



## The Effect on Antioxidant Defense System of Chondroitin-4-Sulphate in Human Erythrocytes Subject to High Glucose Levels: In The Laboratory Conditions

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### Abstract

The purpose of this research was to evaluation the effects on antioxidant defense system of chondroitin-4-sulphate (C4S) in high glucose treated human erythrocytes. 10-20 ml venous blood samples from ten healthy volunteers ages 20-40, nonsmoker, were accumulated in ethylenediamine tetraacetic acid (EDTA) vials for the preparation of red blood cell (RBC) suspensions. Blood for *in vitro* treatments was divided into six groups. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), acetylcholinesterase (AChE), the levels of glutathione (GSH) and malondialdehyde (MDA) were calculated to determine the effects on antioxidant defense system in all groups. As a result of the statistical evaluations, the significant increases ( $p<0.001$ ) in GSH-Px and AChE enzyme activities were observed in the groups in which C4S applied compared to the groups in which glucose alone applied. At the same time, significant decrease ( $p<0.001$ ) in MDA levels was obtained in the groups in which C4S applied compared to the groups in which glucose alone applied. These acquired findings have shown that in the erythrocytes to which high amount of glucose has been treated; C4S may have a positive impact upon antioxidant defense systems. As the results of this study, C4S can be said to be benefited as an antioxidant.

*Keywords:* Human erythrocytes, Antioxidant defense, Chondroitin-4-sulphate, Glucose, Antioxidant enzymes.

## **Laboratuvar Koşullarında Yüksek Glukoza Maruz Kalmış İnsan Eritrositlerinde Kondroitin-4-Sülfatın Antioksidan Savunma Sistemi Üzerine Etkisi**

### **Özet**

Bu çalışmada, yüksek glukoza maruz kalmış insan eritrositlerinde kondroitin-4-sülfatın (C4S) antioksidan savunma sistemine olan etkisi araştırılmıştır. Eritrosit süspansiyonu hazırlamak için, sigara içmeyen, 20-40 yaş aralığında sağlıklı gönüllülerden 10-20 ml venöz kanı etilendiamin tetraasetik asitli (EDTA) tüplere toplanmıştır. *In vitro* uygulamalar için kanlar 6 gruba ayrılmıştır. Tüm gruplarda antioksidan savunma sistemi üzerine olan etkilerin belirlenmesi için süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GSH-Px), asetilkolinesteraz (AChE) enzim aktiviteleri, glutatyon (GSH) ve MDA düzeyleri ölçülmüştür. İstatistiksel değerlendirmeler sonucunda, C4S uygulanan gruplar ile sadece glukoz uygulanan gruplar karşılaştırıldığında GSH-Px ve AChE enzim aktivitelerinde önemli ölçüde artış ( $p<0,001$ ), MDA düzeylerinde de önemli ölçüde azalış olduğu ( $p<0,001$ ) tespit edilmiştir. Elde edilen bu bulgular, yüksek glukoz içeren eritrositlerde C4S'nin uygulanabileceğini ve C4S'nin antioksidan savunma sistemleri üzerine olumlu etkileri olabileceğini göstermektedir. Sonuç olarak C4S'nin etkili bir antioksidan olduğu söylenebilir.

*Anahtar Kelimeler:* İnsan eritrositleri, Antioksidan savunma, Kondroitin-4-sülfat, Glukoz, Antioksidan enzimler.

### **1. Introduction**

The most important effect in the beginning and progression of diabetic complications, is hyperglycemia [1]. One of the instruments connecting hyperglycemia to diabetic complexities is increased reactive oxygen species (ROS). Extreme creation of ROS leads to oxidative damage and change in structures/functions of membrane lipids, proteins and DNA [2]. The negative effects are called oxidative stress. To manage the increased ROS, cells have built up their own defense framework which contains enzymic and non-enzymic components called the antioxidant system. The antioxidant system

consists of antioxidant enzymes and antioxidant molecules such as glutathione (GSH). For instance, some of the enzymes are catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) [3].

Healthy people have a balance between ROS and antioxidants. However, hyperglycemia disturbs ROS/antioxidant balance, in favor of ROS and causes diabetic complications. To reduce these complications, antioxidant defense system activates with used exogenous antioxidants [4].

Human erythrocytes are essential for transport of oxygen and carbon dioxide eradication [2]. Erythrocytes are highly vulnerable to attack by ROS because of the high amount of polyunsaturated fatty acid content (PUFA) in their membranes and the metal catalyzed oxidation reactions because of hemoglobin Fe. Oxidative attack on erythrocytes may alter its function and membrane structure (e.g. fluidity, permeability, and enzyme activity) and can create oxidative stress. Oxidative damage in erythrocytes occurs as hemolysis and lipid peroxidation (LPO), causing normal red cells to age and pathological red blood cells to be shorter in life [5]. Thus, the effects of oxidative stress caused by the hyperglycemia in the erythrocytes can only be reduced or removed by only antioxidant materials [6].

Most biological molecules have more than one function. Particularly, many molecules can operate as antioxidants in tissues and cells by directly/indirectly scavenging ROS. The elevated level of these molecules seems to be a biological response which may prevent oxidation in cells, along with other antioxidant defense systems during oxidative stress. For instance, one of these formation is the glycoaminoglycan. Chondroitin-4-sulphate (C4S), a biomolecule, because of its antioxidant activity has the focused interest of many research groups [7-9]. Chondroitin sulphate (CS) which can be extracted and purified from various tissues, is a complex glycosaminoglycan. CS consisting of galactosamine and glucuronic acid varying disaccharide units is added to the serine residues of protein cores by a tetrasaccharide linkage. Means of sulphation may be used to distinguish CS structures. Sulphate at the C-4 position of galactosamine gives C4S while C6S may be obtained with sulphate at the C-6 position [10]. In the studies conducted to this day, the antioxidant effects of C4S on liver and kidney tissues have been researched. However, the effects of antioxidants on erythrocytes have not been studied.

In the present study, the main objective was to research the antioxidative effect of C4S in human erythrocytes subjected to high glucose levels.

## **2. Materials and Methods**

### **2.1 Preparation of Human Erythrocytes**

Whole blood samples were collected from 10 healthy volunteers aged 20-40 years who were not smoking, were not obese, and did not use any medicines or alcohol. The anticoagulant used was Ethylenediamine tetra acetic acid (EDTA) and blood samples were centrifuged for 10 min at 2500 rpm and 4°C. After that, buffy coat layers and the plasma were discarded. The erythrocytes layers were washed three times with 0.9% NaCl and centrifuged at the same values as specified above. After each centrifugation, the supernatants were discarded. The erythrocytes layers were re-suspended (1:10) in phosphate-buffered saline (PBS, pH:7.4, 0.01 M) [11].

### **2.2 *In Vitro* Treatment of Erythrocytes with Glucose and Chondroitin-4-Sulphate (C4S)**

Three milliliters of diluted and washed erythrocytes was incubated with an equal amount of PBS, 20 and 40 mM glucose levels. C4S at concentrations of 25 and 50 mM were added. The reaction mixtures, using a shaking water bath at 37°C, were incubated for 24h. In some tests, the erythrocytes suspension was preincubated with C4S for 30 min before the addition of glucose to the suspension. All incubations included 10 µl penicillin-streptomycin solution/ml cell suspensions, to avert microbial growth. The penicillin-streptomycin solution contained 500 mg streptomycin in 10 ml PBS and 300 mg penicillin G [12]. Each treatment included three replicates.

Experimental design;

For the *in vitro* treatment of erythrocytes, the erythrocytes suspensions were randomly divided into 6 groups consisting of ten erythrocyte samples;

1. Group: Treatment at 20 mM glucose
2. Group: Treatment at 40 mM glucose

3. Group: Treatment at 20 mM glucose and 25 mM C4S
4. Group: Treatment at 20 mM glucose and 50 mM C4S
5. Group: Treatment at 40 mM glucose and 25 mM C4S
6. Group: Treatment at 40 mM glucose and 50 mM C4S

### **2.3 Estimation of Hemolysis**

The hemoglobin content of the samples centrifuged at 2500 rpm for 10 minutes was recorded at 540 nm after incubation ( $A$ ). A value of 100% lysis was assigned to the supernatant of the sample with control hemolysate, obtained by freezing and thawing the cell suspensions ( $A_1$ ) [13]. The percentage of hemolysis ( $H\%$ ) in each assay tube was calculated by the equation: Percent hemolysis ( $H\%$ ) for each sample was computed by the equation:

$$H\% = A/A_1 * 100$$

### **2.4 Preparation of Hemolysates**

The supernatants were discarded in the reaction mixtures. The tubes were washed 3 times with 0.9% NaCl (1:10) and they were centrifuged at 2500 rpm for 10 min. after each washing. After the centrifugation, the distilled cold water was added to the suspension (1:20) and then vortexed at 15 min. The tubes were centrifuged at 2500 rpm for 10 min [14]. The hemolysates were used for estimated of hemoglobin (Hb), and GSH contents, SOD, CAT, GSH-Px, and acetylcholinesterase (AChE) enzyme activities.

### **2.5 Estimation of Erythrocyte Hemoglobin (Hb) Content**

Hb was estimated according to the Drabkin's method by the cyanomethemoglobin method [15]. The absorbance was measured at 540 nm against a reagent blank. Hb content was represented as g/ml.

### **2.6 Estimation of Erythrocyte Glutathione (GSH) Content**

GSH content in erythrocytes was estimated using the standard method of Beutler et al. [16]. To the 0.7 ml hemolysate, 0.7 ml 1 M phosphate solution (pH 7.8) and 0.05 ml

of fresh prepared 5, 5-dithiobis 2-nitro benzoic acid (DTNB) were added. The absorbance is measured at 412 nm. The GSH content was represented as nmol/g Hb.

### **2.7 Measurement of Erythrocyte Superoxide Dismutase (SOD) Activity**

SOD (E.C. 1.15.1.1) activity in erythrocytes was assayed spectrophotometrically at 560nm as described by Sun et al [17]. One SOD unit is defined as the amount of enzyme leading 50% inhibition in the NBTH<sub>2</sub> reduction ration. SOD activity was also represented USOD g<sup>-1</sup>Hb.

### **2.8 Measurement of Erythrocyte Catalase (CAT) Activity**

CAT (E.C. 1.11.1.6) activity in erythrocytes was determined according the protocol of Aebi [18]. The CAT activities determined by the decrease in absorbance at 240 nm when hydrogen peroxide was added to the sample were expressed as g<sup>-1</sup>Hb.

### **2.9 Measurement of Erythrocyte Glutathione Peroxidase (GSH-Px) Activity**

GSH-Px (E.C. 1.11.1.9) activity in erythrocytes was measured according to the Paglia and Valentina method [19]. In this process, when NADPH is oxidized to NADP<sup>+</sup>, oxidized glutathione is turned to the reduced configuration in the presence of glutathione reductase and NADPH. The reduction in the absorbance of NADPH at 340 nm is measured. GSH-Px activity was represented U GSH-Px g<sup>-1</sup> Hb.

### **2.10 Measurement of Erythrocyte Acetyl Cholinesterase (AChE) Activity**

AChE (E.C. 3.1.1.7) activity in erythrocytes was assayed by following the process of Ellman et al. [20]. The catalytic activity at 412 nm is measured. AChE activity was represented U AChE g<sup>-1</sup> Hb.

### **2.11 Preparation of Erythrocyte Ghosts and Measurement of Erythrocyte Malondialdehyde (MDA)**

Erythrocyte ghosts were prepared from washed erythrocytes as stated by Dodge et al. [21]. MDA is a secondary product of LPO. Sodium dodecyl sulphate (SDS) (8.1%) was added to the erythrocyte ghosts, mixed well as well as being incubated at room temperature. The samples to which thiobarbituric acid (0.6%) and acetic acid (20%) were

added were incubated in a water bath at 100°C. Butanol-pyridine (15:1) mixture was used to treat the cooled samples. After centrifugation, the absorbance of the colored layer was recorded at 532 nm. MDA levels were represented as nmol/g Hb [22].

## 2.12 Statistical Analyses

Statistical analysis of the data was made using the SPSS program. Wilcoxon T test was used in depend groups' comparison and means were compared with Friedman test in multiple groups. The results obtained were represented as Mean±SD. The level of significance was set at  $p<0.05$  for all statistical tests.

## 3. Results

### 3.1 Effects of Chondroitin-4-Sulphate (C4S) on Hemolysis

In Table 1, the effects of C4S on percent hemolysis in erythrocytes treated with glucose are shown. The level of percent hemolysis with 20mM glucose didn't determine any significant change compared to erythrocytes treated with 40mM glucose. The addition of C4S (25 and 50 mM) with 20 mM glucose (3. and 4. Groups) significantly decreased ( $p<0.001$ ) percent hemolysis with respect to erythrocytes treated only with 20 mM glucose (1. Group). Similarly, C4S (25 and 50 mM) with 40 mM glucose (5. and 6. Groups) significantly decreased ( $p<0.001$ ) percent hemolysis with respect to erythrocytes treated only with 40mM glucose alone (2. Group).

**Table 1.** Effects of C4S (25 and 50 mM) on percent hemolysis in erythrocytes treated with 20 mM and 40 mM glucose

| Groups   | Hemolysis%              |
|----------|-------------------------|
| 1. Group | 7.70±1.75               |
| 2. Group | 7.82±2.46               |
| 3. Group | 4.76±1.54 <sup>#a</sup> |
| 4. Group | 3.44±1.86 <sup>#a</sup> |
| 5. Group | 5.46±1.99 <sup>3a</sup> |
| 6. Group | 3.19±1.56 <sup>3a</sup> |

<sup>#</sup>: 1. Group with 3. and 4. Group, <sup>3</sup>: 2. Group with 5. and 6. Group, <sup>a</sup>:  $p<0.001$

### 3.2 Hemoglobin (Hb) Content

Hb contents obtained from all samples are shown Table 2. The Hb values were between 11.08-13.10 g/ml.

**Table 2.** Hemoglobin levels in all samples

| Sample No | Hb (g/ml) |
|-----------|-----------|
| 1         | 12.50     |
| 2         | 11.20     |
| 3         | 11.20     |
| 4         | 12.76     |
| 5         | 13.06     |
| 6         | 12.05     |
| 7         | 11.30     |
| 8         | 13.10     |
| 9         | 11.08     |
| 10        | 12.30     |

### 3.3 Effect on Chondroitin-4-Sulphate (C4S) on Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GSH-Px), Acetylcholinesterase (AChE) Activity, Glutathione (GSH) and Malondialdehyde (MDA) Content

SOD activities of erythrocytes in all groups are offered in Table 3. As seen Table 3, these enzyme activities in all groups compared to each other didn't show any significant change.

CAT activities of erythrocytes in all groups are presented in Table 3. There were not any significant changes observed in CAT activities of erythrocytes.

In Table 3, the effect of C4S on GSH-Px activity in erythrocytes treated with glucose is shown. A significant decline in GSH-Px activity was observed with 2. Group (40 mM glucose) compared to 1. Group (20 mM glucose) ( $p < 0.007$ ). The addition of C4S

(25 and 50 mM) with 20 mM and 40 mM glucose significantly increased GSH-Px activity compared to erythrocytes treated with 20 and 40 mM glucose alone ( $p<0.001$ ).

The effects of C4S on AChE activity in erythrocytes treated with glucose are shown in Table 3. A significant reduction in AChE activity was observed with 2. Group (40 mM glucose) compared to 1. Group (20 mM glucose) ( $p<0.005$ ). C4S(25 and 50 mM) with 20 mM and 40 mM glucose significantly increased AChE activity compared to erythrocytes treated with 20 and 40 mM glucose alone ( $p<0.001$ ).

GSH contents of erythrocytes in all groups are shown in Table 3. While the content in the groups applied C4S didn't show any significant change, the content in 40 mM glucose alone (2. Group) compared to 20 mM glucose alone (1. Group) showed a significant decline ( $p<0.009$ ).

In Table 3, the effect of C4S on MDA content in erythrocytes treated with glucose are shown. As shown in Table 3, a significant enhancement in MDA content was determined with 40 mM glucose (2. Group) compared to 20 mM glucose (1. Group). C4S (25 and 50 mM) with 20 and 40 mM glucose caused decreases in MDA content compared to erythrocytes treated with 20 and 40 mM glucose alone.

**Table 3.** Effects of C4S (25 and 5 mM) on SOD, CAT, GSH-Px, AChE activity, GSH and MDA content in erythrocytes treated with 20 mM and 40 mM glucose

| Groups   | SOD        | CAT        | GSH-Px                     | AChE                        | GSH                      | MDA                        |
|----------|------------|------------|----------------------------|-----------------------------|--------------------------|----------------------------|
| 1. Group | 0.91±0.12  | 59.09±4.45 | 0.12±0.001                 | 0.017±0.004                 | 14.41±0.72               | 261.24±41.89               |
| 2. Group | 0.84±0.02  | 57.80±4.56 | 0.06±0.002 <sup>*c</sup>   | 0.013±0.004 <sup>*b</sup>   | 12.24±1.22 <sup>*c</sup> | 357.42±41.22 <sup>*d</sup> |
| 3. Group | 0.94±0.11  | 60.91±2.71 | 0.42±0.01 <sup>#a</sup>    | 0.022±0.003 <sup>#a</sup>   | 15.66±2.10               | 155.62±34.76 <sup>#a</sup> |
| 4. Group | 1.30±0.36  | 59.89±3.48 | 0.32±0.005 <sup>#aad</sup> | 0.027±0.002 <sup>#aaf</sup> | 13.74±2.88               | 130.07±23.12 <sup>#a</sup> |
| 5. Group | 0.92±0.072 | 0.03±3.35  | 0.22±0.012 <sup>3a</sup>   | 0.020±0.004 <sup>3a</sup>   | 15.11±1.85               | 155.52±49.70 <sup>3a</sup> |
| 6. Group | 0.97±0.10  | 59.13±3.17 | 0.07±0.003 <sup>3aβc</sup> | 0.023±0.005 <sup>3a</sup>   | 14.35±2.49               | 188.94±17.54 <sup>3a</sup> |

\*: 1. Group with 2. Group, <sup>α</sup>: 3. Group with 4. Group, <sup>β</sup>: 5. Group with 6. Group, <sup>#</sup>: 1. Group with 3. and 4. Group, <sup>3</sup>: 2. Group with 5. and 6. Group, <sup>a</sup>:  $p<0.001$ , <sup>b</sup>:  $p<0.005$ , <sup>c</sup>:  $p<0.007$ , <sup>d</sup>:  $p<0.008$ , <sup>e</sup>:  $p<0.009$ , <sup>f</sup>:  $p<0.01$

Enzyme activities (U/g.Hb), GSH and MDA contents (nmol/g.Hb)

#### 4. Discussion

Hyperglycemia likely induced oxidative stress owing to surplus oxygen radical production caused by the auto-oxidation of glucose [23]. However, hyperglycemia is known to cause membrane harm and cell death of erythrocytes [24]. In recent years, different substances are investigated their potential antioxidant effects to elimination or prevention of oxidative damage [2]. In the present study, the antioxidant effects of C4S were evaluated on human erythrocytes exposed to high glucose induced oxidative stress. In previous studies, the antioxidant effects of C4S were studied on experimental animals [8, 9]. However, the antioxidant effects of C4S on erythrocytes have not yet been investigated.

To indicate the antioxidant effect of C4S in our study, hemolysis% values were firstly determined in erythrocytes. Hemolysis is due to erythrocytes destruction which resulted from lysis of membrane lipid bilayer [25]. MDA, a highly reactive and bifunctional molecule, a well-characterized product of the LPO of erythrocytes, cross-links erythrocyte phospholipids and proteins to disrupt various membrane-related functions that lead to hemolysis [23]. In this study indicated that the hemolysis (%) of the groups of treated glucose with C4S significantly decreased compared to the groups of treated glucose alone (Table 1). Under the light of these data, C4S having a regenerative effect on the hemolysis% value can be said. In the previous studies, it has been stated that the hemolysis% value in only glucose-treated groups may have been caused by lipid peroxidation or increase of oxidative stress damage [1, 26].

GSH is an antioxidant that is found in abundance in the human body. GSH plays a very important role in the protection of erythrocytes versus the oxidative stress because erythrocytes contain more than 95% excess of blood GSH [27]. While GSH provides the primary antioxidant defense of stored erythrocytes, it can cause an increase in the oxidative modification of membrane lipids and proteins, leading to a weakening of the membrane skeleton thereby compromising erythrocyte survival [23]. GSH acts as oxidant scavenger for elimination LPO and H<sub>2</sub>O<sub>2</sub> [28]. In this study, GSH contents at the group of 40mM glucose (2. Group) were significant decreased compared to the group of 20mM glucose (1. Group) (Table 3). Similar findings were reported in previous studies showing

that glucose contents at high doses reduce GSH levels [27, 29]. The GSH contents in the groups applied C4S didn't show any significant change (Table 3).

The oxidative damage caused by hyperglycemia causes LPO due to increased ROS. Uncontrolled LPO is a toxic process that causes deterioration of biological membranes. LPO products e.g. MDA has accepted as a biomarker to oxidative stress in biological system. The ROS occurred with oxidative stress are scavenged by antioxidant enzymes such as SOD, CAT, GSH-Px and AChE [30]. SOD and CAT are the first line of defense against ROS. The SOD function is to catalyse the change of superoxide radicals to H<sub>2</sub>O<sub>2</sub>. The H<sub>2</sub>O<sub>2</sub> produced by SOD is excreted as H<sub>2</sub>O based on the activity of GSH-Px and CAT [31]. High concentration H<sub>2</sub>O<sub>2</sub> is converted into H<sub>2</sub>O by GSH-Px, low concentration H<sub>2</sub>O<sub>2</sub> is converted into H<sub>2</sub>O by CAT. Another enzyme, AChE, which supports the hydrolysis of the neurotransmitter acetylcholine, has a necessary role in regulating many vital functions, an important event that has been related to pathogenesis and progression of hyperglycemia [32].

In this study, any significant changes in SOD and CAT activities didn't showed in all groups compared to each other's (Table 3). The role of antioxidant defense systems in hyperglycemia is controversial [4]. There are reports of both increased [33] and decreased [34, 35] SOD and CAT activities in hyperglycemia, while a few studies couldn't find any significant change SOD and CAT activities [36, 37]. When compared to only glucose given groups, the group with higher glucose (40mM) GSH-Px and AChE enzymes was inhibited these two enzymes. The low GSH content in hyperglycemia can reduce GSH-P activity because GSH is the cofactor and substrate of this enzyme [4]. In the studies conducted by De Bona et al. [32], contrary to our findings, increasing AChE activity during high glucose levels has been reported. When groups treated with C4S as well as glucose compared to groups treated with only glucose, activity of the GSH-Px and AChE enzymes have been reported to increase (Table 3). In many of the researches done, the activity measurements of antioxidant enzymes giving an idea of on oxidative stress and the damage caused by it have been illustrated [19, 28, 38, 39].

In our study when we compared the MDA values between groups, the high value corresponded to C4S usage as well as glucose while only glucose resulted in low C4S levels ( $p < 0.001$ ). At the same time, if we had compare the groups with different glucose

levels, the one that has been applied more glucose showed more MDA than the one applied less glucose (Table 3). As with many other studies done in the past as well as in our research, higher glucose levels indicated higher MDA level [10, 20, 39, 40]. Because high glucose levels can lead to oxidative stress and generation of MDA [24]. The findings that we have obtained indicate towards the fact of C4S can be used to reduce the LPO caused by oxidative stress by glucose.

## 5. Conclusion

C4S treatment should be considered in the treatment of hyperglycemia. C4S supplementation can be beneficial for human in order to reduce the harmful effects of hyperglycemia, such as oxidative damage.

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