which 0 represent cells without DNA damage and 2, cells with maximum DNA damage and (B) 0–4, in which 0 represent cells without DNA damage and 4, cells with maximum DNA damage.(32)

Material analysis:

The damaging cells can be analyzed by methods of automated or visual counts and then were classification (33). The methods of automated used to identify the released fluorescence density and geometrical appearance of the nucleoids, such as comet area, tail length and head diameter (34). The visual methods consist in analysing 100 nucleoids in each slide, which are classification depending on two systems: 0–2 (35,36) or 0–4 (37) (Fig.1), where nucleoids with no damaging DNA are classified in 0 on other hand those with higher damage in 2 or 4, according to the system were used. Count of the number of comets observed in each of class in a total of nucleoids, the outcome is acquired depending on formula (38,39). The visual process is preference eligible than the automates process because overlap of nucleoids can be seen and classification as a unique comet (38). On other hand the automatized methods is the scoring of comets hedgehog. Cells that show elevate DNA damage, then computer programs was analyze the head as separate to the tail comet, classified the comets as a zero class (38). Based on the data by the automates method , the visual process is preferably in our work and also used by the most of laboratories (31,40,41). The assay measures of total anti oxidant capacity in which Fe^{3+} -TPTZ is reduced by anti- oxidant to Fe^{2+} -TPTZ the enzyme catalysed reaction products Fe^{2+} -TPTZ can be measured at colorimetric readout at 593 nm.Total antioxidant capacity microplate assay kit was used to measured TAC ,two ml from blood was collected in gel tube to detection and quantification of total antioxidant capacity (TAC).which are produced in oxidation reaction in the cell and then measured for standard , 1ml distilled water was added to dissolve standerd before used the concetration is 100 mmol/L and stored at 4 ºC, centrifuged the samlpes in gel tube at 10,000 rpm at 10 minute then take the serum , 15 µl from serum was placed into the microplate then added 150 µl from substrate diluent into microplate (sample, standard and

blank), placed 15µl substrat in to micrioplate with mixed well , For each sample ,standard and blank were added 15µl from dye reagent then distrubuted 10 µl of sample to microplate except blank, after that adetion 10µl from each standard and distilled water to microplate were mixed for 5 minutes, then measured absorbance on the reader of plate at 593 nm and record the absorbance, and finally calculated according to equation:

$$TAC = 20 * (OD_{sample} - OD_{Blank}) / (OD_{Standard} - OD_{Blank})$$

1 mmol/l of Fe^{+2} per minute according to the volume of serum were calculated the total antioxidant capacity (TAC).

Statistical Analysis:

The statistical analysis system-SAS (2012) program was used to effect of difference factors in study parameters. least significant difference – LSD test (ANOVA) was used to significant compare between means. Estimate of correlation coefficient between variables in this study(42).

Result and Discussion:

Single cell gel (SCG) can used for detection of types DNA alternative, such as double and single strand breaks, alkali-labile sites, imperfect repair sites, cross links and repair in individual cells (43). In this study we used the visual process because of is preferably than the automates method. This assay was used by a researcher and investigators to trace the defects of DNA also used to determine the quantity of DNA by measuring the exchanges between the DNA of the nucleus and the consequent of tail as shown in Fig.2. A sample of 100 individuals (40 non-smoker and 60 smokers) distribution in to three age groups , were used to comet study.

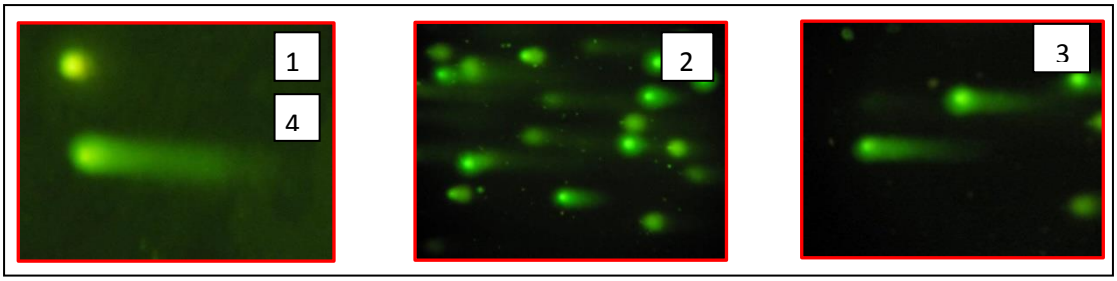

Figure (2): Three examples for comet assay (1: Normal, 2: low DNA damage (LD); 3: Medium DNA damage (MD); 4: High DNA damage (HD)(400X).

The results in Table (2) and figures(3) indicates that the comet high damage HD and MD comet medium damage was significantly higher in the smoker individuals group with a mean values (18.63 ± 0.72) (17.68 ± 0.62) as compared with nonsmoker workers (12.46 ± 0.98) (13.01 ± 0.91) because of the Smoking is a major cause the changes in the genomic of the human single or double strand breaks , which leads to infection with many diseases such as respiratory diseases and cancer (particularly lung cancer) (44,45) comet figures of nonsmoker workers as shown in(Fig.4) . Damaged of DNA increased in smokers than non -smokers as result of oxidative stress and produced free

radicals or reactive oxygen species (ROS) that lead to base damage or breaks of strand , in our result found no significantly difference in comet low damage LD in smoker (14.67 ± 0.84) when compared with nonsmoker (14.28 ± 0.71) the LSD – value was (2.645) and P-value (0.769), the results of the changes in comet no damage ND was higher in nonsmoker than smokers as normal results (60.12 ± 2.42) (48.98 ± 1.06) with high significant result ** (P<0.01) as shown in table 2. When calculate the DFI of non-smokers we deduction the HD in some of cells are caused by the factors especially when exposed to low dose of IR when compared with result of control was the high damage (4.16 ± 0.55) (21).

Table (2): Effect of smoking in ND, LD, MD and HD.

Smoking	Mean ± SE			
	ND	LD	MD	HD
Yes	48.98 ± 1.06 b	14.67 ± 0.84 a	17.68 ± 0.62 a	18.63 ± 0.72 a
No	60.12 ± 2.42 a	14.28 ± 0.71 a	13.01 ± 0.91 b	12.46 ± 0.98 b
LSD-value	8.244 **	2.645 NS	3.126 **	3.386 **
P-value	0.0089	0.769	0.004	0.0005

**** (P<0.01) , NS: Non-Significant.ND:no damage ,LD: low damage ,MD ,medium damage, HD :high damage .**
Means having with the different letters in same column differed significantly

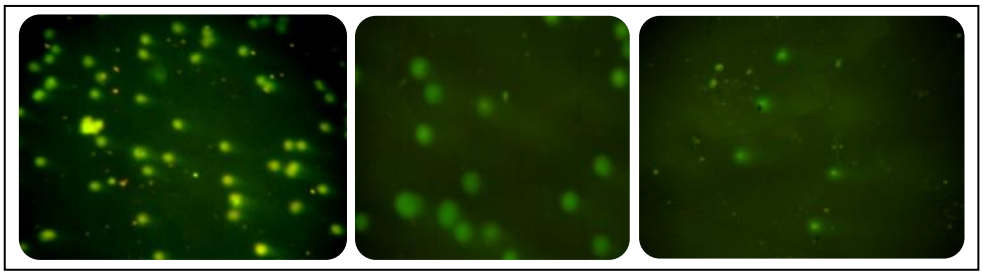

Figure (3): Three examples for comet assay different smoker individuals shown different level of DNA damage.

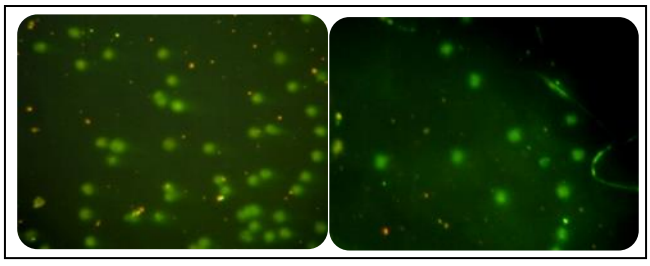

Figure (4): Two examples for comet assay different nonsmoker individuals shown different level of DNA damage.

The age is one of the intrinsic factors' in this study we reported significant result of HD in (50-65 years) and (40-50 years old) groups when compared with age group less than 40 the result of HD and MD were (17.37 ± 1.48) (16.89 ± 1.31) (14.16 ± 1.32) (14.09 ± 1.22) and (12.67 ± 1.28) (13.29 ± 1.14) respectively, as shown in table (3) and figure (5). It is generally assumed that DNA damage accumulates with age, and that therefore the comet assay should show higher levels of single break strands and oxidised DNA

from older age-groups (20, 21, 22). The high comet in first age group may be causes by the environmental and occupational exposure in work place such as inhalation of chemicals reagent or physical factors.

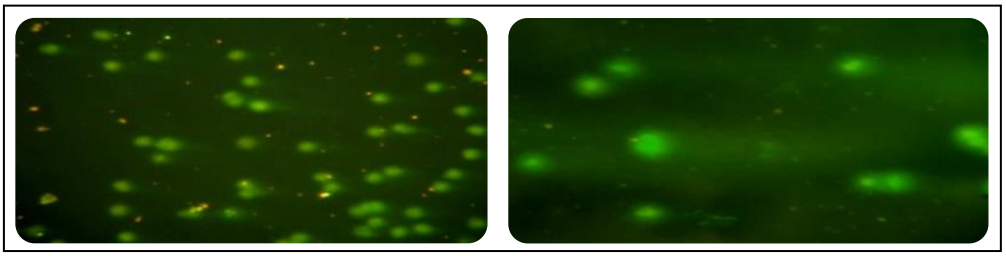

Figure (5): Examples for comet assay in different age groups of individuals shown different level of DNA damage

The account of comet no damage (ND) and comet low damage (LD) in three age groups were recorded non significant result when compared between them (Table 3), but usually the number of ND and LD more than HD and MD, if the reverse happened the exposure workers were abnormality health because of enormous amount from the cells were damaged. This situation occurs when exposed to highest dose of ionizing radiation.

Table (3): Effect of age groups in ND :no damage ,LD: low damage ,MD ,medium damage, HD: high damage.

Age Group (year)	Mean ± SE			
	ND	LD	MD	HD
Less than 40	59.66 ± 3.12 a	13.97 ± 0.97 a	13.29 ± 1.14 a	12.67 ± 1.28 b
40-50	57.24 ± 3.05 a	14.65 ± 0.87 a	14.09 ± 1.22 a	14.16 ± 1.32 ab
More than 50	51.04 ± 3.20 a	14.81 ± 1.13 a	16.89 ± 1.31 a	17.37 ± 1.48 a
LSD-value	9.626 NS	3.088 NS	3.651 NS	3.949 *
P-value	0.253	0.820	0.186	0.0414
* (P<0.05) , NS: Non-Significant. Means having with the different letters in same column differed significantly				

Thus the DNA defects visualize is less than the fact DNA damage indicating an sacrificial estimated for level of DNA defects. However, since significant effect of DNA damage is only observed when DNA fragmentation index (DFI) is greater than 27 to 30 per cent, this technique proves to be ideal in diagnostic workup of idiopat patients and couples with bad obstetric history (46).

Measuring antioxidant:

In our study was measured the level of total antioxidants capacity(TAC) in serum that isolated from individuals blood, we found non significantly result of TAC in smoker individuals and non- smoker (0.685 ± 0.03, 0.905 ± 0.11) respectively as shown in Table 4. the different in concentration it was found but non- significant, this result was highly significant decreased when compared with result of TAC in control group 1.571 ± 0.18, non- smoker no hard work and no high free radicals concentration. This a condition leads to fact that the environmental factors such as inhalation of chemical fumes, exposure to occupationally ionizing radiation it were hard effective on

DNA structure more than effect of smoking, this due to consumption of large quantity from total antioxidant against the free radicals(18).

Table (4): Effect of smoking in TAC.

Smoking	Mean ± SE
	TAC
Yes	0.685 ± 0.03 a
No	0.905 ± 0.11 a
LSD-value	0.321 NS
P-value	0.173
NS: Non-Significant. Means having with the different letters in same column differed significantly	

On the other hand, we recorded the non-significant effect of age in three groups as in Table 5. The level of TAC in 30- 39 years and 40-50 years groups, were somewhat similar with little difference inverse the third group 50-65 years it was appear decreased in TAC when compared with other age groups as shown in Table 5, this result lower than normally level of TAC in younger living in environment free from contaminants ,the aging, metabolism, stress and also carbon tetrachloride, were increased the level of free radicals(47) then lead to decreased the concentration of TAC.

Table (5): Effect of age groups in TAC

Age Group (year)	Mean ± SE
	TAC
⩽40	0.891 ± 0.12 a
40-50	0.861 ± 0.13 a
⩾50	0.612 ± 0.05 a
LSD-value	0.403 NS
P-value	0.389
NS: Non-Significant. Means having with the different letters in same column differed significantly	

Also we found an negative correlation between TAC level (enzymes catalase, vitamin C and carotenoids) and oxidised bases DNA measured by comet assay (single and double strands break) in different measurement (48, 49), also inverse correlation of result between TAC and different age groups may be because of the increasing the damaging and reducing of metabolic activity with aging the result shun in Table 6.

Table (6): Correlation coefficients between parameters study**.**

	Correlation coefficients-r	Level of sig.
TAC & CDN	0.84	**
TAC& CLD	-0.64	**
TAC & CMD	-0.79	**
TAC & CHD	-0.79	**
AGE & TAC	-0.17	NS
** (P<0.01), NS: Non-Significant.		

Conclusion:

The result that there is possibility of using the change in the comet assay as biodose metric of damage in human lymphocyte for the detection in smoker individuals exposed to IR.

Reference:

1. M. Valverde, E. and Rojas, (2009). Environmental and occupational biomonitoring using the Comet assay. *Mutat. Res*., 681: 93–109.
2. Piperakis, S.M.; Petrakou, E. and Tsilimigaki, S. (2000). Effects of air pollution and smoking on DNA damage of human lymphocytes. *Environ. Mol. Mutagen*, 36: 243– 249.
3. Malling, H. (2004). History of the science of mutagenesis from a personal perspective, Environ. *Mol. Mutagen*. 44: 372–386.
4. Muller, H. (1928). The production of mutations by X-rays. *Proc. Natl. Acad. Sci. U. S. A*. 68 : 59.
5. Miyamae, Y.; Yamamoto, M.; Sasaki, Y.; Kobayashi, H.; Igarashi-Soga, M.; Shimoi, K., *et al*. (1998). Evaluation of a tissue homogenization technique that isolates nuclei for the in vivo single cell gel electrophoresis (comet) assay: a collaborative study by five laboratories. *Mutat. Res*., 418 : 131–140.
6. Blachford S.L. (2002). The Gale Encyclopedia of Genetic Disorders. Detroit Gale Group. *Thomson learning. U.S.A*., 1: 1345.
7. AL-Faisal A.M.; Gaaib, J.N.; Al-Alwan, N. and Ghanim, M. (2014). Evaluation the Diagnostic and prognostic value of cytokeratin-19 (CK19) gene Cytokeratin Expression in Iraqi Breast Cancer Patients. *International J. of Current Research*, 6: 6346-6351.
8. Shen, H.; Spitz, M.; Qiao, Y.; Guo, Z.; Wang, L.; Bosken, C., *et al*. (2003). Smoking, DNA repair capacity and risk of nonsmall cell lung cancer. *Int. J. Cancer*, 107: 84-88.
9. Lee, K.W and Pausova, Z. (2013). Cigarette smoking and DNA methylation. *Front Genet*., 4: 132.
10. Hartmann, A. (2004). Use of the alkaline in vivo Comet assay for mechanistic genotoxicity investigations. *Mutagenesis*, 19 51–59.
11. Snyder, R. and Green, J. (2001). A review of the genotoxicity of marketed pharmaceuticals. *Mutat. Res*., 488: 151-116.
12. ANVISA, (2013). Guia para a conduc¸a˜o de estudos na˜o cl´ınicos de toxicologia e seguranc¸a farmacolo´ gica necessa´ rios ao desenvolvimento de medicamentos.
13. FDA, (2008). Guidance for industry impurities in drug substances and products: recom-mended approaches.
14. OECD, (2014). Test No. 489: *In vivo* mammalian alkaline comet assay.
15. Moller, P. and Loft, S. (2010). Oxidative damage to DNA and lipids as biomarkers of exposure to air pollution, Environ. *Health Perspect*., 118: 1126–1136.
16. Cheeseman, K. H. and Slater, T.F. (1993). An introduction to free radicals chemistry. Br Med Bull, 49:481–93.
17. Young, I.S. and Woodside, J.V. (2001). Antioxidants in health and disease. *J Clin Pathol*., 54:176–86.
18. Mark Percival (1998). Antioxidants. *Clinical Nutrition Insights*, 31: 01-04.
19. Dusinska, M.; Staruchova, M.; Horska, A.; Smolkova, B.; Collins, A.; Bonassi, S., *et al.* (2012). Are glutathione S transferases involved in DNA damage signalling? Interactions with DNA damage and repair revealed from molecular epidemiolo-gy studies. *Mutat. Res*. 736 :130–137.
20. Staruchova, M.; Collins, A.R.; Volkovova, K.; Mislanova, C.; Kovacikova, Z.; Tulinska, J., *et al*, (2008). Occupational exposure to mineral fibres. Biomarkers of oxidative damage and antioxidant defence and associations with DNA damage and repair. *Mutagenesis*, 23: 249–260.
21. Dusinska, M. and Collins, A.R. (2008). The comet assay in human biomonitoring: gene–environment interactions. *Mutagenesis*, 23: 191–205.
22. Moller, P. (2006). Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA. *Mutat. Res*. 612 :84–104.
23. Tice, R.R.; Agurell, E.; Anderson, D.; Burlinson, B.; Hartmann, A.; Kobayashi, H., *et al.* (2000)."Single Cell Gel/Comet Assay: Guidelines for *In Vitro* and *In Vivo* Genetic Toxicology Testing. *Environmental and Molecular Mutagenesis*, 35:206 –221.
24. Boyum, A. (1968). Separation of lymphocyte from blood and bone marrow.

Scand. J. clin. lab. invent, 21(suppl .97): 77-89.
25. Ostling, O. and Johanson, K.J. (1984). Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun*., 123: 291–298.
26. Singh, N.P.; McCoy, M.T.; Tice, R.R. and Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res*. 175: 184–191.
27. Collins, A.R.; Duthie, S.J. and Dobson, V.L. (1993). Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* 14: 1733–1735.
28. Dusinska, M. and Collins, A. (1996). Detection of oxidised purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay. *Altern. Lab. Anim*., 24 405–411.
29. Olive, P.L.; Banath, J.P. and Durand, R.E. (1990). Heterogeneity in radiation induced DNA damage and repair in tumor and normal cells using the Comet assay. *Radiat Res*., 122: 86-94.
30. De Boeck, M.; Touil, N.; De Visscher, G.; Vande, P.A. and Kirsch–Volders, M. (2000). Validation and implementation of an internal standard in comet assay. *Mutat. Res*., 469, 181-197.
31. A-Collins, A.R.; Oscoz, A.A.; Brunborg, G.; Gaiva, I.; Giovannelli, L.; Kruszewski, M., *et al*. (2008). The comet assay: topical issues. *Mutagenesis*, 23 (3): 143–151.
32. Araldi, R.P.; de Melo, T.C.; Mendes, T.B.; de Sá Júnior, P.L.; Nozima, B.H.; Ito, E.T., *et al.*(2015). Using the comet and micronucleus assays for genotoxicity studies: A review. *Biomedicine & Pharmacotherapy*, 72 :74–82.
33. Godschalk, R.; Ersson, C.; Riso, P.; Porrini, M.; Langie, S.; van Schooten, F.; *et al*., (2013) .DNArepair measurements by use of the modified comet assay: an inter-laboratory comparison within the European Comet Assay Validation Group (ECVAG). *Mutat. Res*., 757: 60–67.
34. McKelvey-Martin, V.; Green, M.; Schmezer, P.; Pool-Zobel, B.; De Me´ o, M. and Collins, A. (1993). The single cell gel electrophoresis assay (comet assay): a European review. *Mutat. Res*., 288: 47–63.
35. Anderson, D.; Yu, T. and D. McGregor, (1998). Comet assay responses as indicators of carcinogen exposure. *Mutagenesis*, 13: 539-555.
36. Araldi, R.; Melo, T.; Diniz, N.; Carvalho, R.; Bec¸ak, W. and Stocco, R. (2013). Bovine papillo- mavirus clastogenic effect analyzed in comet assay. *Biomed. Res. Int*., 2013: 1–7.
37. Azqueta, A. and Collins, A. (2013). The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch. Toxicol*., 87 949-968.
38. Celik, A.; Ekinci, S.; Gu¨ ler, G. and Yildirim, S. (2014). In vitro genotoxicity of fipronil sister chromatid exchange, cytokinesis block micronucleus test, and comet assay. *DNA Cell Biol*., 33: 148–154.
39. Yasuhara, S.; Zhu, Y.; Matsui, T.; Tipirneni, N.; Yasuhara, Y.; Kaneki, M., *et al*. (2003). Comparison of comet assay, electron microscopy, and flow cytometry for detection of apoptosis. *J. Histochem. Cytochem*., 51 :873-885.
40. Araldi, R.; Rechiutti, B.; Mendes, T.; Ito, E. and Souza, E. (2012). Mutagenic potential of Cordia ecalyculata alone and in association with Spirulina maxima for their evaluation as candidate anti-obesity drugs. *Genet. Mol. Res*., 13: (2014) 5207–5220.
41. Corte´s-Gutie´ rrez, E.; Herna´ ndez-Garza, F.; Garcı´a-Pe´ rez, J.; Da´ vila-Rodrı´guez, M.; Aguado-Barrera, M. and Cerda-Flores, R. (2012). Evaluation of DNA single and double strand breaks in women with cervical neoplasia based on alkaline and neutral comet assay techniques. *J. Biomed Biotechnol*., 385245.
42. SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C.USA.
43. Gontijo, A.M.; Elias, F.N.; Salvadori, D.M.; de Oliveira, M.L.; Correa, L.A. and Goldberg, J. (2001). Single-cell gel (comet) assay detects primary DNA damage in nonneoplastic urothelial cells of smokers and ex-smokers. *Cancer Epidemiol Biomarkers Prev*., 10(9): 987-993.
44. Shen, H.; Spitz, M.; Qiao, Y.; Guo, Z.; Wang, L.; Bosken, C., *et al*. (2003). Smoking, DNA repair capacity and risk of nonsmall cell lung cancer. *Int. J. Cancer*, 107: 84-88.
45. Lee, K.W. and Pausova, Z. (2013). Cigarette smoking and DNA methylation. *Front Genet*., 4: 132.

46. Olive, P.L.; Durand, R.E.; Banath, J.P. and Johnston, P.J. (2001). Analysis of DNA damage in individual cells. *Methods Cell Biol*, 64: 235-249.
47. Mohammed, M.T.; Kadhim, S.M.; Jassim, A.M.N. and Abbas, S.I. (2015). Free Radicals And Human Health, *International Journal of Innovation Sciences and research*, 4: 218-223.
48. Markovic, J.; Garcia-Gimenez, J.L.; Gimeno, A.; Viña, J. and Pallardo, F.V. (2010). Role of glutathione in cell nucleus. *Free Rad. Res*., 44) 721–733.
49. Duthie, S.J.; Ma, A.; Ross, M.A. and Collins, A.R. (1996). Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Res*., 56: 1291–1295.