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AGE-RELATED CHANGES IN THE MITOCHONDRIAL SUPEROXIDE PRODUCTION BY PERIPHERAL PHAGOCYTES IN WHOLE BLOOD

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ABSTRACT

PURPOSE: Ageing is a complex multi-factorial process. There are data published on reactive oxygen species (ROS) and oxidative stress as the factors that determine the lifespan of organisms. The oxidative injury of mitochondrial enzymes and the mitochondrial genome itself have been considered to play a role in a number of age-related degenerative processes. The aim of the present work was to study the changes that occur with age in the mitochondrial superoxide production by zymosan-stimulated phagocytes in whole blood. METHODS: Forty normal adult subjects having age range 40-70 years were included in the study. The mitochondrial superoxide production by peripheral phagocytes was evaluated by lucigenin chemiluminescence (LgCL). RESULTS: It was found that the velocity of phagocyte activation assessed as the reciprocal of the time to the peak of LgCL kinetic curve decreased with age. The maximum oxidative activity and the total oxidative capacity of the cells initially increased and then remained unchanged after the age of 50 years. CONCLUSION: We believe it is time now to revise the mitochondrial theory of ageing and to answer the question of whether ROS are just a peripheral target correlating with longevity or central regulators of the ageing process.

Key words: reactive oxygen species, respiratory chain, zymosan-stimulated phagocytes, lucigenin chemiluminescence

INTRODUCTION

Ageing is a biological process of age-related decline in the regulatory systems and homeostatic capacity of the organism. Aged individuals demonstrate reduced resistance to infections, increased incidence of autoimmune diseases and cancer, that is a less competent immune system. Despite the accumulated data regarding immune senescence, the age-related changes in the immune function and the specific role of immune system in ageing are not fully clarified (1).

Reactive oxygen species (ROS) and oxidative stress have been considered as the factors contributing to ageing and longevity of organisms (2). The phagocytes themselves perform their bactericidal function through the reactive oxygen species generation under stimulation. Mitochondria, on the other hand, are the main intracellular source of ROS in mammals. The function of mitochondria has been supposed to be compromised in old age, thus leading to metabolic insufficiency and oxidative stress (3). Even a mild deficit in the mitochondrial function may cause weakness, fatigue and cognitive disturbances. Compounds that influence mitochondrial function are strong toxins.

Hence, it may be supposed that both the adequate immune system and the proper energy production in mitochondria can serve as foundation for health and welfare. We aimed to investigate the mitochondrial superoxide production of stimulated peripheral phagocytes in whole blood as a function of age.

MATERIALS AND METHODS

Study population

Forty normal adult subjects aged 40-70 years were included in the study. All individuals were in good health with no clinical evidence of acute or chronic infection. They did not receive vitamins, minerals or any other food additives with antioxidant properties. All experiments were conducted in accordance

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with the rules and regulations approved by the University research ethics committee.
Peripheral venous blood, anti-coagulated with heparin (20 U/ml), was taken in a fasting state. The experiments were carried out within 30 min of blood collection.

Blood parameters
The following laboratory parameters were determined: total leukocyte, erythrocyte and platelet numbers, differential blood count and hemoglobin concentration.

Lucigenin chemiluminescence
The activity of opsonised zymosan-stimulated phagocytes to generate superoxide was studied in whole blood by lucigenin chemiluminescence (LgCL) (4, 5). The LgCL kinetics were registered by a computer chemiluminometer (6). The tested samples contained: 0.1 ml (1:10) whole blood, lucigenin (10^{-5} mol/l), zymosan (4 mg/ml) and Krebs Ringer phosphate buffer in a total volume of 2 ml. The chemiluminescent responses recorded from the blood of different individuals were normalized with respect to the total phagocyte number and erythrocyte absorption in the blood samples (7).

The following aspects of phagocyte activity to produce superoxide were studied:

- *Velocity of phagocyte activation*, which is inversely proportional to the parameter $\mathbf{T_{max}}$ (the time of the peak appearance after the start of LCL response by phagocyte stimulation). The results were expressed in minutes.
- *Maximum oxidative phagocyte activity*, which is proportional to the parameter $\mathbf{I_{max}}$ (the maximum achieved value of LgCL kinetics). The results were expressed as counts per second (cps) per 10^4 phagocytes.
- *Integral oxidative phagocyte capacity*, which is proportional to the parameter **S** (the area under the kinetic curve over a 25-min interval after the start of the response). The results were expressed as counts per 10^4 phagocytes.

Statistical analysis
The statistical analysis was performed with the Statistical Package for Social Sciences 12.0 (SPSS Inc., Chicago). Normality of data was checked with the Shapiro-Wilk test. Depending on age, the study population was classified into 4 groups, as follows: 1 group (40-49 years), 2 group (50-59 years) and 3 group (60-69 years). Kruskal Wallis test was applied to study the effect of the age factor on the parameters of LgCL kinetic curves. The value of $p<0.05$ was taken to be the threshold of statistical significance.

RESULTS
The representative LgCL kinetic curves recorded from the blood of individuals of different ages are given in **Figure 1**

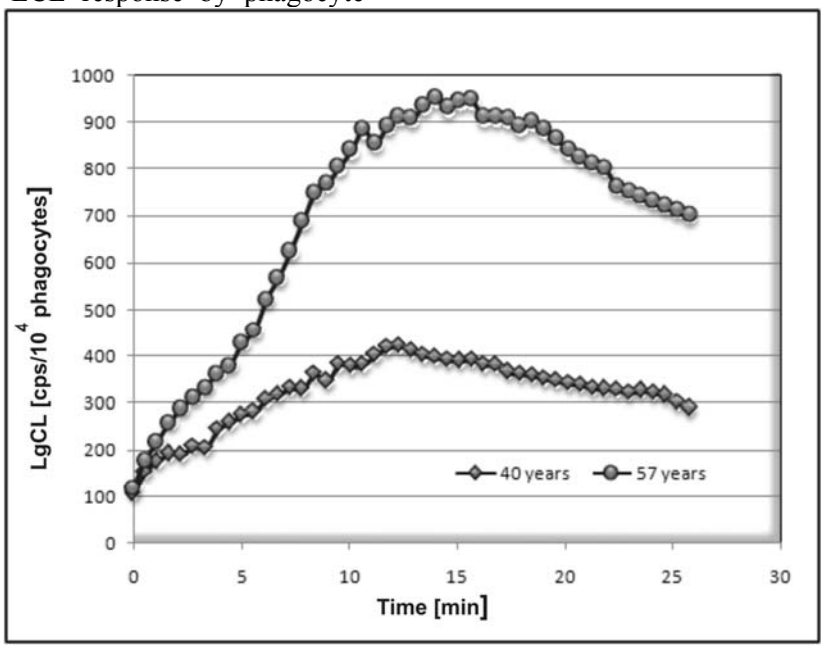

Figure 1. Representative LgCL kinetics from phagocytes of a 40-year old individual and of a 57-year old individual.

Velocity of phagocyte activation

We found that the age factor was significant with respect to the velocity of phagocyte activation, assessed as the reciprocal of the time to the peak of LgCL response $\mathbf{T_{max}}$ (*Kruskal Wallis test,* χ^2 *=6.646, p=0.010*). The value of $\mathbf{T_{max}}$ significantly increased with age, that is the velocity of activation of the cells decreased (**Figure 2**).

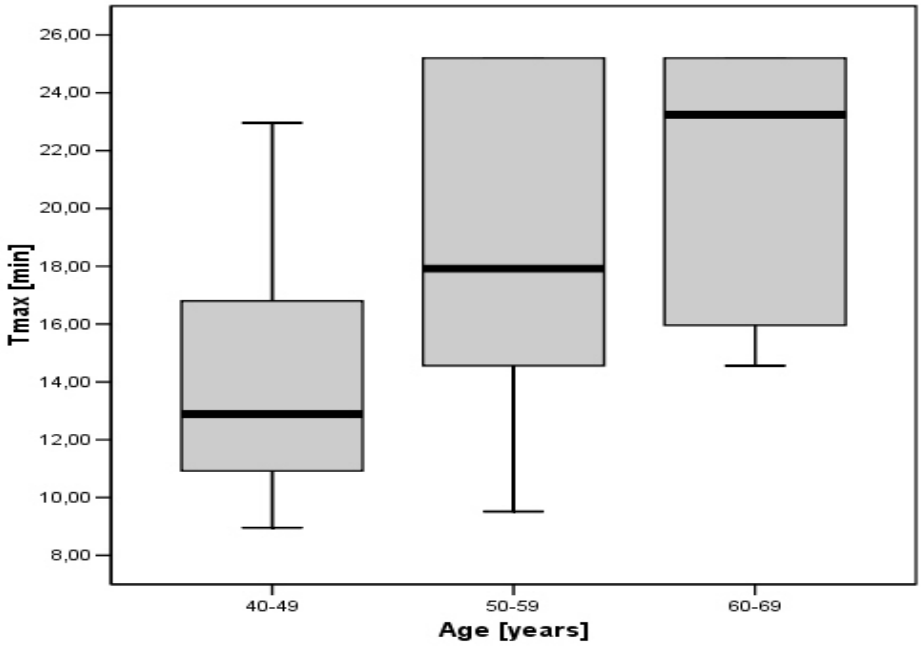

Figure 2. Time to the peak T_{max} of zymosan-stimulated LgCL depending on age. The data are represented as median (minimum-maximum value).

Maximum oxidative activity and integral oxidative capacity of peripheral phagocytes

The age factor approached but did not achieve a statistical significance with respect to the maximum phagocyte oxidative activity, assessed by the maximum achieved value of LgCL kinetics $\mathbf{I_{max}}$ (*Kruskal Wallis test,* χ^2 *=5.610, p=0.061*) (Fig. 3). The maximum oxidative activity of the cells initially increased with age and then remained unchanged within the age range of 50–69 years (**Figure 3**). The integral oxidative capacity of the cells showed a similar age-related pattern (*Kruskal Wallis test,* χ^2 *=4.823, p=0.090*) (**Figure 4**).

DISCUSSION

Mitochondrial dysfunction has been considered to contribute to the process of ageing (3). There is a tendency in the literature to automatically relate the respiratory chain in mitochondria with the enhanced ROS production but the experimental evidence for such a link is rather weak. Animal models having impaired mitochondrial function show no evidence for oxidative stress. On the other hand, the immune system also demonstrates some age-related changes associated with increased morbidity and mortality from infectious diseases among adult population (1).

The aim of the present work was to study the changes that occur with age in the mitochondrial superoxide production by stimulated peripheral phagocytes in whole blood.

Lucigenin chemiluminescence is widely used technique to evaluate both the extracellular superoxide generation by phagocyte NADPH oxidase and the phagocyte mitochondrial superoxide production (4, 5). The LgCL kinetic registered in the present study will therefore visualize these two different processes.

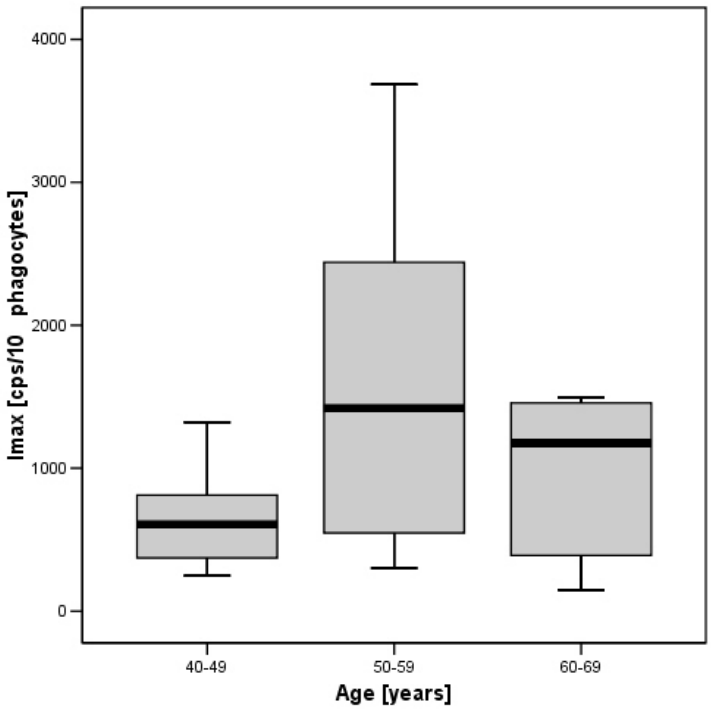

Figure 3. Maximum intensity I_{max} of zymosan-stimulated LgCL depending on age. The data are represented as median (minimum-maximum value).

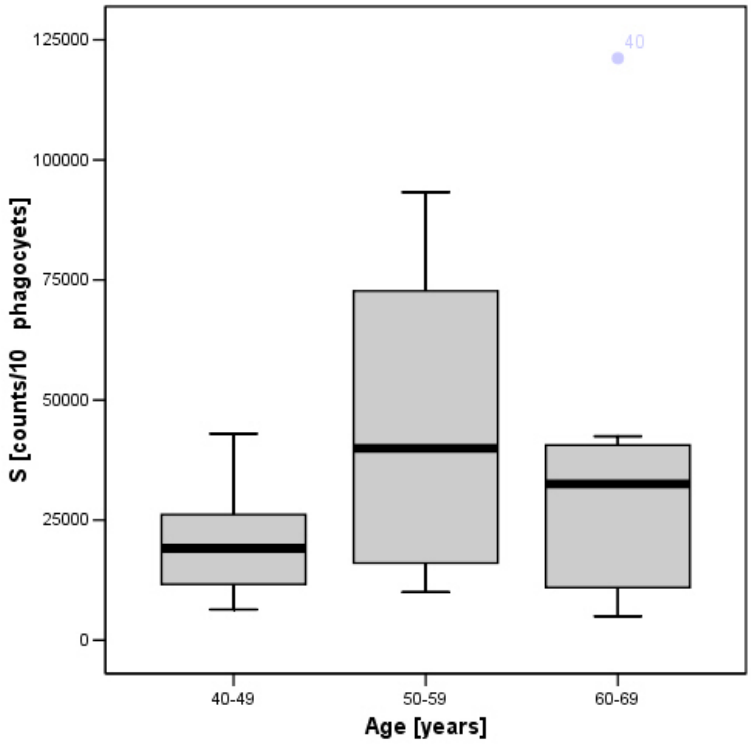

Figure 4. Area S under the zymosan-stimulated LgCL curve depending on age. The data are represented as median (minimum-maximum value).

We obtained that the time to the peak of LgCL kinetic curve increased with age. In our previous study we reported age-related reduced extracellular NADPH oxidase-mediated superoxide production by stimulated phagocytes in whole blood (8, 9). Hence, the increased time to the peak observed may point out changed proportions between the NADPH-oxidase mediated extracellular superoxide production and the intracellular mitochondrial superoxide production in favor of the mitochondrial one. Such a conclusion is supported by other authors who have reported that the rate of mitochondrial production of superoxide significantly increases with age due to the progressive oxidative modification of the mitochondrial enzymes (10, 11).

Also, we established that the maximum oxidative activity and the integral oxidative capacity of the cells initially increased with age and after the age of 50 years did not change. Our results suggest that the organism may have mechanisms to strictly control the cellular redox status and at a certain age or rather at certain levels of net radical production they tend to establish a new redox steady state. Thus, it is likely that the popular mitochondrial theory of ageing will have to be revised.
According to the initially proposed free radical theory, ageing and the age-related degenerative processes are associated with deleterious radical overproduction in the cells. Since the mitochondrial respiratory chain is the main intracellular source of radicals, mitochondria have been considered to be the main potential target of oxidative injury and the accumulation of defective mitochondria may contribute to ageing of the organism (3). In support of the mitochondrial theory of ageing, data have been published on the age-related increase in the levels of oxidatively damaged lipids, proteins and DNA (12).

If ROS production is important for the ageing process, any manipulation that can decrease this production should increase longevity. The interventions aimed at reducing ROS levels or mitigating their effects have not produced convincing evidence in this respect (13). Thus, for instance, experimental models deficient in antioxidant enzymes display increased sensitivity to oxidative stress but do not have reduced lifespan (14). Experimental models lacking mitochondrial superoxide dismutase have increased levels of oxidatively damaged proteins and DNA in mitochondria, increased incidence of cancer but do not have reduced lifespan (13). What is more, the antioxidant food supplements may even reduce longevity in humans (15). Obviously the steady-state levels of oxidatively damaged molecules depend both on net ROS production and clearance of oxidatively modified molecules from the cell. It is possible for the level of damaged molecules to increase at normal rate of ROS generation if the clearance of the damaged molecules is defective and vice versa. We consider that the mitochondrial theory of ageing is to be revised as the link between ageing and progressive enhancement of ROS generation is questioned. ROS are toxic agents and may damage a variety of cellular components. The organism, however, may cope with enhanced ROS injury without developing premature ageing. The question then arises whether the oxidative injury is sufficient to limit the lifespan of organisms under normal condition. Furthermore, ROS have been proposed to be important signal molecules. There are data published on the existence of a complex network of signaling pathways that coordinatively regulate the ROS levels in the cell. For example, the tumor suppressing factor p53 has been found to regulate the antioxidant gene expression (16). That is why, instead of changing the level of some antioxidant enzymes, a better approach to increase the lifespan could be manipulation of signaling pathways that control the intracellular redox status.

CONCLUSIONS

Ageing is a complex process comprising a consecutive activation of many cellular events necessary to develop and maintain the redox homeostasis of the cell. Mitochondrial functioning and ROS generation are the important elements of this process. Investigations of the relationship between mitochondrial function, oxidative stress and ageing have produced conflicting results. The data of the present study does not support the idea of the age-related increase in the mitochondrial superoxide production. Mitochondrial function and ROS generation probably belong to the same tightly regulated program with important consequences for the fate of the cell. It may be that very old age is achieved by individuals having a well preserved immune system and mitochondrial function due to an improved general homeostasis of the organism. Future investigations are needed to clarify the relative

importance of mitochondrial dysfunction and radical generation in the processes of ageing.

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