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Age-related changes in haptoglobin phenotypes, some non-enzymatic antioxidants and electrophoretic profiles of serum proteins in rats

[Ratlarda serum haptoglobin fenotipi, bazı nonenzimatik antioksidan ve elektroforetik profilin yaşlanmaya bağlı değişimi]

Gülay Ciftci[1],
Gül Fatma Yarım[1],
Sena Cenesiz[1],
Arzu Fındık[2]

University of Ondokuz Mayis, Departments of [1]Biochemistry and [2]Microbiology, 55210, Samsun, Turkey

Yazışma Adresi
[Correspondence Address]

Gülay CIFTCI

Department of Biochemistry
Faculty of Veterinary Medicine
University of Ondokuz Mayis
55139, Samsun, Turkey
Tel. +90 362 3121919-3908
Fax. +90 362 4576922
E-mail. gciftci@omu.edu.tr

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ABSTRACT

Aim: Determination of age-related changes in levels of some non-enzymatic antioxidants such as serum haptoglobin, haemoglobin and albumin, serum haptoglobin phenotype and serum protein profile was aimed with this study.

Methods and results: Fifty Sprague-Dawley male rats were divided equally into five groups as 1 (one-month old), 2 (six-months old), 3 (twelve-months old), 4 (eighteen-months old) and 5 (twenty-four-months old). The serum protein concentrations were measured by autoanalyzer. A decrease was determined in the concentrations of the total protein and albumin related to age. However, no clear decrease in the serum globulin concentrations occurred. Serum haptoglobin concentrations were measured by ELISA and an increase was detected with age. Serum haemoglobin concentrations were increased up to 3 months old, but decreased from 3 to 24 months old of age. The electrophoretic profiles of serum total protein were determined by native-PAGE. No differences were observed in total serum profiles between age groups. To determine serum haptoglobin profiles, western blot was performed and haptoglobin patterns were designated as "aa" (single band) or "ab" (two bands) patterns. The "ab" pattern was more frequently observed in older rats. Also, 15 protein bands ranged between 19 to 244 kDa, were detected by SDS-PAGE. After SDS-PAGE, western blot were performed and three haptoglobin bands (10, 37 and 47 kDa) were detected.

Conclusion: The age-related changes in total serum protein, albumin, haemoglobin and haptoglobin concentrations as well as the prevalent haptoglobin phenotypes may be considered as indicators in some age-related diseases.

Key Words: aging, serum protein, haptoglobin, haemoglobin, rat

Conflict of Interest: The authors declare that there was no conflict of interest in this work.

ÖZET

Amaç: Bu çalışmada serum haptoglobin, hemoglobin, albumin gibi non enzimatik antioksidant düzeyleri ile serum haptoglobin ve serum protein profilinin yaşlanmaya bağlı olarak değişimlerinin belirlenmesi amaçlanmıştır.

Yöntem ve Bulgular: Çalışmada 50 adet Sprague-Dawley ırkı erkek rat kullanıldı. Ratlar her grupta 1, 6, 12, 18 ve 24 aylık 10'ar hayvan olacak şekilde 5 gruba ayrıldı. Serum protein ve hemoglobin konsantrasyonları otoanalizörde ölçüldü. Total protein ve albümin konsantrasyonlarının yaşlanmaya bağlı olarak anlamlı bir azalma olduğu belirlendi. Serum globülin düzeyinde ise anlamlı bir değişiklik yoktu. Serum haptoglobin konsantrasyonları ELISA ile ölçüldü ve yaşlanmaya bağlı olarak anlamlı bir artış gösterdiği belirlendi. Serum hemoglobin konsantrasyonunun 3 aylık yaşa kadar artış gösterdiği ve sonrasında düşüş gösterdiği belirlendi. Serum total protein profilinin elektroforetik olarak belirlenmesi amacıyla natif-PAGE yapıldı ve yaş grupları arasında farklılık görülmedi. Serum haptoglobin profilinin saptanması için natif-PAGE sonrasında western blot yapıldı. Haptoglobin paternleri tek (aa) ya da çift (ab) bant görülme kriterine göre değerlendirildi ve yaşlı ratlarda "ab" profili daha çok görüldü. SDS-PAGE yöntemi ile de 244 ile 19 kDa arasında değişen 15 tane protein bantı görüldü ve sonrasında yapılan western blot yönteminde 10, 37 ve 47 kDa ağırlığında haptoglobin bantları belirlendi.

Sonuç: Total serum protein, hemoglobin ve haptoglobin konsantrasyonlarının ve profilinin yaşa bağlı olarak değişim gösterdiği ve yaşlanmaya bağlı olarak gelişen hastalıklarda belirteç olarak kullanılabileceği kanaatine varıldı.

Anahtar Kelimeler: serum protein, yaşlanma, haptoglobin, hemoglobin, sıçan

Çıkar Çatışması: Yazarlar bu çalışmada hiçbir çıkar çatışması bulunmadığını beyan ederler.

Introduction

Aging is defined as the progressive accumulation of diverse deleterious changes in cells and tissues with advancing age that increase the risk of disease and death [1]. One of the aging theory is "Free Radical Theory" that is the most popular theory defined by Dr. Denham Harman [2]. According to this theory, one of the causes of aging is oxidative stress.

Antioxidants have a role in protection against oxidative stress. Non-enzymatic antioxidants, a group of antioxidants, include haptoglobin (Hp), haemoglobin and albumin. These non-enzymatic antioxidants protect cells from harmful effects of free radicals by capturing them within cells [3]. Albumin is found in the circulatory system, abundantly [4]. It has a 66 kDa of molecular weight and carries fatty acids, cholesterol, bile pigments, and drugs in the organism. Also, it plays role in regulating the osmotic pressure [5].

Haemoglobin is the iron-containing protoporfirin and composed of four -hem and a globin. Haemoglobin can bind oxygen reversibly and four oxygen molecules can bind to each haemoglobin. It is responsible for the transport of oxygen to tissues. It plays both oxidant and antioxidant roles in the organism. In a tetrameric form, it is an oxidant because oxygen can only bind iron molecule [6]. It has been reported that the antioxidant nature of haemoglobin is based both on its peroxidase activity and its interaction with the superoxyde anion [7].

Haptoglobin is a tetrameric plasma protein that binds haemoglobin with high affinity. It is a strong polymorphic antioxidant and produced mostly by liver [8]. Hp can be used both as a diagnostic and prognostic marker [9]. Three phenotypes (Hp1-1, Hp2-2 and Hp2-1) of Hp have been identified in humans. It has been reported that the Hp phenotype identified in rats is similar to Hp1-1 phenotype in human and had a heterotetrameric ($\alpha 2\beta 2$) structure [10]. The structural and functional differences between Hp phenotypes in humans have important biological and clinical consequences. Hp polymorphism has been reported to associate with the prevalence and clinical evolution of several infectious diseases and autoimmune disorders [11].

In this study, it is aimed to investigate the age-related changes in concentrations of some non-enzymatic antioxidants, haptoglobin phenotypes and serum electrophoretic profiles in rats.

Material and Methods

Animals

Fifty Sprague-Dawley male rats were supplied from the Center of Medicinal and Surgical Research, Ondokuz Mayıs University. Ethical approval for the study was received from the Laboratory Animals Local Ethical Committee (HEK/214/28.12.2006). Experimental groups of rats were divided as follows: 10 rats of one-month-old, 10 rats of six-months-old, 10 rats of twelve-months-old, 10 rats of eighteen-months-old and 10 rats of twenty four-months-old. Pre- experimental conditions were accepted as optimal zone for rats such as temperature (23 ± 2 °C), humidity (50 ± 10 %) and light (12 h light/dark cycles). All animals were allowed free access to standard chow and ad-lib freshwater. Same management condition had been applied for all experimental groups during the experimental period.

Serum samples

Blood samples were collected from all rats of each group and centrifuged at 1500 x g at 4 ^{o}C for 10 min. The sera were stored at -80 ^{o}C until assayed.

Measurement of serum haptoglobin, haemoglobin, albumin, globulin and total protein

Serum haptoglobin concentrations were determined by highly sensitive two-site enzyme linked immunoassay (ELISA) using commercial kit (SCY Tek, SHP-IFU).

Haemoglobin, albumin, globulin and total protein in sera were measured in autoanalyzer (Autolab, AMS Srl, Autoanalyzer, Netherlands) with using commercial autoanalyzer test kits (Audit Diagnostics, Ireland).

Determination of serum protein profiles and haptoglobin phenotypes

Serum protein profiles were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE [12]. Serum haptoglobin phenotypes were determined by western blot method after SDS-PAGE and native electrophoresis [13].

SDS-PAGE of serum samples

Serum samples were diluted with saline solution and mixed with sample buffer (0.062 M Tris- HCl, pH 6.8, 10 % glycerol, 2 % sodium dodecyl sulfate, 5 % β-mercaptoethanol and 0.002 % bromophenol blue), then heated for denaturation in boiling-water-bath. The protein concentrations of sera were determined with spectrophotometer and equalized before used. Twenty µl of samples were loaded on two separate stacking gels (4 %). Proteins were separated under reducing conditions on 7.5 % SDS-PAGE gels and run for about 1.5 h at 100 V. One of these gels was stained by Blue Silver staining according to standard protocols [14] and the other gel was transferred to polyvinyl difluoride membranes (PVDF) (Millipore) for western blotting. Molecular mass standards (Sigma, S8445) or prestained marker (Sigma, SDS7B2) were run in parallel in order to calculate the molecular weights of proteins by using Molecular Imaging Software (Kodak).

Native-PAGE

Six µl serum samples were mixed with 1 µl of a 10 % haemoglobin solution and 20 µl of the sample buffer (0.062 M Tris- HCl, pH 6.8, 10 % glycerol and 0.002

% bromophenol blue). The mixtures were incubated for 5 min at room temperature to permit formation of Hp-haemoglobin complex. The samples were run on two separate 7.5 % native acrylamide gels without boiling at 20 mA. Then, one of these gels was stained with Blue Silver and the other gel was transferred to PVDF at 85 mA for 45 min.

Western Blotting

After SDS- and native-PAGE, gels were transferred to PVDF membranes according to a standard procedure as previously described [15]. After the transfer process, PVDF membranes were blocked with blocking buffer containing 5 % skimmed milk (w/v), 0.1 % Tween 20 in PBS (pH 7.4) for 1 h at room temperature. After blocking, the membranes were incubated with a primer rabbit polyclonal antibody against human haptoglobin (rabbit anti-human haptoglobin immunoglobin G) diluted 1:1000 in blocking buffer for 2 h at room temperature and washed. Then, the membranes were incubated with anti-rabbit Ig peroxidase conjugate (Sigma) diluted 1:3000 in blocking buffer at room temperature and washed with PBS (pH 7.4) containing 0.1 % Tween and finally the bands were visualized with a substrate, 3,3'-diaminobenzidine (DAB).

Statistical analysis

The analysis of variance was used to compare the concentrations of haptoglobin, haemoglobin, albumin, globulin, total protein and albumin/globulin in serum from rats of different ages. Duncan test was used for the statistical significance of differences among the groups' means. Pearson Correlation Test was used to determine the relationship between serum haemoglobin and haptoglobin.

Results

Serum haptoglobin concentrations were found as 2.0 (± 0.2), 2.5 (± 0.2), 2.7 (± 0.1), 2.9 (± 0.2), 3.5 (± 0.6) ng/ml in 1, 6, 12, 18 and 24 months old rats, respectively ($P<0.05$). Serum haemoglobin concentrations were found as 62.4 (± 8.6), 82.9 (± 9.2), 67.3 (± 7.3), 63.4 (± 7.9) and 33.1(± 2.1) g/dl in 1, 6, 12, 18 and 24 months old rats, respectively. These results were summarized in Table 1. According to Pearson Correlation Test results, there was a negative correlation between the serum haemoglobin and haptoglobin values ($r = -0.298$) ($P<0.05$). The results of the variance analysis and Duncan's Test to evaluate the relationship between age groups were summarized in Table 1.

In the process of aging, the concentration of total protein, albumin, globulin were found as 7.2 (± 0.08), 3.4 (± 0.05), 3.78 (± 0.1); 7.3 (± 0.25), 3.2 (± 0.08), 4 (± 0.05); 7.0 (± 0.11), 3.2 (± 0.05), 3.7 (± 0.11); 6.5 (± 0.07), 3.1 (± 0.04), 3.3 (± 0.2); 6.3 (± 0.07), 2.84 (± 0.07), 3.4 (± 0.1) in 1, 6, 12, 18 and 24 months-old rats, respectively. These results were presented in Table 1.

Serum protein profiles were determined by SDS-PAGE and a total of 15 bands with molecular weights of about 19, 42, 47, 52, 54, 57, 65, 88, 97, 112, 128, 156, 184, 216 and 244 kDa were observed. The densities of these bands were observed to decrease by aging.

In native serum protein profile, pre-albumin, albumin, α-1 globulin, α-2 globulin, β-1 globulin, β-2 globulin and gamma-globulin fractions were clearly observed.

Western Blot Analyses were performed after SDS- and native-PAGE. In western Blotting performed after SDS-PAGE, three bands with molecular weights of 10, 37 and 47 kDa were observed. There were no differences in the number and/or molecular weights between age groups. But, it was observed that the densities of the bands increased by aging. On the other hand; two bands were observed and called "a" and "b" in Western Blotting performed after native-PAGE. According to include "a" and/or "b" bands, two profiles were seen. Having only "a" band was called as "aa" and having both "a" and "b" band was called as "ab". In the study, "aa" band profile was more frequent in young rats than older rats (Table 1 and Figures 1-4).

Discussion

Haptoglobin is a variety of plasma N-glycoprotein. It has a clinical importance, since the levels of haptoglobin increase in various pathological conditions both in human and animals [16,17]. In several studies, a positive relationship was reported between the increase in Hp and aging [18,19]. Compared to the reference value for adults, the haptoglobin concentration was found as low in the young [20]. In parallel to these studies, we found that the level of haptoglobin in rat sera increased with aging, significantly ($P<0.05$).

Haemoglobin concentration may show variability according to age, sex, and race as well as by altitude [21]. It has been suggested that the variation of haemoglobin in rats is similar with the variations in the human. Several studies indicated a decrease in the level of serum haemoglobin with aging. In this study, we observed that the level of haemoglobin increased up to 3 months-old, but its level decreased from 3 to 24 months old [22-24]. We also found a negative correlation between haemoglobin and haptoglobin concentrations during aging ($P<0.05$).

The concentrations of the total serum protein, albumin and globulin changes in normal aging duration. It has been reported that the concentrations of serum albumin and total protein decreased, however the concentrations of some globulins increased in aging process [25,26]. In our study, no significant decrease in the serum globulin concentration was determined. We found that the total protein, albumin and albumin/globulin levels decreased with aging and especially the decrease in 24 months-old group was significant ($P<0.05$). Serum albumin is synthesized in the liver, and a number of potential associations between its plasma concentrations and mortality have been observed [27]. Recent observation

Table 1. Serum albumin, globulin, total protein, albumin/globulin, haptoglobin and haemoglobin concentrations of rats with different ages and percentage of haptoglobin phenotypes in groups (mean ± S.D).

	1 month	6 months	12 months	18 months	24 months	F value
Hg concentration (g/dl)	62.4±27.4 [a]	82.9±29.2 [a]	67.3±23 [a]	63.4±25 [a]	33.1±6.7 [b]	5.780
Hp concentration (ng/ml)	2.0±0.8 [a]	2.5±0.8 [ab]	2.7±0.6 [ab]	2.9±0.7[ab]	3.5±0.6 [b]	2.332
Albumin (g/dl)	3.4±0.1 [a]	3.2±0.2 [ab]	3.2±0.1 [b]	3.1±0.1 [b]	2.84±0.2 [c]	12.363
Globulin (g/dl)	3.7±0.3[ab]	4.0±0.1 [c]	3.7±0.3 [bc]	3.3±0.2 [d]	3.4±0.3 [ad]	7.188
Total protein (g/dl)	7.2±0.2 [ab]	7.3±0.2 [b]	7.0±0.3 [a]	6.5±0.1 [c]	6.3±0.2 [c]	26,8
Albumin/globin(g/dl)	0.9±0.1 [b]	0.8±0.0 [ab]	0.6±0.3[a]	0.8±0.3 [ab]	0.04±0.1[ab]	1.920
Hp “aa” (n)	8/10	5/10	3/10	3/10	2/10	
Hp “ab” (n)	2/10	5/10	7/10	7/10	8/10	

a,b; the difference between the mean values bearing the different letters in the same row is statistically significant (P<0.05)

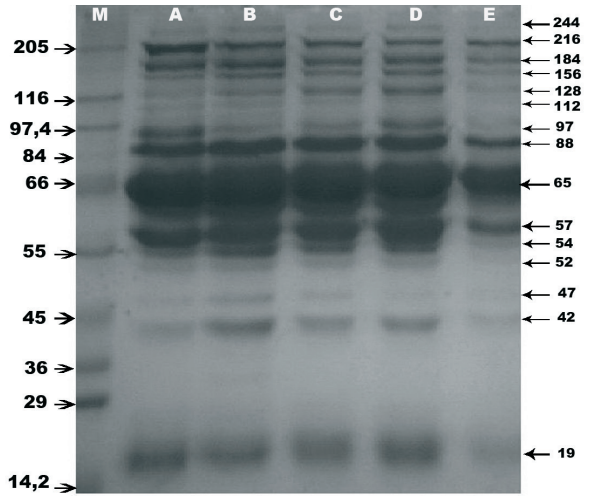

Figure 1. Protein profiles of the representative serum samples of rats generated on 7.5% SDS-PAGE. A:1 month old, B: 6 months old, C: 12 months old, D: 18 months old, E: 24 months old, M= marker

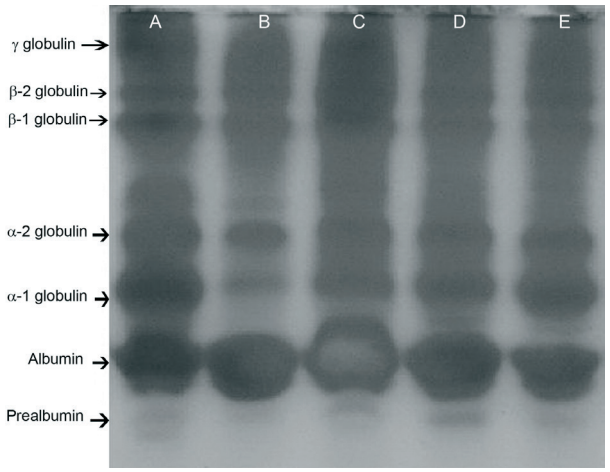

Figure 2. Protein profiles of the representative serum samples of rats generated on native PAGE. A: 1 month old, B: 6 months old, C: 12 months old, D: 18 months old, E: 24 months old

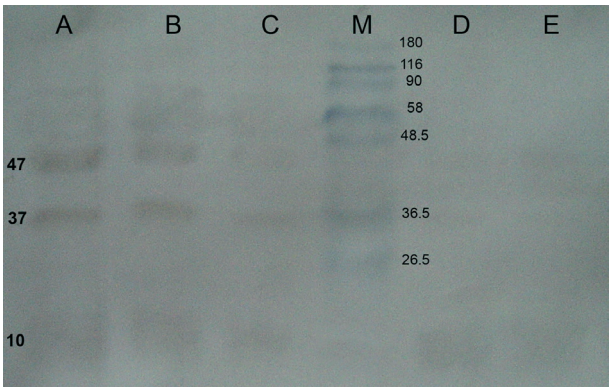

Figure 3. Western blot analysis (after 7.5% SDS-PAGE) of haptoglobin in rat serum pools. A: 24 months old, B: 18 months old, C: 12 months old, D: 6 months old, E: 1 month old

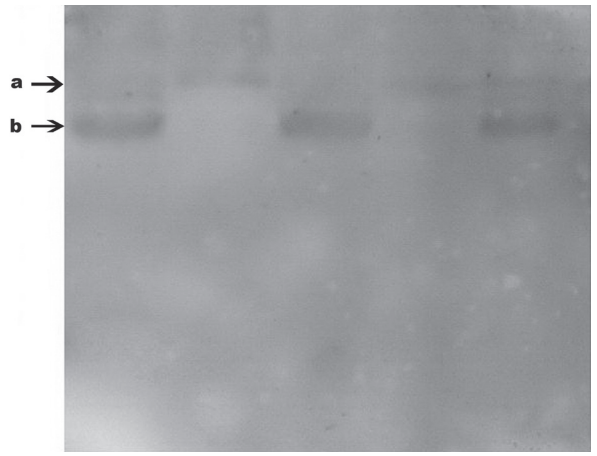

Figure 4. Western blot analysis (after 7.5% native PAGE) of haptoglobin in rat serum pools. ab: western blot profile having both a and b bands, aa: western blot profile having only a band.

revealed that serum albumin plays an important role in the host defense mechanisms. It is one of the important and most abundant antioxidants in vivo. Low albumin concentrations was stated as a marker of protein malnutrition and this situation commonly observed in older persons [28]. In parallel to our study, it was reported in human that albumin was decreased significantly at the age of 65 and over [27]. The decrease in the concentration of serum albumin in the aging process may be associated with the decrease in the weight of the liver and the hepatocyte amount. Furthermore, this event is also associated with the decreasing of total serum protein because albumin forms the large proportion of all serum protein.

Total serum profile was determined by both SDS- and native-PAGE. In both SDS-PAGE and native-PAGE, there were no differences seen in the number of protein bands but the densities of these bands were found to be decreased with aging. In native-PAGE, the fastest proteins running on the gel were pre-albumin and albumin and it was followed by α-1 globulin, α-2 globulin, β-1 globulin, β-2 globulin and gamma-globulin. This was consistent with the results of the study performed by McCormick *et al.* [29].

In human, three main haptoglobin phenotypes were known. Among them, Hp1-1 has been reported to be better antioxidant than other phenotypes. However the angiogenic effect of Hp 1-1 has been reported to be weaker than the other phenotypes [30]. Hp1-1 phenotype has been found to be more common in some cancer types, some haematological diseases and allergies [31]. Haptoglobin phenotypes identified in rats has been reported to be similar with Hp1-1 phenotype in human and have a heterotetrameric (α2β2) structure [32,33]. In rat haptoglobin, haptoglobin α-subunit is lack of carbohydrates ($M_{r=}$ 9,500 kDa) while β subunit contains several asparagin-linked carbohydrate side chains ($M_{r=}$ 37,500 kDa). Hanley *et al.* [34] have reported that the molecular weight of α subunit and β subunit of prohaptoglobin, were about 48, 38 and 9.5 kDa, respectively. In their study, 10 and 40 kDa bands corresponding to α- and β-chains of haptoglobin, respectively, have been obtained in western blot analysis of rat plasma. In western blot analyses performed under denatured conditions, we found that the densities of haptoglobin bands increased with aging whereas there was no difference determined in the number of bands. In western blot analyses performed after native- PAGE analysis, two haptoglobin bands referred as a and b were obtained and it was shown that "aa" profile was the most common profile in young rats and "ab" profile increased with age. No literature considering the determination of haptoglobin phenotypes by western blot analysis performed after SDS/native- PAGE could be found.

In conclusion, the age-related changes in the concentrations of albumin, total protein, haemoglobin and haptoglobin as well as the prevalent haptoglobin phenotypes may be considered as indicators in some age-related diseases. Thus, investigation of albumin level with aging becomes an important marker for promoting health among elder persons. Furthermore, it was showed that western blot analysis performed after native-PAGE was a useful method for determining the haptoglobin phenotypes. Age-related variations of these values can be used by clinicians with an additional diagnostic aid.

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Conflict of interest

The authors declare that there was no conflict of interest in this work.

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