ASSESSMENT OF AGE-RELATED CARDIO-VASCULAR DISEASE USING REDOX STRESS MARKERS

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Abstract

The general ageing of the population is considered one of the most important issues of the current medical research, several studies being devoted to finding the parameters that would allow a precise evaluation of the health status of the patients. Cardio-vascular disease (CVD) is the main co-morbidity of the ageing process. Glycation of proteins and redox mechanisms are mainly involved in its development. In the present study we were focused on evaluating the level of advanced glycation end products (AGEs) as well as the susceptibility of serum lipids to induced peroxidation in samples from patients diagnosed with cardio-vascular disease compared to controls. Results show that the two evaluated markers are significantly increased in CVD patients.

Rezumat

Îmbătrânirea generală a populaţiei este considerată una dintre problemele medicale majore în cercetarea medicală; în prezent se află în desfăşurare numeroase studii care au ca scop identificarea unor markeri care să permită evaluarea cât mai precisă a stării de sănătate a pacienţilor vârstnici. Maladiile cardio-vasculare reprezintă principala co-morbiditate asociată îmbătrânirii. Glicarea proteinelor şi mecanismele redox sunt direct implicate în dezvoltarea acestor afecţiuni. În prezentul studiu ne-am propus evaluarea nivelului produşilor finali de glicare avansată precum şi a susceptibilităţii lipidelor serice la peroxidarea indusă exogen, la pacienţi diagnosticaţi cu maladii cardio-vasculare, comparativ cu subiecţi de control. Rezultatele obţinute au demonstrat că cei doi markeri sunt semnificativi crescuţi la pacienţii cu boală cardio-vasculară.

Keywords: ageing, glycation, lipid peroxidation, fluorimetric probes

Introduction

Ageing is commonly characterized as a progressive impairment of function, resulting in an increased vulnerability to environmental challenge and a growing risk of disease and death; the so-called "geriatric impairments" in vision, hearing, mobility and cognition become increasingly prevalent with age [1].

According to current World Health Organization (WHO) statistical analysis, around 8% of the world's population is aged over 65 years, but this proportion varies between nations, being higher in countries with increased income (over 20% in Japan). The proportion of those aged over 65 years,

and over 80 years respectively, is projected to globally increase substantially within the next 20 years. In China alone, the absolute number of individuals aged over 65 years and 80 years is expected to be over 330 million and 100 million by the year 2050 [2]. WHO pointed out, at the end of 2011 that over 2 billion people worldwide will be over 60 years old by 2050.

The incidence of disease, particularly chronic disease, for which increasing age is an independent risk factor, is much higher in the aged population; also, in older adults, multi-morbidity is common.

Cardiovascular disease is the major cause of morbidity and mortality worldwide; age is a major risk factor for cardiovascular disease and older age is associated with an increasing prevalence of coronary artery disease, heart failure and cerebrovascular disease [2, 3].

Glycation is the major cause of spontaneous damage to cellular and extracellular proteins in physiological systems; advanced glycation end products (AGEs) form when proteins interact with aldose sugars for an extended period of time (weeks, months). There are multiple types of glycation adducts identified *in vivo* in tissues and blood or other body fluids; they form as a result of a nonenzymatic reaction between reducing sugars and amino groups. The initial glycation reaction is followed by a cascade of chemical reactions resulting in the formation of intermediate products (Schiff base, Amadori and Maillard products) and finally to a variety of derivatives named advanced glycation end products (AGEs). In hyperglycemic environments and in natural aging, AGEs alter cell structure and function [4].

Protein glycation is exacerbated in diabetes as a consequence of the increase in glucose in plasma and at the sites of vascular complications. Increased levels of AGE are also associated with macular disease, arthropaties, Alzheimer disease, cancers, etc. Specifically, AGEs are considered to be formed slowly and in small quantities throughout life and their concentration in the blood and tissues is considered to represent the life-long accumulation of glycation adducts. So, the AGE level is associated with the ageing process.

The AGEs level in serum originates either from diet or from endogenous production; the AGE-restricted dietary intervention has been reported to be an effective inhibitor of atherogenic processes in hyperlipidemic apolipoprotein E–deficient mice, or diabetes patients [2].

In this context, the aim of the study was to assess the relevance of AGEs level in serum as well as the susceptibility of serum lipids to induced peroxidation in the evaluation of patients with age-related cardio-vascular disease.

Materials and Methods

Study design

We selected 40 patients:

- 30 patients diagnosed with cardiovascular diseases (CVD), aged 60 to 86, and
- 10 control healthy subjects, ages 17 to 40 years old.

The patients were selected from Humanitas Medical Bucharest. The diagnosis for CVD (cardio-vascular disease) was established after clinical and imagistic evaluation. Patients with severe renal, hepatic or hematological disease, or malignancy were excluded from the study. None of the subjects had taken known antioxidants containing supplements (vitamin C, vitamin E, probucol, etc). Informed consent was obtained from each subject participating in the study. The protocol was approved by the ethics committee of Humanitas Medical Bucharest.

From the selected patients, fasting venous blood samples were drawn without using any anticoagulant, and the serum was separated by centrifugation.

Biochemical evaluation. Fasting serum glucose (Gli), total cholesterol (TC), HDL-cholesterol and triglycerides (TG) were assayed on an Olympus 400 analysor with BIORAD and RANDOX commercial kits.

Measurement of lipid susceptibility to peroxidation.

DPPP (diphenyl-1-pyrenylphosphine, Molecular Probes, Invitrogen Inc.) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mM. After addition of DPPP at a final concentration of 5 μM, serum samples were incubated at room temperature, for 20 minutes in the dark. DPPP labeled samples were treated with 10μM cumene peroxide to induce DPPP-oxide formation. The process was assessed by registering the fluorescence emission spectra between 360nm and 410nm, with excitation at 351nm, every minute, for 5 minutes. Maximum fluorescence intensity was measured at 380nm [5].

Evaluation of AGEs. The plasma level of advanced glycation end products (AGEs) was evaluated using a fluorimetric method, since AGEs are fluorescent markers. Plasma samples were diluted 1:20 with PBS, excited at 350 nm. The emission was registered between 350nm and 550nm, with the maximum at 450nm. Fluorescence of PBS alone was subtracted from each data set [6].

Statistical Analysis Results are expressed as means ± standard deviation (SD). Differences between groups and comparisons between healthy controls and CVD patients were evaluated using *t Student* test. Differences were considered significant for $p < 0.05$.

Results and Discussion

For the recruited patients, we evaluated the general metabolic profile (Table I).

Table I

Biochemical parameters evaluated for the control group as well as for the patients with cardio-vascular disease (HDL – high density lipoproteins, LDL-low density lipoproteins)

Biochemical parameter	Control group	Cardio-vascular disease group
Fasting serum glucose (mg/dL)	88.62±23.49	119.82±56.02*
Total cholesterol (mg/dL)	188.38±35.68	192.55±41.85
HDL/cholesterol (mg/dL)	59.38±19.52	52.53±14.09
LDL/cholesterol (mg/dL)	102.58±21.33	115.47±34.91
Triglycerides (mg/dL)	123.00±29.93	150.37±74.28
Uric acid (mg/dL)	4.39±1.02	4.92±1.13

*$p<0.05$

The statistical analysis of the results revealed the fact that the only parameter that is significantly different between the two groups is the fasting serum glucose level. The parameters of the lipid metabolism had similar values for the two groups, and did not constitute an indicator of the cardio-vascular status for the analyzed patients.

Several methods for the evaluation of lipid peroxidation are mentioned in the literature, the most frequently used being the evaluation of malondialdehyde (MDA), 4-hydroxynonenal, isoprostanes, or the evaluation of oxidized LDL particles [7].

Recent literature mentions DPPP (diphenyl-1-pyrenylphosphine) as a fluorescent marker that allows the evaluation of the lipid peroxidation processes kinetics *in vitro*. DPPP has been used for hydroperoxide analysis in different models (imaging, HPLC, electrophoresis, etc), because DPPP-oxide that results under the effect of peroxides is highly fluorescent [8].

In the present study we aimed at evaluating the susceptibility of plasma lipids and lipoproteins to induce lipid peroxidation using DPPP. The proposed method is characterized by low-cost and simplicity. Diphenyl-1-pyrenylphosphine (DPPP) is a probe that lacks fluorescence and directly reacts with lipids; conversely, the DPPP-oxide, which is specifically generated in reaction with hydroperoxides (generated from lipids, under the effects of exogenously added prooxidants), has a strong fluorescence. According to literature data, cumene hydroperoxide (CuOOH) generates the most effective reaction with DPPP in the lipid membranes, compared to other oxidative stress generating compounds; for example, hydrogen

peroxide oxidizes DPPP at a very small extent and is not useful for inducing oxidative stress in the case of DPPP assay [9].

In the present study we used diphenyl-1-pyrenylphosphine DPPP as a fluorescent probe for lipid peroxidation in lipoprotein rich serum samples for the selected patients.

The kinetics of DPPP-oxide pointed out a higher tendency to oxidation in the case of samples isolated from CVD group (figure 1).

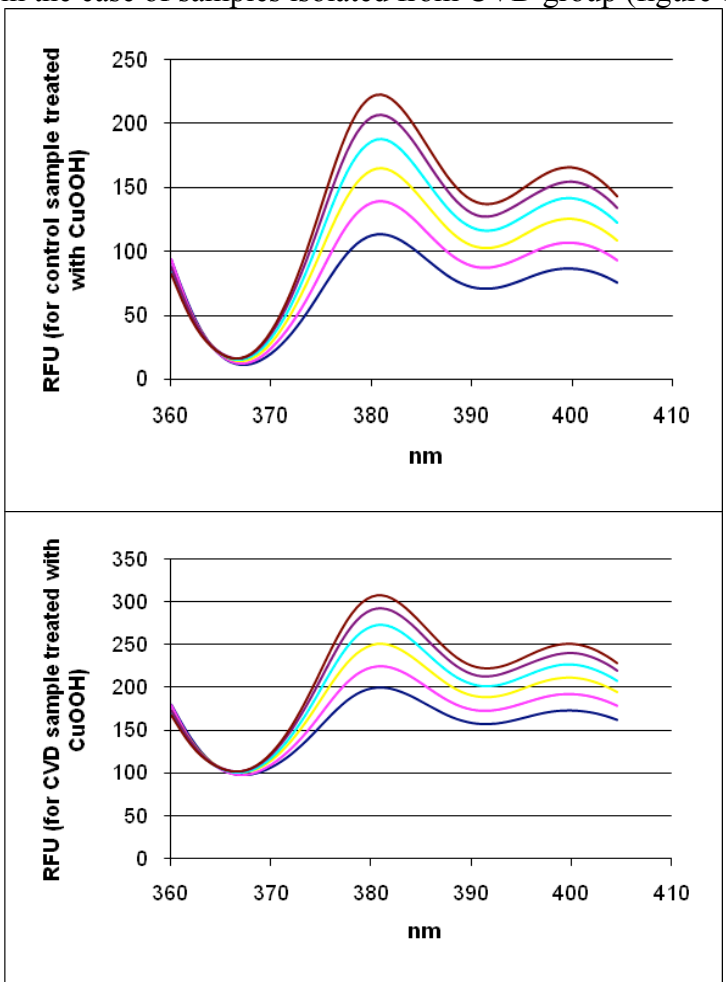

Figure 1

Relative fluorescence units (RFU) coresponding to DPPP-oxide kinetics for one sample in the control group and one in the CVD group, after CuOOH (cumene hydroperoxyde) oxidantion has been induced.

The evaluation of the total level of hydroperoxides generated in serum samples, after treatment with CuOOH (5 minutes), for the control and

CVD groups revealed the fact that the fluorescence intensity is significantly higher for the pathological group, at 380nm.

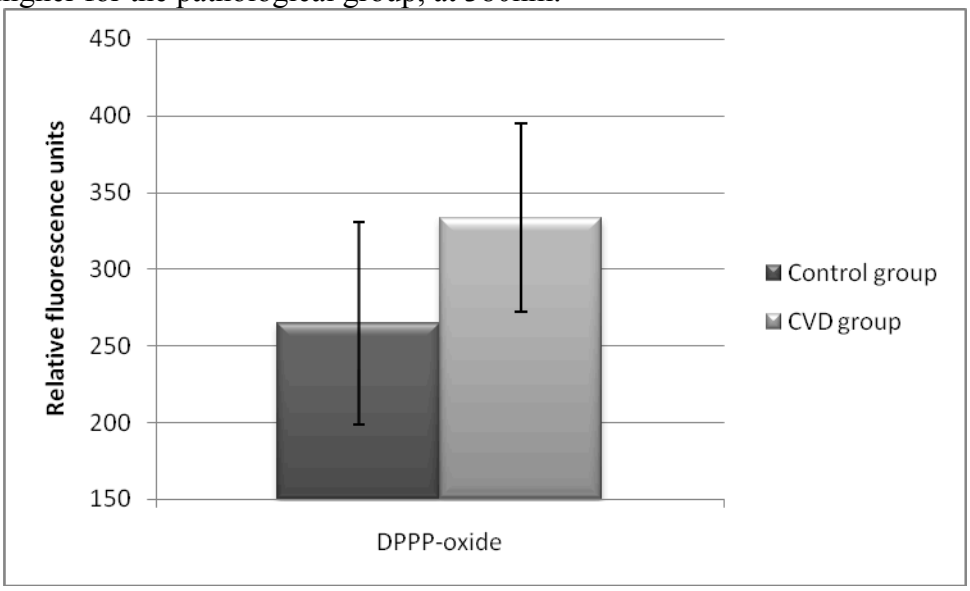

Figure 2

The level of hydroperoxides (evaluated as diphenyl-1-pyrenylphosphine –oxide, DPPP-oxide) generated in serum samples isolated from control subjects is significantly higher compared to cardio-vascular (CVD) ones (p=0.032)

We can conclude that the susceptibility of serum lipids and lipoproteins is significantly impaired in CVD patients, probably due to the depletion of lipid antioxidants. Also, the statistical analysis of the data revealed that there is a positive, significant correlation between the total cholesterol level and the DPPP-oxide generated in the serum samples ($r=0.308$, $p<0.01$). This might be interpreted in view of the fact that this method for the evaluation of lipid susceptibility to induced peroxidation is reliable for the assay of the peroxidation of cholesterol rich particles.

The assay of the AGEs level showed that the formation of advanced glycation end products is significantly higher in the case of CVD patients, compared to controls.

According to literature data, advanced glycation end products (AGEs) generated in excess have been identified as having proatherogenic properties, through redox-dependent oxidant stress mechanisms [2].

AGEs accumulate in the vessel wall especially in diabetes but also in euglycemic situations, in the latter case, driven by oxidative stress.

Studies show that AGEs impair the vascular function by accumulating in the vessel wall and by quenching the nitric oxide released

as endogenous vasodilatatory and antythrombotic molecule, thereby potentially impacting on vascular relaxation and function. AGE-bound to their specific receptors (RAGE) in the endothelium induce the production of reactive oxygen intermediates, this phenomenon being triggered, at least in part, through the activation of NADPH oxidase [10, 11, 12].

The results obtained for the AGEs level are correlated with that for DPPP-oxide, confirming the fact that AGE act as lessional agents at the vascular level through redox mechanisms.

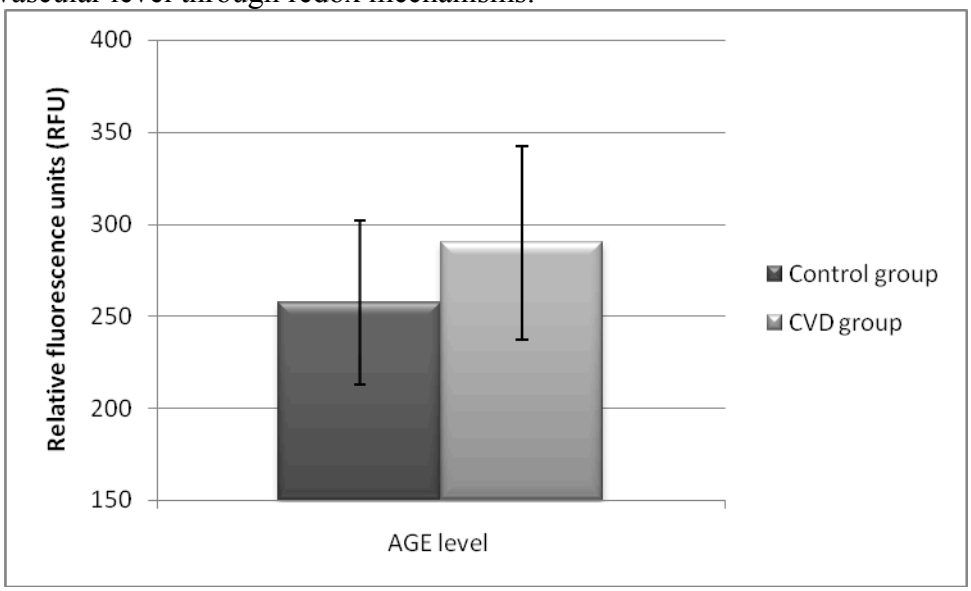

Figure 3
The advanced glycation end products (AGEs) is significantly higher in the patients with cardio-vascular disease compared to controls (p=0.036)

Conclusions

The two parameters (AGE and DPPP-lipid peroxidation) are higher in the patients with cardio-vascular disease and can be used for the assessment of the risk CVD, especially in aged patients. The proposed fluorimetric methods proved to be very sensitive for the evaluation of the redox status in the biological samples.

The method proposed for the evaluation of serum lipid and lipoprotein susceptibility to induced peroxidation is sensitive and reliable for the assay of the peroxidation of cholesterol rich particles.

In hyperglycemic environments and in natural aging, AGEs alter cell structure and function.

Understanding AGE formation and biochemistry as well as AGE-induced effects on extracellular and intracellular functions will serve in the process of finding effective therapies that block excessive accumulation of these species and their pathological outcomes.

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