Scientific Papers. Series D. Animal Science. Vol. LX, 2017
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

SPIN-SPIN PROTON TRANSVERSE RELAXATION TIMES STUDIES OF RED BLOOD CELL MEMBRANE IN RABBITS WITH EXPERIMENTAL ATHEROSCLEROSIS

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Abstract

Nuclear magnetic resonance (NMR) is a modern and accessible technique for studies of erythrocyte membrane permeability in physiological and pathological conditions. In this paper we investigated in rabbits fed on high reach cholesterol diet, by nuclear magnetic resonance method (NMR) the following parameters: the proton life time in erythrocyte, in erythrocyte sediment proton transverse relaxation times (T2a), proton transverse relaxation times in plasma (T2b) and erythrocyte membrane permeability (EMPW). Investigations were carried out on 12 male rabbits aged 20 months old divided into two groups of 6 rabbits each: group A controls and group B fed on cholesterol reach diet (animal origin) for 8 weeks. 1H NMR measurements of the above parameters were performed with an Aremi`78 Spectrometer at 25 mHz frequency. There was a decrease in proton transverse relaxation times in red blood cells from rabbits fed on cholesterol reach diet which suggests an accelerated proton exchange. The activation energy of water exchange through erythrocyte membrane (E_a) is decreased in cholesterol fed rabbits versus controls. This means that at higher levels of cholesterol the exchanges of water become more accelerated and the processes of membrane exchange are partially disconnected under the influence of thermal processes with heat liberation. In other words, in controls the water exchange processes through erythrocyte membranes increases in parallel with the increase in local or global temperature due to metabolic reactions with heat liberation in intracellular environment. Erythrocyte membrane permeability to water can be taken into consideration as an index of cardiovascular system recovery, important in maintaining a dynamic equilibrium with vascular destruction phenomenon due to high blood pressure.

Key words*: erythrocyte membrane permeability, nuclear magnetic resonance (NMR), spin-spin proton transverse relaxation times, atherosclerosis, arterial hypertension.*

INTRODUCTION

High blood pressure and its complications is one of the major problem of medical research, the attention being concentrated towards elucidation of path physiology mechanisms which interfere during evolutionary stages of the disease.

Recent investigations regarding atherosclerosis origin have initiated a strong debate upon the main preponderent role of hypercholesterolemia in the onset of this desease, in counterpart with the 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 (Balta, 2009).

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 (Balta, 2009).

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 (Balta, 2009). These aspects are well revealed by the most modern nuclear magnetic resonance (NMR) techniques (Gatina et al., 1998; Petcu et al., 1995).

Water crosses cell membranes by two routes: by diffusion through the lipid bilayer and through water channels (namely aquaporins) (Benga, 2012), which 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 (Herrera et al., 2006; Zhang et al., 2007). Different aquaporins have different functionally important specialty (Rutkovskiy et al., 2013). 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 plaques (Shanahan et al., 2000). 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. Moreover, the role of arginin vasopressin and atrial natriuretic peptide in the aquaporine regulation of water channel activity (Schrier et al., 2001) consolidates this assumption. These aspects facilitate the evaluation of the cardiovascular status by NMR relaxometry 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 hypertension experimentally induced feeding rabbits on reach cholesterol diet.

Erythrocyte membrane illustrates the functional state and the capacity of cell to renew during the life span (120 days) and imagistic methods (Stoian et al., 2012) allow the evidence for modifications in water permeability and the results may contribute to a better understanding of pathological mechanisms of arterial hypertension (Gatina et al., 1998).

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

MATERIALS AND METHODS

1. Biological material – 12 white male rabbits aged 20 months old divided into two groups of 6 rabbits each: (group A and B) for 8 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 rabbits 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 erythrocyte-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 intra erythrocyte 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 doping with paramagnetic ions.

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

Determinations of erythrocyte membrane proton transverse relaxation times (T_{2a} and T_{2b}) Nuclear Magnetic Resonance were done on an Aremi`78 Spectrometer in impulses at 25 MHz frequency, using the standard sequence CARR-PURCELL-MEIBOOM-GILL with the interval between impulses of 1 ms.

The measurement of T_{2a} and T_{2b}in intracellular compartment was done in the presence of water exchange between intracellular and extracellular compartments doped with Mn^{2+} obtaining in such a way the apparent relaxation time T_{2a}'. Representation as a function of time of transverse magnetisation is:

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

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

After 10 min centrifugation of blood samples at 1000 g, 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.

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

The 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:

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

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

The mean erythrocyte volume has been calculate by formula:

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

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:

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

D is 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 and

42^0C, and the obtained values for membrane permeability to water (PMEA) have been compared at 37^0C.

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 and 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:

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

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

From the graph $\ln\tau_{\exp} = functie\left(\frac{1}{kT}\right)$ is calculated Ea, after filtering with a (line) 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 AND DISCUSSIONS

Nuclear Magnetic Resonance data

The study of experiemtaly induction of arterial hyperthension by overdosing cholesterol in feding the rabbits 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 (Gatina et al., 1998) 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 (Gatina et al., 1998).

Aquaporin AQP1 is the most widely found in organism being present in erythrocyte membrane, in artery, arterioles, venes, cappilaries endothelium, as well as in certain smooth vascular muscle from human atherosclerotic plaques and which assure the active water transport through cell membranes, is responsible for water exchange through vascular walls (Benga, 2012).

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.

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

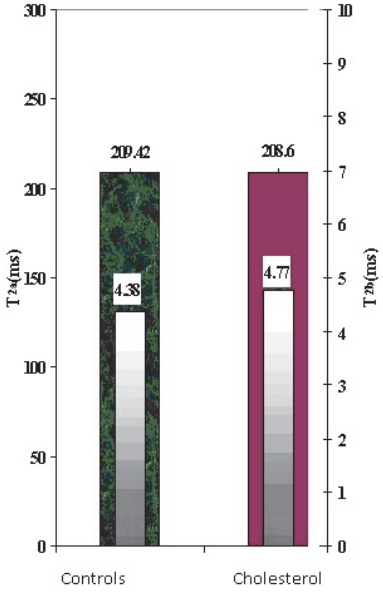

Figure 1. Proton transverse relaxation time of intra erythrocyte water (T2a) and of plasma water (T2b) from controls and cholesterol fed rabbits

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 rabbits of group B. Activation energy of water exchange through erythrocyte membrane (E_τ) is decreased in cholesterol fed rabbits 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.

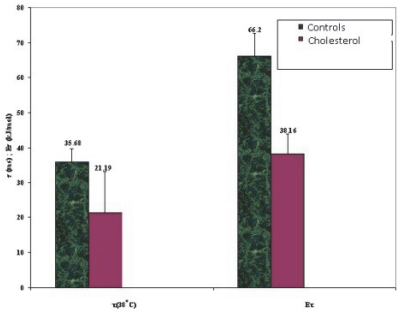

Figure 2. Exchange time of water through erythrocyte membrane(τ) and activation energy of water exchange through erythrocyte membrane (E_τ) in controls and cholesterol fed rabbits

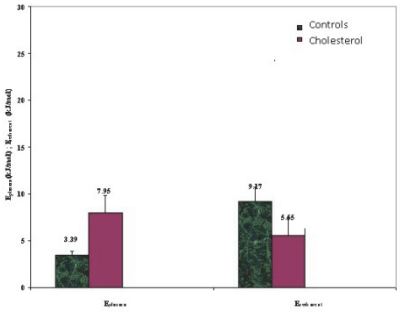

Figure 3. Activation energy of water exchange through plasma and activation energy of water exchange through erythrocyte pellet in controls and cholesterol fed rabbits

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 rabbits versus controls. There is a significant decrease in activation energies inside erythrocytes from cholesterol fed rabbits versus controls. Erythrocyte membrane permeability to water (PMEA) is a parameter which accounts for the exchange of water through erythrocyte membrane, as well as for those processes which take place in vascular

walls. This correlation is allowed by the presence of the same type of aquaporine-AQP1- both in erythrocyte membrane and in vascular endothelial membranes at all levels.

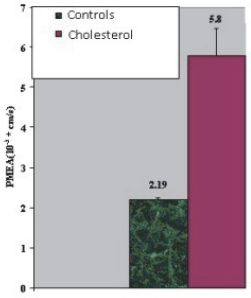

Figure 4. Permeability to water increases very much in hyper cholestorelemic rabbits (for example: from the value of 2,19cm-s*10^{-3} in controls, to 5,8cm-s*10^{-3} in hyper cholesterolemic rabbits

The study of experiemtaly induction of arterial hyperthension by overdosing cholesterol in rabbits feding 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, in artery, arterioles, venes, capillaries, 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 (Benga, 2012).

Because the aquaporine synthesis is altered, the action of some hormones, such as arginin-vasopresin (activator of synthesis) or natriuretic peptide (inhibitor of synthesis), determine modifications in membrane permeability to water at the level erythrocyte which are sincronous with those from the cardiovascular system which are produced in the same way. Also, the permeability to water can be modified 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 rabbits versus controls.

This means that at higher levels of cholesterol the exchanges of water becames 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 investigations in pararel with the increase in local or global temperature (in presence of metabolic reactions with heat liberation in intracellular environment.

From our previous data (Gatina et al., 1998) resulted the fact that permeability to water is increased in the onset stages of the disease as an adapting mechanism to the increased arterial hypertension value and if after maintaining it at an increased level occurs a sudden decrease, this is correlated with an increased risk for stroke onset.

Therefore, not always the reduction to normal values of an increased physiological parameter in the context of a pathological state is indicated because the adaptation of organism has created a new state of equilibrium; if the primary cause of the disturbance of normal equilibrium is not corrected then a more severe situation is achieved.

It is mentioned that a single dose administration of drugs in hypertensive patients which decreases permeability to water of red blood cell membranes is risky and it 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 rabbits fed on cholesterol reach diet versus age matched controls.

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

There is a decrease of proton life time in erythrocyte, which suggest an accelerated proton exchange in hypercholesterolemic rabbits.

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 rabbits versus controls. This may be taken into account as an index of recovery of cardiovascular system, important in mentaining a dynamic equilibrium with phenomena of vascular destruction due to the increased arterial blood pressure.

NMR relaxometric 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 hypertension.

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TECHNOLOGIES OF ANIMAL HUSBANDRY