SPIN –SPIN PROTON TRANSVERSE RELAXATION TIMES OF RED BLOOD CELLS FROM AGING RAT WITH EXPERIMENTAL CARDIOVASCULAR PATHOLOGY

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Abstract. Background: Nuclear magnetic resonance (NMR) is modern and accessible technique for studies of erythrocyte membrane permeability in physiological and pathological conditions. Aim: In this paper we investigated by nuclear magnetic resonance method (NMR) the following parameters:the proton life time in erythrocyte, erythrocyte sediment proton transverse relaxation times (T2a), proton transverse relaxation times in plasma(T2b) and erythrocyte membrane permeability(EMPW) in rats fed on high reach cholesterol diet . Material and methods: Investigations were carried out on 14white male Wistar rats aged 24 months old divided into two groups of 7 rats each:group A controls and group B fed on cholesterol reach diet(animal origin) for 6 weeks. 1HNMR measurements of the above parameters were performed with an Aremi Spectrometer at 25 mHZ frequency . Results:There is a decrease in proton transverse relaxation time in red blood cells from rats fed on cholesterol reach diet which suggest an accelerated proton exchange Conclusion: Erythrocyte membrane permeability to water can be taken into consideration as an index of cardiovascular system recovery ,important in mantaining a dynamic equilibrium with vascular distruction phenomeneon due to high blood pressure. Key words:erythrocyte permeability, nuclear magnetic resonance(NMR),spin-spin proton transverse relaxation times,aging,aterial hyperthension.

Rezumat. Introducere: Rezonanta Nucleara Magnetica (NMR) este o tehnica modrna si accessibla pentru studiile de permeabilitate ale membranei eritrocitare in conditii fiziologice si patologice. Scop:In aceasta lucrare am investigat cu ajutorul tehnicii de RMN urmatorii parametri:timpul de viata al protonilor in eritrocit, timpii de relaxare protonica transversa in sedimentul eritrocitar (T2a), timpii de relaxare protonica transversa in plasma (T2b) si permeabilitatea eritrocitara pentru apa (PMEA) la sobolanii varstnici hraniti cu colesterol de origine animala in exces. Material si metoda: Investigatiile au fost effectuate pe un numar de 14 sobolani albi Wistar in varsta de 24 de luni impartiti in doua loturi a cate 7 sobolani fiecare:LotulA de control si lotul B hranit cu colesterol de origine animala in exces timp de 6 saptamani Masuratorile de 1H RMN ai parametrilor sus mentionati au fost efectuate cu un Spectrometru Aremi la o frecventa de 25 mHZ . Rezultate:S-a observat o scadere in timpii de relaxare protonica transversa in eritrocitele sobolanilor hraniti cu cholesterol in exces ceeea ce indica un schimb protonic accelerat la nivelul membranei eritrocitare. Concluzii: Permeabilitatea de membrana pentru apa poate fi luata in considerare ca un index pentru recuperarea sistemului cardiovascular ,important in mentinerea unui echilibru dinamic cu fenomenul de distrugere vasculara datorita hipertensiunii arteriale induse experimental de dieta hipercolesterolemica . Cuvinte cheie:permeabilitatea eritrocitara,Rezonanta Nucleara Magnetica(RMN), timpii de relaxare protonica transversa spin-spin, imbatranire,hipertensiunea arteriala

INTRODUCTION

High blood pressure and its complications is one of the major problemes of medical research, the attention being concentrated towards elucidation of physiopathological mechanisms which interfere during evolutionary stages of the desease.

The recent investigations upon atherosclerosis origin have initiated a strong debate upon the main preponderent role of hypercholesterolemia în the onset of this desease, în counterpart with ideea that atherosclerosis could have the origin in an inadequate immune response to the appearance of vascular alterations .Despite the fact that the role of the immune system has been studied, an impresive quantity of experimental studies clearly have shown that atherogenesis is innitiated under the reciprocal influence between cholesterol, cytokine cellular secretion (esspecialy IL-6), apolipoprotein E and arterial wall[1] .

Recent studies have shown that the cells posses two types of sensors for cholesterol:

- Ck receptors which are sensitive for extracellular colesterol and initiate the sygnaling pathway responsible for gene regulations implicated in cell cycle ,cell death and homeostasis of cell cholesterol and cytokines including (IL-6) and
- LxR alfa receptors, which are sensitive to intracellular oxysterols and control genes implicated in cell death, cellular cholesterol homeostasis and cytokine IL-8. [1] .

The understanding of the cell membrane permeability mechanisms to water and of changes in the intracellular water structure will might improve the actual view about various diseases in which water transport is directly involved, or the medication influences the cellular water state[1]. These aspects are well revealed by the most modern nuclear magnetic resonance (NMR) techniques[2,3].

Water crosses cell membranes by two routes: by diffusion through the lipid bilayer and through water channels (aquaporins)[4]. The aquaporins are intrinsic membrane proteins that have been characterized as facilitators of water flux. Originally termed major intrinsic proteins (MIPs), they are now also known as water channels, glycerol facilitators and aqua-glyceroporins, yet recent data suggest that they facilitate the movement of other low-molecular-weight metabolites as well[5,6].

Different aquaporins have different functionally important specialty[7]. The AQP-1 is found in the erythrocyte membranes, as well as in the epithelia, its expression being recently confirmed in the arterial and the capillary endothelia, in the smooth muscle vascular cells and in the atherosclerotic plagues[8]. Taking into account this distribution we might suppose that the vascular cells and the erythrocyte membrane permeability to water are well correlated; they are modulated by the same AQP-1, controlled by the same circulating factors. More over, the role of arginine vasopressin and atrial natriuretic peptide in the aquaporine regulation of water channel activity[9] consolidates this assumption. These aspects facilitate the evaluation of the cardiovascular status by NMR relaxometric measurements on blood.

Nuclear magnetic resonance (NMR) is an accessible technique for studies of erythrocyte membrane permeability in physiological and pathological conditions such as arterial hyperthension experimentally induced feeding rats on reach cholesterol diet.

Erythrocyte membrane illustrates the functional state and the capacity of cell to renew during the life span(120 days) and imagistics[10] allows the evidence for modifications in water permeability and the results may contribute to a better understanding of aging process as well as pathological mechanisms of arterial hyperthension[2]

OBJECTIVE

The aim of this study was to investigate in an experimental model of arterial hyperthension induced in aging rats fed on cholesterol reach diet,the proton transverse relaxation times of intracellular water protons and membrane permeability for water, by 1H NMR method.

MATERIAL AND METHODS

1.-Biological material – 14 Wistar white male rats 24 months old divided into two groups of 7 rats each (group A and group

B) for 6 weeks, with high reach cholesterol(animal origin).

2 – Determinations of nuclear magnetic resonance

Biological material used was the peripherial blood harvested on heparin by exsanguination of rats and dopped with an adequate volume of $MnCl_2$ in such a way to obtain in extracellular compartment a concentration of 20 mM $MnCl_2$.

The method used consists in determination by means of 1 H NMR technique of proton transverse relaxation times of intra and extra erythrocyte water, determination of protons exchange time through erythrocyte membrane and the calculus of permeambility for water.[3]

The principle of the method consists in characterisation în a system composed of two compartments - A and B –of two relaxation times - T_{2a} and T_{2b} – of the same type of nuclei originating from the same compartment.

Nuclear relaxation times are the parameters which characterise the returning to the equilibrium of the nuclei after appling of an adequate perturbations of radiofrequency. For the system erythrocite-plasma we are dealing with the same type of molecules distributed in A and B compartments which have corresponding relaxation times different T_{2a} and T_{2b}. A compartment represents intracerythrocyte compartment, and B represents extracellular compartment, respectively blood plasma , and nuclei of interest are water protons from the two compartments. Because there is a relatively rapid exchange process between the two compartments, and the relaxation times have the closer values, the result is the perception of a single medium global relaxation time. Therefore, for differentiation of relaxation times between the two compartments is necessary a method which makes $T_{2a} >> T_{2b}$.One of the possibilities is dopping with paramagnetic ions .

If a paramagnetic ion is added, for example Mn, to cell suspension, then T_{2b} relaxation times of water molecules în suspension solution decrease considerably because of some processes of interactions electron-proton, resulting in such a way the possibility of separation of the two relaxation times.

Have been realised determinations of nuclear magnetic resonance on an Aremi spectrometer in impulses at 25 MHz frequency, using the standard sequency CARR-PURCELL-MEIBOOM-GILL with the interval between impulses of 1 ms. Have been measured the proton transverse relaxation times in intracellular compartment in the presence of water exchange between intracellular and extracellular compartments dopped with Mn^{2+} obtaining in such a way the appearent relaxation time T_{2a}'. Representation as a function of time of transverse magnetisation is:

$$M(t) = A^* \exp(-t/T_{2a}') + B^* \exp(-t/T_{2B}) \quad [1]$$

Where the slow component of magnetisation with appearent relaxation time T_{2a}' separates significantly from the fast decreasing component , after introduction of experimental data in a filtring programm of the two exponentials.

After 10 min centrifugation of blood samples at 1000g , has been collected the supernatant for NMR measurements ,obtaining in such a way the intrinsic relaxation time T_{2b} of doped plasma which represents the extracellular water compartment . Then the erythrocytes have been washed 3 times with phosphate buffer saline (PBS) and centrifuged at the above mentioned parameters.The sediment has been measured in order to obtain the intrinsic relaxation time of intracellular compartment of water T_{2a} .Using these data, has been calculated the life time of a water molecule□□ in intracellular compartment.

$$\frac{1}{\tau} = \frac{1}{T'_{2a}}(1-h) - \frac{1}{T_{2a}}(1-h)^2 - \frac{1}{T_{2b}}h(1-h)\left[1 + \frac{(1-h)\frac{\left(\frac{1}{T_{2a}} - \frac{1}{T_{2b}}\right)^2}{\frac{1}{T_{2b}}}}{\frac{1}{T'_{2a}} - h\frac{1}{T_{2a}} - \frac{1}{T_{2b}}(1-h)}\right] \quad [2]$$

h parameter represents the ratio between the intracellular water volume and the total volume of water in blood . It is obtained from hematocrit, taking into account that 71,5% from the medium erythrocyte volume (vem) and respectively 94,5% from the volume of blood plasma is occupied by water. In our experiments the integral blood samples have been reconstituted by resuspending erythrocytes in plasma , using in all cases a 55% proportion of erythrocyte pellet.

The value of erythrocyte membrane permeability is obtained from t using formula:

$$P = \left(\frac{V}{A}\right)\left(\frac{1}{\tau}\right) \quad [3]$$

Where V and A represent volume, and respectively erythrocyte area .

The mean erythrocyte volume has been calculate by formula:

$$V = \frac{h*10}{N} \quad [4]$$

where h is the percentige measured value of haematocrite , and N is the number of erythrocyte /mm^3, experimentally determined.

The mean erythrocyte surface is obtained from :

$$A = \pi * \frac{D^2}{2} + 2 * \frac{V}{D} \quad [5]$$

D being the medium erythrocyte diameter measured.

RMN measurements have been done on a range of temperatures between 0-42^{0}C, respectively at 0^{0}C, 22^{0}C, 30^{0}C, 37^{0}C şi 42^{0}C, and the obtained values for membrane permeability to water (PMEA)have been compared at 37^{0}C.

There are many pathways of water transport (lipid and protein), to each being associated a certain specific activation energy of transmembrane water diffusion process (Ea^L şi Ea^P), in such a way that the determined transmembrane exchange time becames:

$$\frac{1}{\tau} = a * \exp\left(\frac{-Ea^L}{kT}\right) + b * \exp\left(\frac{-Ea^P}{kt}\right) \quad [6]$$

a and *b* being constants dipending on the membrane structure.

In this context is defined the activation global energy of transmembrane water exchange processes (Ea) as being :

$$\frac{1}{\tau_{\exp}} = c * \exp\left(\frac{-Ea}{kT}\right) \quad [7]$$

where *c* is a constant.By logaritming the expression, results:

$$\ln\left(\frac{1}{\tau_{\exp}}\right) = \ln c - \frac{Ea}{kT} \quad , \quad \text{deci}$$

$$\ln \tau_{\exp} = \ln c - \frac{Ea}{kT} \quad \text{sau} \quad \ln \tau_{\exp} \frac{Ea}{kT} - \ln c \quad [8]$$

From the graph $\ln \tau_{\exp} = functie\left(\frac{1}{kT}\right)$ is calculated Ea, after filtering with a(line) dreaptă of experiemntaly obtained points. In all above formula *k* is the Boltzmann constant.

Using the logarithmic representations of variations of proton relaxation times of plasma water and respectively of erythrocytes, as a function of temperature,

we have deduced analogous activation energies of water relaxation processes from extracellular and intracellular water compartment.

RESULTS

Nuclear Magnetic Resonance data

The study of experiemtaly induction of arterial hyperthension by overdosing cholesterol in rats feeding was intended to point out vascular system dysfunctions ,respectively at the level of red blood cells membrane permeability for water.

In our laboratory, the previous research data [2] have pointed out that any of the modifications produced at the level of coronary or periferic circulation, are accompanied by changing in the permeability for water of vascular walls ,which brings about a modification in hydration state of tissues supply by the vascular affected bed. These deviations from the equilibrium state is reflected in modifications of proton transverse relaxation times parameters which are accesible for NMR for the investigated biological material.

Modern studies in the molecular biology field have pointed out the presence of some proteins which are implicated in water channels 9, aquaporins.[2] Aquaporin AQP1, is the most widely found in organism being present in erythrocyte membrane, în artery arterioles, venes, cappilaries endothelium , as well as in certain smooth vascular muscule from human atherosclerotic plaques.and which assure the active water transport through cell membranes, is responsible for water exchange through vascular walls.[4]

Because the aquaporine synthesis is altered, the action of some hormones ,such as arginin-vasopresin (activator of synthesis) or natriuretic peptide (inhibitor of synthesis), result in erythrocyte membrane permeability to water alteration which are sincronous with those from the cardiovascular system and are produced in the same way.Also, the permeability to water can be modified by chainging the proportion and distribution of lipid membranes.

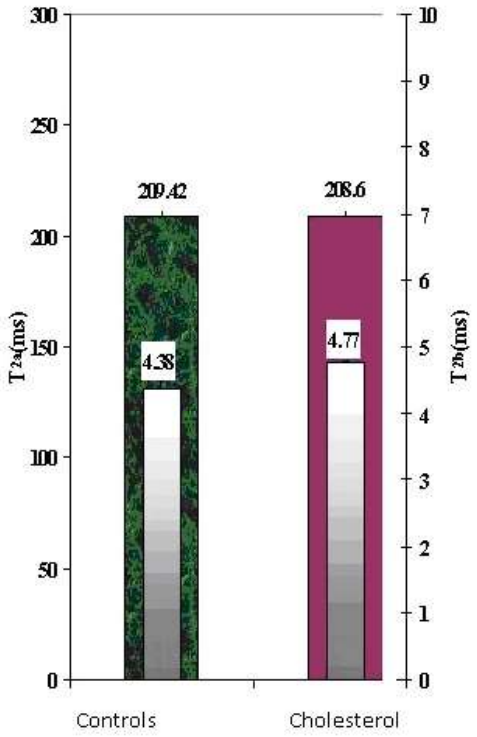

Fig. 1. Proton transverse relaxation time of intraerythrocyte water (T2a) and of plasma water (T2b) from controls and cholesterol fed rats

The proton transverse relaxation time of intraerythrocyte water (T_{2a}) decreases very slightly, in hypercholesterolemic rats, versus controls, while Proton transverse relaxation time in case of plasma erythrocyte water (T_{2b}) increases slightly in cholesterol fed rats (*figure 1)*.

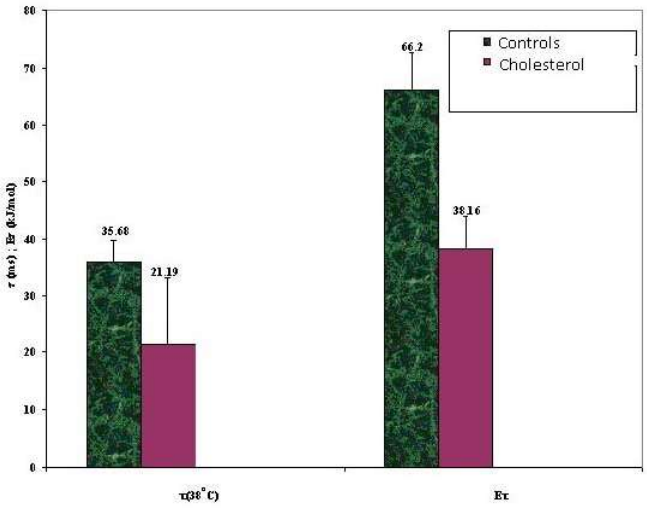

Fig. 2. Exchange time of water through erythrocyte membrane(τ) and activation energy of water exchange through erythrocyte membrane (E_τ) in controls and cholesterol fed rats

Figure 2 presents the aspects related to the dynamics of protons through erythrocyte membrane and modifications of water exchange energetics. There is a decrease of erythrocyte proton life time (τ), which suggests an accelerated proton exchange , in cholesterol fed rats of group B. Activation energy of water exchange through erythrocyte membrane (E_τ)is decreased in cholesterol fed rats versus controls. This means that at higher levels of cholesterol the exchanges of water became more accelerated , and the process of membrane exchange is partially deconected under the influence of thermic processes with heat liberation. In other words, in controls the water exchange processes through erythrocyte membrane increases in paralell with the increase in local or global temperature due to metabolic reactions with heat liberation in intracellular environment.

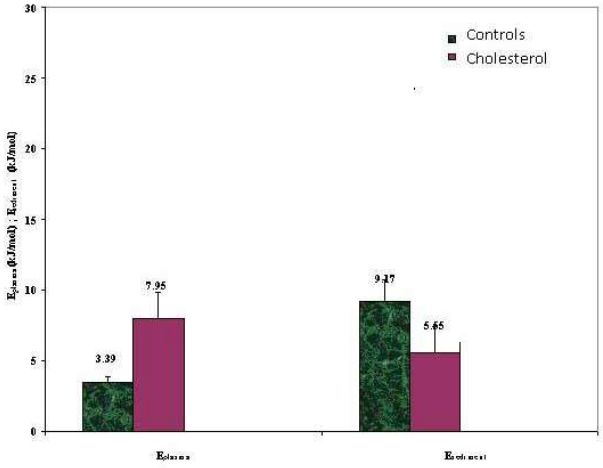

Fig. 3. Activation energy of water exchange through plasma and activation energy of water exchange through erythrocyte pellet in controls and cholesterol fed rats

When specific energetic processess of an exchange between the two compartments are affected, this means that either one of these compartments is responsable for a certain „turn" on of energetic domain or, both compartments are implicated in this process.

Investigating the situation of biocompartimental system plasma-erythrocyte from the point of view of activation energy of proton relaxation processes (*figure 3*), it is observed a significant increase of activation energy in plasma (E_{plasma}) of cholesterol fed rats versus controls.There is a significant decrease in activation energies inside erythrocytes from cholesterol fed rats versus controls. Erythrocyte membrane permeability to water (PMEA) is a parameter which characterise the exchange of water through erythrocyte membrane, as well as those processes which take place in vascular walls. This correlation is permited by the presence of the same type of aquaporine-AQP1- both in erythrocyte membrane and in vascular endothelial membranes at all levels.

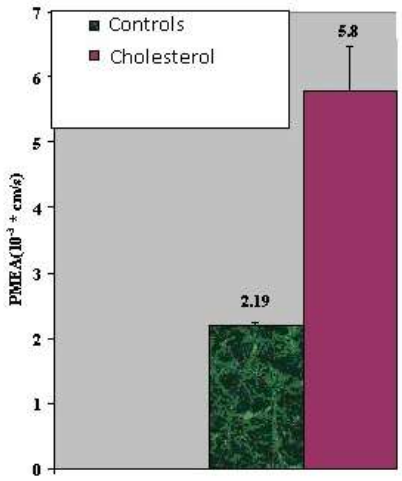

Fig. 4. Erythrocyte membrane permeability for water (EMPW) in Controls and Cholesterol treated rats

Permeability to water increases very much in hypercholestorelemic animals (from the value of 2,19cm-s*10^{-3} in controls, to 5,8cm-s*10^{-3} în hypercholesterolemic animals.

DISCUSSION

The study of experiemtaly induction of arterial hyperthension by overdosing cholesterol in rats feeding diet was intended to point out vascular system dysfunctions ,respectively at the level of blood.

In our laboratory, the research data have pointed out that any of the modifications produced at the level of coronary or periferic circulation, are accompanied by changing in the permeability to water of vascular walls ,which brings about a modification in hydration state of tissues supply by the vascular bed affected. These deviations from the equilibrium state is reflected in modifications of proton transverse relaxation time, parameters which are accesible for NMR for the investigated tissue.

Modern studies in the molecular biology field have pointed out the presence of some proteins which are implicated in water channels 9, *aquaporins*. Aquaporin AQP1, is the most widely found in organism being present in erythrocyte membrane, în artery arterioles, venes, cappilaries endothelium , as well as in certain smooth vascular muscule from human atherosclerotic plaques.and which

assure the active water transport through cell membranes, is responsible for water exchange through vascular wallls.[4]
Because the aquaporine synthesis is altered, the action of some hormones ,such as arginin-vasopresin (activator of synthesis) or natriuretic peptide (inhibitor of synthesis), rezults that modifications in membrane permeability to water at the level erythrocyte are sincronous with those from the cardiovascular and are produced in the same way.Also, the permeability to water can be modified also by chainging the proportion and distribution of lipid membranes.
There is a decrease of erythrocyte proton life time (τ), which suggests an accelerated proton exchange , in group B.(Figure 2). Activation energy of water exchange through erythrocyte membrane (E_τ)is decreased in cholesterol fed rats versus controls. This means that at higher levels of cholesterol the exchanges of water became more accelerated , and the process of membrane exchange is partially deconected under the influence of thermic processes with heat liberation. In other words, in controls the water exchange processes through erythrocyte membrane increase in paralell with the increase in local or global temperature(în presence of metabolic reactions with heat liberation in intracellular environment.
From our previous data[2] resulted the fact that permeability to water is increased in the onset stages of disease as an adapting to the increased arterial hyperthension and if after maintaining it in increased platau takes place a sudden decrease, this is correlated with an increased risc for stroke onset. Therefore,not always the reduction to normal values of a increased physiological parameter în context of a desease is wellcomed, because adaptatively the organism has created a new state of equilibrium; if the primary cause of dysregulation of normal equilibrium is not corrected then a more severe situation occurs.It is mentioned that single administration of drugs in hyperthensive patients which decrease permeability to water of cell membranes is riscky and is recommended that this administration to be associated with drugs that have an effect on membrane permeability.

CONCLUSIONS

-Modern investigations with NMR methodology have pointed out modifications in erythrocyte membrane function in hyperthensive aging rats fed on cholesterol reach diet versus age matched controls.
-The proton transverse relaxation time of intraerythrocyte water (T_{2a}) decreases very slightly, in hypercholesterolemic rats, versus controls, while Proton transverse relaxation time in case of plasma erythrocyte water (T_{2b}) increases slightly in cholesterol fed rats
-There is a decrease of proton life time in erythrocyte , which suggest an accelerated proton exchange in hypercholesterolemic rats.
-In controls, water exchange through erythrocyte membrane is accelerated as a function of increase in local or global temperature (determined by the activation of metabolic reactions with heat liberation from intracellular medium.)
-Membrane permeability to water (PMEA) increases significantly in cholesterol fed rats versus controls.This may be taken into account as an index of recovery of cardiovascular system, important în mentaining a dynamic equilibrium with phenomena of vascular destruction due to the increased arterial blood pressure.
-1NMR method could be a useful tool in functional evaluation of erythrocyte membrane permeability in normal and pathological conditions and these results bring a valuable contribution to a better understanding of aging process as well as of pathological mechanisms of arterial hyperthension.

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