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التغيرات الشكلية الناتجة عن تعرض كريات الدم الحمراء للرايبوفلافين المعرض للضوء المرئي

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Abstract

Background: Reactive oxygen species (ROS) are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times of oxidative stress, ROS levels can rise dramatically. This may result in significant damage to cell structures. In this work we are interested to show the effect of different ROS on the morphology of fresh human RBCs. **Methods:** The RBCs were incubated with different reaction mixtures at room temperature and exposed to cool fluorescent light (800 lux). Then, cells were isolated and scanned by a scanning electron microscope. **Results:** When incubated with photoilluminated riboflavin, RBCs lost their biconcave shape and adopted a spherocytes shape. The formation of spherocytes is usually associated with spectrin deficiency. In the presence of Cu(II) and riboflavin, RBCs appeared with spikes of different sizes on their surface showing the formation of "acanthocytes", which is usually prevalent in abetalipoprotienemia. Moreover, addition of NaN_3 to riboflavin-Cu(II) system resulted in completely damaged RBCs. Away from the above combinations, when RBCs are incubated with

riboflavin-aminophylline combination, they appeared with spikes of equal lengths and sizes on their surface "echinocytes", which usually appear in different diseases like pyruvate kinase deficiency and uremia. **Conclusion**: Red blood cells undergo different morphological changes when incubated in each of the above combinations, most probably due to the formation of different ROS and these ROS could be involved in different pathological consequences.

Keywords: Riboflavin, ROS, RBC, Morphology, Scanning Electron Microscope.

الملخص

تتأثر كريات الدم الحمراء بالظروف السابقة بطرق مختلفة نتيجة لتكون انواع مختلفة من جذور الاكسجين الحرة وهذا يطرح تساؤل عن الدور التي تلعبه جذور الاكسجين الحرة في آلية حدوث الامراض المختلفة. **أرضية البحث:** جذور الاكسجين الحرة تتكون كنواتج جانبية لكثير من العمليات الحيوية في جسم الانسان، ولها دور اساسي في تنظيم كثير من العمليات. أثناء اوقات الاكسدة تتغلب جذور الاكسجين الحرة على موانع الاكسدة بشكل كبير، وهذا قد يؤدي الى تدمير الخلايا. في هذه الدراسة قمنا بدراسة آثار جذور الاكسجين الحرة على شكل كريات الدم الحمراء. **طريقة العمل:** تم تعريض كريات الدم الحمراء لعدة انواع من التفاعلات على درجة حرارة الغرفة وبوجود قوة ضوء مرئي مقداره 800 لوكس. تم عزل كريات الدم الحمراء ودراسة التغيرات باستخدام الميكروسكوب الالكتروني. **النتائج:** عندما تعرضت كريات الدم الحمراء ل رايبوفلافين المعرض للضوء فقدت كريات الدم الحمراء شكلها القرصي المقعر الوجهين واصبحت كروية. وهذا الشكل الكروي يحدث ايضاً كنتيجة لنقص بروتين ال "سبكترين". أما بوجود النحاس بالاضافة الى الرايبوفلافين والضوء، فأن كريات الدم الحمراء يظهر على سطحها نتوءات بأطوال مختلفة وهذا الشكل يسمى "اكانثوسايت" وعادة تظهر هذه الاشكال في حالات ابيتالايبوبروتينيميا. أما عند اضافة ازايد الصوديوم الى التفاعل الاخير نتج التكسر التام لكريات الدم الحمراء. وبعيدا عن ما ذكر، عند تعرض كريات الدم الحمراء لخليط من الرايبوفلافين و الامينوفيلين وبوجود الضوء، ظهرت اشكال جديدية لكريات الدم الحمراء تسمى "ايكاينوسايت" وهذه الاشكال تظهر في حالات مرضية كنقص البايروفيت واليوريميا.

Background

Recent evidence suggests that oxidative stress contributes significantly to the regulation of hematopoietic cell homeostasis (Ghaffari, 2008). In particular, red blood cells (RBCs) and hematopoietic stem cells are highly sensitive to deregulated accumulation of reactive

oxygen species (ROS). Unchecked ROS accumulation often leads to hemolysis and the premature destruction of RBCs. In addition, the process of erythroid cell formation is also sensitive to ROS accumulation and oxidative stress (Ghaffari, 2008). Oxidative stress is a state where increased formation of ROS overwhelms the body's antioxidant protection, and subsequently induces DNA damage, lipid peroxidation, and protein modification. Oxidative stress is thought to be the cause of numerous diseases including cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders and chronic inflammation (Jomova & Valko, 2011; Touyz, 2004).

Erythroid precursors synthesize and accumulate hemoglobin as they mature, and this process includes the insertion of iron to make heme within the mitochondria of erythroid precursors. The presence of iron within the circulating erythrocytes that carry oxygen makes the cells susceptible to Fenton reaction and consequently, highly prone to oxidative damage (Marinkovic et al., 2007). Erythrocytes are exposed to high level of oxidative stress and due to the occasionally compromised protection against ROS, the life span of RBCs is shortened and premature hemolysis occurs leading to anemia (Kong et al., 2004; Lee, Chan, Kan, & Johnson, 2004).

The objective of this work is to examine the effect of different ROS on human RBCs morphology and to compare these changes with the changes induced by certain diseases. This comparison may open the door to understand how different diseases affect the human RBCs and causing their dysfunction.

Materials and Methods

Riboflavin, cupric chloride and NaN_3 were obtained from Sigma Chemical Co., USA. Aminophylline was obtained from Priya pharmaceutical Co., India. All other chemicals used were of the highest purity grade available commercially.

Preparation of RBC

Red blood cells were prepared from fresh human blood taken from a healthy volunteer. The blood samples were collected in acid citrate dextrose and centrifuged for 10 minutes at 1500 xg at room temperature. The cells were washed three times with 5 volumes of isotonic NaCl solution. The 0.5% hematocrit reactions were carried in a suspension of 3 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The cells were treated with riboflavin, Cu(II), NaN_3 and/or aminophylline. The concentrations used in these reactions were standardized from our previous work (I. Ali, Gatasheh, & Naseem, 2000; I. Ali, Sakhnini, & Naseem, 2005; L. Ali & Naseem, 2002). The reaction mixtures were incubated at room temperature in front of 800 lux of cool fluorescent light. During the incubation period, the reaction tubes were gently inverted every 10 min to prevent cell sedimentation.

Scanning Electron Microscopy

The reaction mixtures containing RBCs treated with 50 μM riboflavin alone and in the presence of different combination are indicated in the legends. After 2 hours of incubation, RBCs from different reactions were placed on glass slides to be processed for electron microscopy. The glass slides were coated with gold by a sputter cotter, and micrographs were taken using a scanning electron microscope (378 × 512), Philips, Japan.

Results

Riboflavin or vitamin B_2 is the prosthetic group of several proteins and enzymes which is reversibly reduced by hydrogen atoms. When exposed to light, riboflavin absorbs energy and reacts via its triplet excited state with other molecules such as protonated substrates or molecular oxygen generating ROS (Frati et al., 1997). In the presence of oxygen and visible light, riboflavin has been shown to be lethal to animal and human cells in culture and induce mutations in microorganisms (I. Ali et al., 2000). Incubation of RBCs with photoilluminated riboflavin resulted in the formation of spherical-shaped RBCs called spherocytes (Fig. 1B). We have previously shown that riboflavin in the presence of

Cu(II) and fluorescent light generate hydroxyl radical ($^{\bullet}OH$) (Hasan, Ali, & Naseem, 2006) that damage calf thymus DNA and supercoiled plasmid DNA (Jazzar & Naseem, 1996), and inhibited protein (Husain, Fatima, Ali, & Naseem, 2006). When RBCs were incubated with both riboflavin and Cu(II), irregular spikes appeared on RBCs' surface (Fig. 1C). These RBCs are called acanthocytes. When NaN_3 was added to the riboflavin-Cu(II) system, it inhibited the photodegradation of riboflavin resulting in increased $^{\bullet}OH$ generation. Also, there is possibility of azide radical formation and its involvement in the reaction (I. Ali et al., 2005). The effect of photoilluminated riboflavin, Cu(II) and NaN_3 combination on RBCs is shown in Fig. (1D) and the cells were completely damaged.

Based on our earlier reports, we suggested that photoilluminated riboflavin generates the singlet and triplet excited states that, upon energy transfer, generate singlet and triplet oxygen (L. Ali & Naseem, 2002). These activated oxygen species probably attack aminophylline leading to its oxidation, generating $^{\bullet}OH$ which presumably cause inactivation and fragmentation of trypsin (Hasan et al., 2006). Figure (1E) shows the structural changes seen when RBCs were incubated with both riboflavin and aminophylline. Equally spaced projections were observed over the entire surface of RBCs and these cells are called "echinocytes".

Parallel reactions with all the above combinations were incubated in dark; in which the RBC's maintained their normal biconcave shape (Table 1).

Discussion

When exposed to light, riboflavin generates ROS and these ROS damage several biological molecules (I. Ali et al., 2000; Hasan et al., 2006; Husain et al., 2006). The present work studied the effect of photoactivated riboflavin alone, and in the presence of Cu(II), Cu(II) and NaN_3, or aminophylline on human RBCs.

The morphological changes on RBCs shape that occurred after treatment with different reaction mixtures were studied by using scanning electron microscopy. When RBCs incubated with photoilluminated riboflavin, they lost their biconcave shape (Fig. 1A) and formed one of their pathological morphology called spherocyte (Fig. 1B). The spherocyte formation occurs due to spectrin deficiency and it is also associated with different disease states like anemia and jaundice (Ayhan et al., 2012). These structural abnormalities may result in partial K^+ loss without causing hemolysis as it has been suggested in our previous study (I. Ali et al., 2000).

Copper is the therapeutic target for the treatment of Wilson's disease, where copper is accumulated in the liver and extra hepatic organs such as the brain and cornea. Patients may present with combinations of hepatic, neurological and psychiatric symptoms (Ala, Walker, Ashkan, Dooley, & Schilsky, 2007). Addition of Cu(II) to photoilluminated riboflavin (Fig. 1C) generate $^{\bullet}OH$ (Hasan et al., 2006), and as a result acanthocytes were formed which are usually found in liver diseases and malabsorptive states (Saibara, 2007), and abetalipoprotienemia (Hasosah, Shesha, Sukkar, & Bassuni, 2010). Acanthocytes formation could accompany the peroxidation of lipids as suggested in our previous report (I. Ali et al., 2005).

Addition of NaN_3 to the riboflavin-Cu(II) combination may result in the formation of azide radical (I. Ali et al., 2005). Azide radical caused a massive damage to RBC's (Fig. 1D) and lead to extensive hemolysis. This extensive damaging effect was seen when RBCs were incubated with riboflavin-Cu(II)-NaN_3 combination. We propose that the enhanced damaging effect of this combination is probably due to the combined

effect of $^{\bullet}OH$ and azide radicals produced within this combination. In other studies, NaN_3 is usually used as a singlet oxygen scavenger, (Han, Hwang, Yoon, & Kang, 2011) but the present study found the opposite effect of NaN_3 which is in agreement with our earlier report (I. Ali et al., 2005).

Aminophylline is an anti-asthmatic drug and a known phosphodiesterase inhibitor (Hasan et al., 2006). In this experiment, we examined the effect of riboflavin-aminophylline on cells using RBCs as the target cells. Echinocytes were formed when RBCs were incubated with riboflavin-aminophylline combination in the presence of fluorescent light (Fig. 1E). There have been an association between the echinocytes formation and different diseases like thromboembolic ischemic stroke (Swanepoel & Pretorius, 2012). On the other hand, aminophylline is excreted in human milk with concentrations approximately equivalent to the maternal serum concentration. This may be critical in infants suffering from jaundice after birth who are usually subjected to phototherapy for a few days. These infants born to mothers treated with aminophylline may face a problem of having aminophylline in their blood and are subjected to phototherapy treatment at the same time. These infants may be at risk of producing large amount of ROS which may interact with aminophylline causing more damage to cells. This condition may further damage the infant's brain as more RBCs will be lyzed leading to more bilirubin production and free iron from heme degradation.

Conclusion

In conclusion, different ROS species are formed in each of the above reaction mixtures (Table1). Each ROS has a different effect on RBCs morphology, ranging from spherocytes, acanthocytes, echinocytes to complete lysis. This indicates that different ROS have different mechanisms of action. The association between various RBC shapes and different diseases, and the appearance of these shapes as a result of ROS action open the eyes on further investigation about these diseases and ROS actions. This work also highlights the possibilities concerning the role of ROS in the etiology or the development of these diseases. These

possibilities require further investigation which may help in the treatment of these diseases or at least minimize their effects on RBCs.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Legends

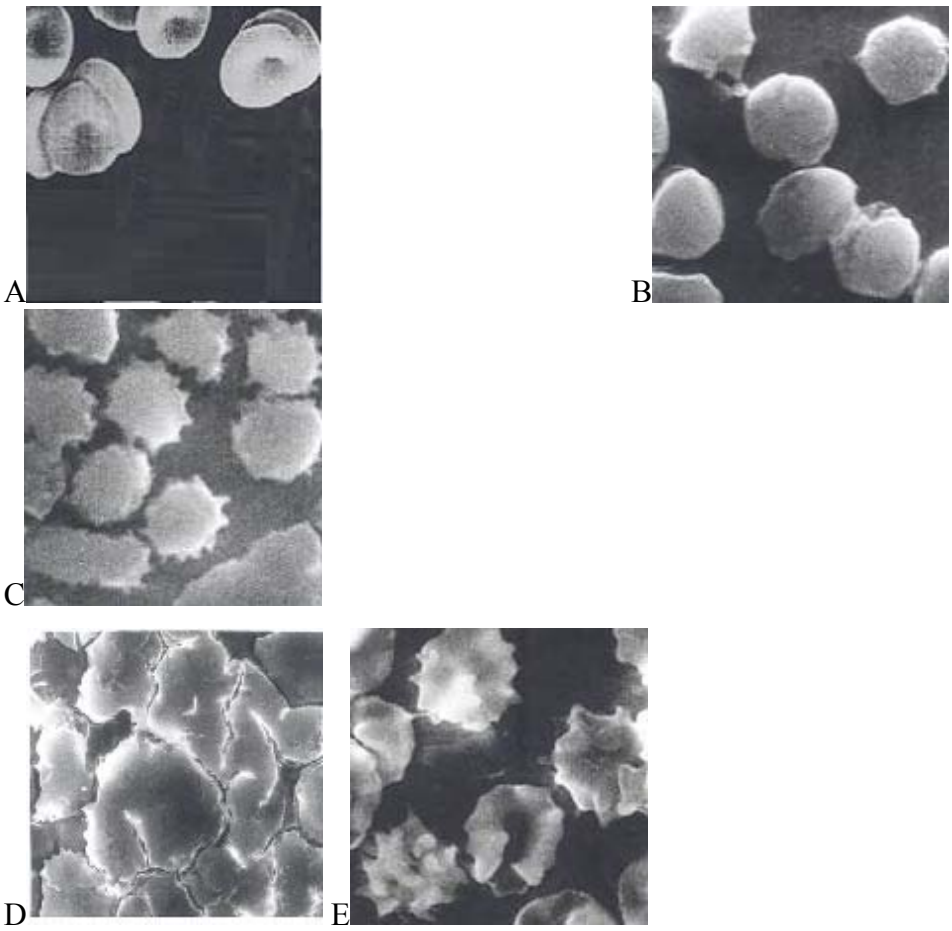

Figure (1): Scan electron microscopy of RBCs incubated with different reaction mixtures. (A) Normal RBCs; (B) RBCs treated with 50µM riboflavin alone; (C)

RBCs treated with 50µM riboflavin and 100µM Cu(II); (D) RBCs treated with 50µM riboflavin, 100µM Cu(II) and 20µM NaN_3; (E) RBCs treated by 50µM riboflavin and 20µM aminophylline. The reaction mixtures were incubated at room temperature in 800 lux of cool fluorescent light for 30 minutes.

Table (1): Comparisons between the changes occur in the RBCs structure when treated with different combinations and incubated in fluorescent light and in dark. (A) Normal RBCs; (B) RBCs treated with 50µM riboflavin alone; (C) RBCs treated with 50µM riboflavin and 100µM Cu(II); (D) RBCs treated with 50µM riboflavin, 100µM Cu(II) and 20µM NaN_3; (E) RBCs treated by 50µM riboflavin and 20µM aminophylline. The reaction mixture was incubated at room temperature in 800 lux of cool fluorescent light for 30 minutes.

Table 1	**(A)**	**(B)**	**(C)**	**(D)**	**(E)**
Combinations Conditions	Normal saline buffer	Riboflavin alone	Riboflavin + Cu(II)	Riboflavin + Cu(II) + NaN_3	Riboflavin + aminophylline
RBCs shape (fluorescent light)	Biconcave shape	Spherocytes	Acanthocytes	Completely damaged cells	Echinocytes
Expected ROS generated (fluorescent light)	Nil	Singlet oxygen, superoxide radical & hydrogen peroxide	Hydroxyl radical	Hydroxyl radical and possibly azide radical	Hydroxyl radical & unknown products
RBCs shape (dark)	Biconcave shape	Biconcave shape	Biconcave shape	Biconcave shape	Biconcave shape
Expected ROS generated (dark)	Nil	Nil	Nil	Nil	Nil

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