The Effects Of Eexogenous Free Radicals And Ultraviolet C Radiation On The Cytoskeleton Membrane Protein Of Human Red Blood Cells.

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Abstract

Objectives: This study aimed to explore the effect of exogenous free radicals and ultraviolet C (UVC) radiation on the protein and peptides of cytoskeleton membranes isolated from human red blood cells

Methods: Cytoskeleton membranes protein of human red blood cells were isolated and incubated with different concentrations of exogenous free radicals notably peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) or hydrochlorate (HOCl) for 5 minutes. In another series of experiments, the cytoskeleton membranes protein were subjected to UVC radiation (λ 254 nm) up to 30 minutes. The total protein was measured spectrophotometrically by Bradford method and the peptides levels were also measured spectrophotometrically.

Results : All the exogenous free radicals completely destroyed the protein of cytoskeleton membranes of human red cells and this effect is associated with increase peptides level in a dose dependent manner with $ONOO^-$ (65.86-329.34 μM), H_2O_2 (14.7-73.52 μM), but not with HOCl (9.52-47.62 μM). Variable damaging effects of UVC radiation were observed on both total protein and peptides. The effect of UVC radiation was not related to the exposure period.

Conclusions : Protein cytoskeleton membrane of human red cells is directly damaged by exogenous free radicals and while the effect of UVC radiation is resulted from both direct radiation effect, and indirect via releasing free radicals in aqueous solution.

Introduction

The topography of red cell membrane is regulated by several proteins including spectrin, actin, ankyrin, band 4.1, band 4.9, tropomyosin and others which are arranged in network [1] . Any alteration in these structural proteins led to loss the function of red cells particularly the deformability, which is needed for the passages of red cells in small blood vessels [2]. Free radicals resulted from several health problems play a role in destruction the cytoskeleton of red cell but not any antioxidant can protect the cell membrane from such damage. Quercetin, but not other polyphenols enhances the resistance of membrane to destruction by free radicals and this effect of quercetin is not directly mediated through antioxidative [3]. Recently, Sánchez-Gallego et al (2010) found that cholesterol depletion reduced the protection provided by both antioxidants against erythrophagocytosis and hemolysis, which is in accordance with the lower degree of preservation against lipid peroxidation observed in oxidized cholesterol-depleted erythrocytes [4]. On the other hand, protein or DNA photo-damage was observed with ultraviolet-C irradiation [5] , partly due to the release of free radicals notably nitric oxide [6] .Therefore, it is worth trial to utilize the red blood membrane ghosts which offer a good model for studying protein damage [7] . The objective of this study is to compare the effects of UVC and exogenous free radicals related to different groups (peroxynitrite, hydrogen peroxide and hypochlorate) on the cytoskeleton membrane protein of human red cells in an attempt to understand, partly, the mechanism protein damage.

Materials And Methods

This study was conducted in Department of Physiology/Medical Physics, College of Medicine, Al-Mustansiriya University in Baghdad, in cooperation with Department of Physiology / Medical Physics, College of Medicine, Diyala University in Diyala, Iraq during September 2010.

Preparation Of Red Blood Cell Ghosts

Human blood was freshly obtained from healthy subjects and collected into ACD anticoagulant (75 mM trisodium citrate, 42 mM citric acid, and 139 mM glucose). The plasma was separated by centrifugation (4000rpm) for 5 minutes, then the remaining leucocytes and blood platelets were removed by repeated washing with phosphate-buffered saline. Erythrocytes ghosts were prepared by adding 25 volumes of lysis solution (10 mM ethylenediaminetetraacetate disodium, 0.15M NH_4Cl, 0.1M $NaHCO_3$). After After lysis, the ghosts were sedimented at 4500 rpm g for 30 minutes and the pellet was washed in lysis buffer until hemoglobin was eliminated [8,9].

Synthesis Of Peroxynitrite ($ONOO^-$)

Peroxynitrite was synthesized as described by Whiteman and Halliwell (1996) [10]. In brief; 1 volume cooled sodium nitrite (1M) was mixed with 1 volume cooled hydrogen peroxide (1M) in dark room, then the reaction was quenched by adding 2 volume sodium hydroxide (1.5M). The upper layer was collected and quantified spectrophotometrically at λ302 nm wavelength (ε =1670M.cm^{-1}) . The yield was 5.269 mM. The stock solution was diluted with 0.1N NaOH when the desired concentration was prepared.

Preparation Of H_2O_2 And HOCl

Hydrogen peroxide (6%, Emirates hydrogen peroxide, Private Clean LLC, UAE) and hypochlorite (6.02%, Fas, Iraq) were obtained from local market and diluted with distilled water when the desired concentration was prepared.

Exposure Of The Membrane Cytoskeleton To $ONOO^-$, H_2O_2, HOCl

The yield of cytoskeleton membrane total protein was 160 µg/mL and the peptide concentration was 19.35 µg/mL and they considered as stock samples.

The membrane cytoskeleton was incubated with $ONOO^-$ (65.86 µM-329.34 µM), H_2O_2 (14.7 µM-73.52 µM), and HOCl (9.52 µM-47.62 µM) for 5 minutes, then the sample was diluted four times with 0.9% sodium chloride just prior to quantify the peptides spectrophotometrically at λ205 nm wavelength. Control sample of membrane cytoskeleton not exposed to any of the above solutions was also included as a reference. Moreover, total protein was quantified in each sample using Bradford assay method [11].

The concentration of peptide is calculated according to the following equation:

Absorbance at λ205 nm = 31 (mL/cm.mg) x [concentration in mg/mL) x 1 cm.

And the concentration of total protein was calculated from the following equation:

The concentration of total protein in sample = (Absorbance of sample/absorbance of standard human albumin) x concentration of standard human albumin in µg/mL

Exposure Of The Membrane Cytoskeleton To Ultraviolet Radiation

Samples of aqueous solution of membrane cytoskeleton were subjected to the ultraviolet radiation (UVC; λ254 nm wavelength. A 1mL of sample in 4 ml test tubes subjected to the UVC radiation from above in closed box. The distance between the UVC lamp to the upper surface of aqueous sample was 14 cm and the samples were subjected to UVC radiation for 5-30 minutes. Control sample of membrane cytoskeleton not exposed to radiation was also included as a reference. Then the peptides were quantified spectrophotometrically at λ205 nm wavelength. Moreover, total protein was quantified in each sample using Bradford assay method [11] . The concentrations of peptides and total protein were calculated as above.

Results

The protein-damage induced by exogenous free radicals; $ONOO^-$, H_2O_2, and HOCl was well observed. The pre exposure total protein that measured by Bradford method was 10µg/mL and completely destroyed by these free radical to be zero values after exposure. On the other hand the effects of free radicals on the peptides were related to the nature and the concentration of free radical as well as the pre-exposure peptides level. Fig. 1A shows that peroxynitrite, in a concentration dependent manner, increased the peptides level and its effect is augmented as the initial peptides level is decreased. The effect of H_2O_2 showed similar pattern to that observed with peroxynitrite [Fig.1 B] and it seems to be more potent than peroxynitrite. Peroxynitrite in a concentration of 329.34 µMol induced an increase of peptides level from 3.628µg/mL to 35.16µg/mL while hydrogen peroxide in a concentration of 73.5µMol (i.e. about 4.5 times less than corresponding concentration of peroxynitrite) increased the peptides level from 3.628µg/mL to 30.064 µg/dL. Fig. 1C shows that the pattern of hypochlorate effect is differed from those peroxynitrite and hydrogen peroxide i.e. bimodal

rather than concentration dependent manner. Also the effect of hypochlorate on the peptides was inferior to that observed with peroxynitrite or hydrogen peroxide.

The effects of ultraviolet-C on the total protein and peptides were not identical to that observed with exogenous free radicals (Fig.2 and Fig.3). The photo-damage effect on the total protein was not related to the period of exposure as shown in Fig. 2. Moreover, the effect of ultraviolet-C on peptides was the net of combined effects; the first increase peptides level as a result of protein destruction as observed in samples exposed to 5, 15 and 30 minutes and the second effect reduction of peptides level as a result of direct damaging effect of UVC on peptides in samples exposed to 10, 20 and 25 minutes.

Discussion

The results reported *herein* revealed that the effect of ultraviolet radiation is differed from that observed with exogenous free radical and its damaging effect is extended beyond the protein photo-damage to peptides degradation.

Previous studies showed that peroxynitrite at physiological pH can be protonated and decomposed to an intermediate with reactivity similar to that of hydroxyl radical and nitrogen dioxide [12,13] and it interacts with cellular protein[14] . The results of this study indicated that peroxynitrite selectively damaged the cytoskeleton protein and released the intermediate substances i.e. peptides which increased in a peroxynitrite concentration dependent manner. These results are in agreement with Di Mascio et al (2000) who found that the cytoskeleton membrane protein of red cells, particularly spectrins, is sensitive to the peroxynitrite [15] . As a result of protein oxidation with hydrogen peroxide, the cytoskeleton membrane protein is damaged and its intermediate substances including peptides are released [16] . And this explained the higher peptides concentration that is observed when the cytoskeleton membrane protein incubated with hydrogen peroxide. The results reported with hypochlorate are similar to that obtained with peroxynitrite and hydrogen peroxide. Recently Wasim Khan et al (2010) reported that HOCl significantly damage the antiprotolytic activity of macroglobulin [17] . Therefore, the differences in the concentration of peptides release as a result of protein degradation is related to the selective action of the free radicals on the protein sites.

In vitro aqueous solution, UV radiation exerts its direct and indirect effects via generation of free radicals whether reactive oxygen or reactive nitrogen species depending on the

constituents of that solution [18] . In this study the photo-damaging effect of UVC against total protein and peptides is not related solely to the generation of free radicals and the possibility of direct damaging effect should be considered. As early as 1998, Menter et al found that UVC (0-2.6 x 10^4 J/m^2) generated reactive oxygen species is likely to be present in sufficient concentration to cause collagen damage [19] . Accordingly, the use of free radical scavengers or antioxidants as protective agents in sunscreen can obviated the indirect effect of ultraviolet radiation but not the direct effect [20,21,22] . It concludes that the protein cytoskeleton membrane of human red cells is easily damaged by different sources of free radicals and the effect of UVC radiation is resulted from both direct protein and its intermediates damage, and indirect via releasing free radicals in aqueous solution.

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References

1. Bennet V. The membrane skeleton of human erythrocytes and its implication for more complex cells. Annual Review of Biochemistry 1985; 54: 273-304.

2. Handelman GJ, Levin NW . Red cell survival: relevance and mechanism involved. J Ren Nutr 2010; 20(5 Suppl):S84-88.

3. Hapner CD, Deuster P, Chen Y. Inhibition of oxidative hemolysis by quercetin, but not other antioxidants. Chem Biol Interact 2010; 186(3):275-279.

4. Sánchez-Gallego JI, López-Revuelta A, Sardina JL, Hernández-Hernández A, Sánchez-Yagüe J, Llanillo M. Membrane cholesterol contents modify the protective effects of quercetin and rutin on integrity and cellular viability in oxidized erythrocytes. Free Radic Biol Med 2010; 48:1444-1454.

5. Kaufmann WK . The human intra-S checkpoint response to UVC-induced DNA damage. Carcinogenesis 2010; 31:751-765.

6. Fotiou S, Fotiou D, Deliconstantinos G. Formation of heme-iron complexes with nitric oxide (NO) and peroxynitrite (ONOO-) after ultraviolet Radiation as a protective mechanism in rat skin. In Vivo 2009 ; 23: 281-286.

7. Hsieh CL, Yen GC, Chen HY. Antioxidant activities of phenolic acids on ultraviolet radiation-induced erythrocyte and low density lipoprotein oxidation. J Agric Food Chem 2005; 53:6151-6155.

8. Homewood CA & Neame KD. A comparison of methods used for removal of white cells from malaria infected blood. Annals of Tropical and Medical Parasitology 1976; 70: 249-251.

9. Tyler TM, Hargraves WR, Branton D. Associations of erythrocyte membrane protein. Proceedings of the National Academy of Sciences(USA) 1980;70: 5192-5196.

10. Whiteman M, Halliwell B. Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants. Free Radic Res 1996;25:275-283.

11. Bradfod, MM. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976; 72: 248-254.

12. Pryor WA, Jin X , Squadrito GL. One-and two-electron oxidations of methionine by peroxynitrite. Proceedings of the National Academy of Sciences (USA) 1994; 91: 11173-11177.

13. Augusto O, Gatti RM, Radi R . Spin-trapping studies of peroxynitrite decomposition and of 3-morpholinosydnonimine N-ethylcarbamide autoxidation: direct evidence for metal-

independent formation of free radical intermediates. Archives of Biochemistry and Biophysics 1994; 310: 118-125

14. Salgo MG, Bermudez E, Squadrito GL, Pryor WA. Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes.Archives of Biochemistry and Biophysics 1995: 322: 500-505.

15. Di Mascio P, Dewez B, Garcia CRS. Ghost protein damage by peroxynitrite and its protection by melatonin. Brazilian Journal of Medical and Biological Research 2000; 33: 11-17

16. Finnegan M, Linley E, Denyer SP, McDonnell G, Simons C, Maillard JY Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. J Antimicrob Chemother 2010 Aug 16.

17. Wasim Khan M, Naqshbandi A, Zubair H, Ahsan H, Khan SA, Khan FH. Nitrite, a reactive nitrogen species, protects human Alpha-2-macroglobulin from halogenated oxidant, HOCl. Protein J 2010 May 28.

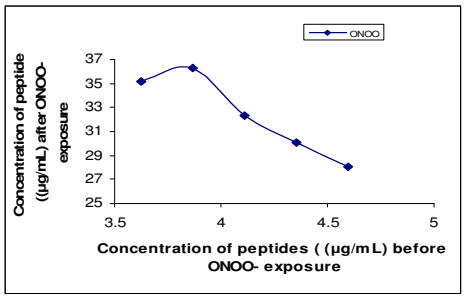
18. Puzanowska-Tarasiewicz H, Kuźmicka L, Tarasiewicz M. Reactive nitrogen and oxygen species. Pol Merkur Lekarski 2009; 27(160):338-40..

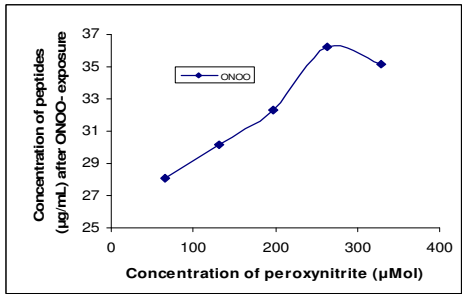
19. Menter JM, Patta AM, Hollins TD, Moore CL, Willis I .Photoprotection of mammalian acid-soluble collagen by cuttlefish sepia melanin in vitro. Photochem Photobiol 1998; 68:532-537.

20. Nakajima A, Tahara M, Yoshimura Y, Nakazawa H. Study of compounds suppressing free radical generation from UV-exposed ketoprofen. [Journal Chem Pharm Bull (Tokyo) 2007; 55(10):1431-8.

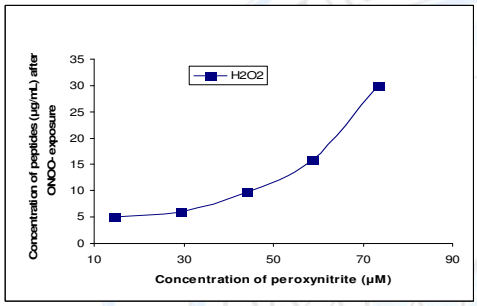
21. Cotter MA, Thomas J, Cassidy P, Robinette K, Jenkins N, Florell SR, Leachman S, Samlowski WE, Grossman D. N-Acetylcysteine Protects Melanocytes against Oxidative Stress/Damage and Delays Onset of Ultraviolet-Induced Melanoma in Mice. Clin Cancer Res 2007; 13:5952-5958.

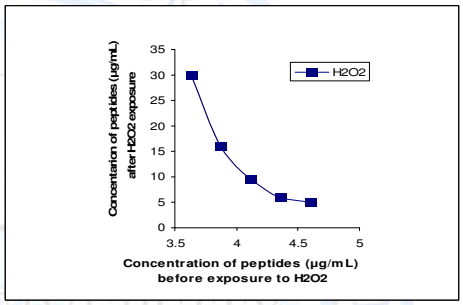
22. Lee BC, Lee SY, Lee HJ, Sim GS, Kim JH, Kim JH, Cho YH, Lee DH, Pyo HB, Choe TB, Moon DC, Yun YP, Hong JT. Anti-oxidative and photo-protective effects of coumarins isolated from Fraxinus chinensis. Arch Pharm Res 2007; 30:1293-301


ONOO
Concentration of peptide ((µg/mL) after ONOO- exposure
37
35
33
31
29
27
25
3.5
4
4.5
5
Concentration of peptides ((µg/mL) before ONOO- exposure

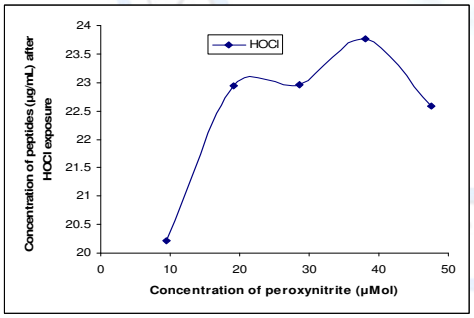

ONOO
Concentration of peptides (µg/mL) after ONOO- exposure
37
35
33
31
29
27
25
0
100
200
300
400
Concentration of peroxynitrite (µMol)

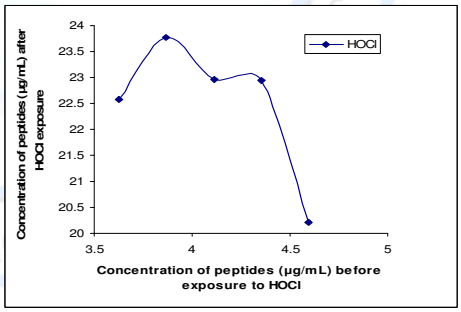
[A]


H2O2
Concentration of peptides (µg/mL) after ONOO- exposure
35
30
25
20
15
10
5
0
10
30
50
70
90
Concentration of peroxynitrite (µM)


H2O2
Concentration of peptides (µg/mL) after H2O2 exposure
35
30
25
20
15
10
5
0
3.5
4
4.5
5
Concentration of peptides (µg/mL) before exposure to H2O2

[B]


HOCl
Concentration of peptides (µg/mL) after HOCl exposure
24
23.5
23
22.5
22
21.5
21
20.5
20
0
10
20
30
40
50
Concentration of peroxynitrite (µMol)


HOCl
Concentration of peptides (µg/mL) after HOCl exposure
24
23.5
23
22.5
22
21.5
21
20.5
20
3.5
4
4.5
5
Concentration of peptides (µg/mL) before exposure to HOCl

[C]

Figure 1. Shows the effect of peroxynitrite (ONOO-) [A], hydrogen peroxide (H2O2) [B] and perchlorate (HOCl) [C] on the cytoskeleton membrane protein of human red blood cells.

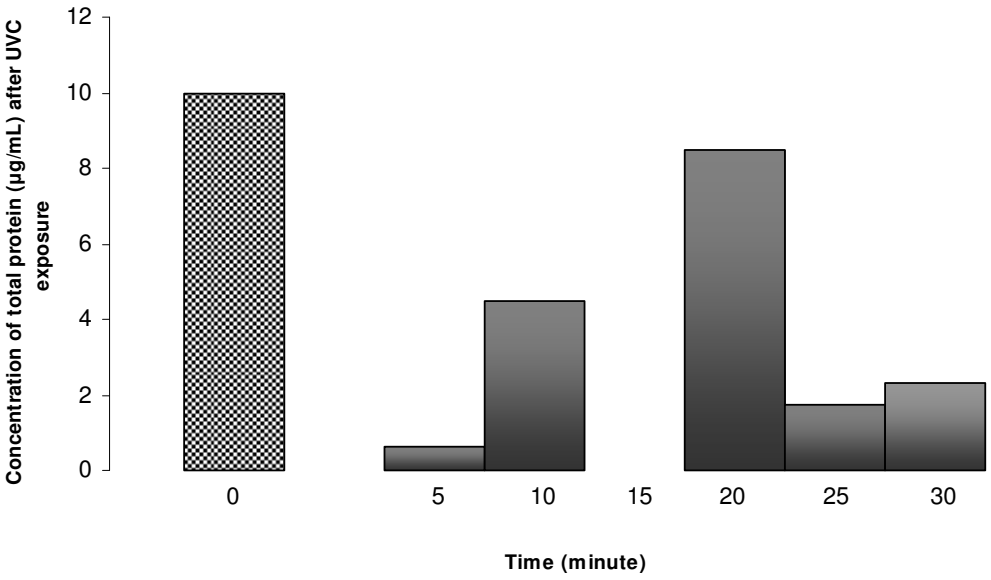


Figure 2. Concentration of total protein (µg/mL) after variable periods of UVC exposure

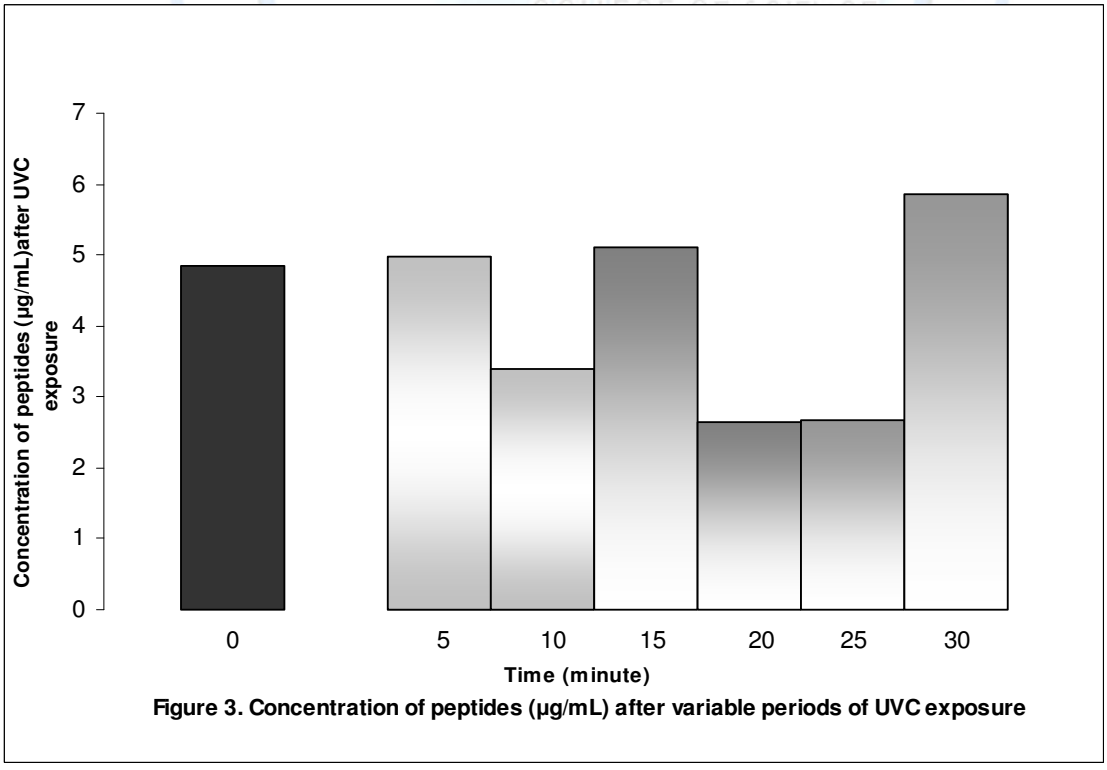


Figure 3. Concentration of peptides (µg/mL) after variable periods of UVC exposure