Atherosclerotic plaque characterization by NMR spectroscopy

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Abstract

Background: High resolution Magnetic Resonance Spectroscopy (MRS) in vitro was used to investigate the correlation between the chemical composition of atheromatous plaques and their stability (vulnerability to rupture) which is of fundamental importance and is a rather controversial point in literature.

Methods: In this study five tissues were collected from ex vivo human abdominal aortas and divided in three groups according to their atherosclerotic lesion using the American Heart Association Committee on Vascular Lesions. Due to the semi-solid nature of the plaques, Magic Angle Spinning (MAS) and heteronuclear Decoupling (DEC) were used to acquire six 13C spectra with appreciable high resolution for five samples and the solvent. The 1H high resolution spectra also were obtained for the same samples.

Results: The most common fatty acids present in the samples were identified and also the relative amounts of polyunsaturated (PUFA), unsaturated (UFA) and saturated fatty acids associated with the progress of the deterioration process of atheromatous plaques.

Conclusions: NMR results show that the lipidic composition of atheromatous plaque evolves from a state with high levels of PUFA and UFA for a subject with lower classification to a new state containing, 6 and 4 times less unsaturated fatty acids respectively than those subjects with more stenotic lesions. In addition, average rate of conversion of PUFA to UFA is approximately two times higher in more stenotic lesions. These results suggest that the lipidic components of atheromatous plaque can be related to its vulnerability to rupture, which clearly demonstrate the importance of the method to study this disease.

Key words
13C-high resolution Nuclear Magnetic Resonance Spectroscopy, Atherosclerosis, Fatty acids, Oxidation, Lipids

1 Introduction

Cardiovascular and cerebrovascular diseases, currently the first and third causes of death in developed countries, will soon be a major burden to public health worldwide [1, 2]. Several investigations have established that the acute clinical complications of these diseases are associated with atherosclerotic plaque disruption and thrombosis [3-5]. Atherosclerotic
plaque composition rather than degree of stenosis determines its instability. Thus, noninvasive in vivo techniques that can provide both chemical and spatial information on the distribution of different plaque components may allow risk stratification in asymptomatic as well as symptomatic patients with cardiovascular and cerebrovascular diseases, plaque monitoring, and possibly guide targeted therapy.

The pathophysiology of atherosclerosis involves many highly interrelated processes, including dyslipidemia, endothelial dysfunction, inflammation, oxidative stress, Vascular Smooth Muscle Cell (VSMC) activation, Extra-Cellular Matrix (ECM) remodeling, thrombosis, homodynamic, genetic and risk factors [6-8]. Fatty dots and fatty streaks, the earliest indications of atherosclerosis, develop when macrophages and VSMCs are recruited to the intima, where they engulf lipids to form scattered foam cells. Within these initial lesions, the lipids are predominantly in the form of a cholesteryl ester oily phase and a cholesterol-phospholipids liquid crystalline phase [9-13]. At an early stage of atherogenesis, the intimal plaque is formed by the aggregation of multiple foam cells, some of which may have undergone apoptosis and released cellular debris and lipids into the extracellular media. As the disease progresses, fibrotic lesions appear by further deposition of collagen and proteoglycans synthesized by VSMCs.

These lesions have a fibrous cap that covers a core of foam cells, debris from necrotic cells, and crystals of cholesterol monohydrate. Eventually, complicated lesions develop. These lesions characteristically contain more necrotic debris and cholesterol crystals. In addition, since these lesions fracture easily, thrombi are often found on the atherosclerotic lesions.

It is thought that plaque vulnerability is highly dependent on its constituents such as lipids, calcification, blood, inflammatory cells, fibrous tissues and also, the physical state of the lipids (crystalline, liquid-crystalline, liquid and the oxidative state of the LDL [14]) in the plaque).

Generally, the presence of necrotic cores, inflammation, hemorrhage, and thinning of the fibrous cap is thought to render a plaque vulnerable. A high cellularity lesion, for example, is found to disrupt plaque stability.

A myriad of established and emerging techniques have been tested for their ability to evaluate atherosclerosis. These modalities include X-ray angiography, Intra-Vascular Ultra Sound (IVUS), angioscopy, Optical Coherence Tomography (OCT), Near Infra-Red (NIR) spectroscopy, Fourier Transform Infra-Red (FTIR) spectroscopy, Raman spectroscopy, thermography, surface and transesophageal ultrasound, Ultra-Fast Computed Tomography (UFCT), nuclear scintigraphy, and high resolution Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS). Currently, all of the techniques cited have limitations to some extent and no universal standard has been set [15-17].

MRI allows serial monitoring with high resolution images of multiple vascular territories in the same individual. It does not involve ionizing radiation and provides noninvasive appraisal of the pathophysiology of atherosclerosis. Another important feature of this technique is that it permits in vivo nondestructive characterization of the chemical constituents of the tissue sample. Special techniques of MRS in vitro allows a highly precise prediction of the chemical constituents of atheromatous plaque in a systematic way, providing a very important method to study the evolution of the pathological process caused by the atherosclerotic disease and pave the way for future in vivo studies.

The aim of the present study is to investigate the feasibility and efficiency of in vitro high resolution MRS to detect the chemical composition of atheromatous plaque in different stages of human atherosclerosis. We hypothesized that plaque vulnerability to rupture is as direct consequence of lipid alterations and can be detected by variations in $^{13}$C and $^1$H spectra patterns. Resonances of Unsaturated Fatty Acids (UFA), Poly Unsaturated Fatty Acids (PUFA), terminal CH$_3$ carbon of Cholesteryl Ester (CE) and the methine carbons of fatty acids ($\text{CH}_2_n$) usually named as “lipid pool”, were analyzed in order to provide more quantitative parameters (relative amounts of fatty acids) and how these parameters are related with atheromatous stability.
2 Materials and methods

Classification - Atherosclerotic lesions were graded according to the American Heart Association’s (AHA’s) Committee on Vascular Lesions classification as shown in table 1 [18].

| Subject | Age (yrs) | Gender | Race | Lesion Classification | Cause of Death | Hypertension | Diabetes |
|---------|-----------|--------|------|-----------------------|----------------|--------------|----------|
| A       | 33        | Male   | Caucasian | I-II                  | Chronic hepatopathy | -            | -        |
| B       | 70        | Male   | Caucasian | V                    | Pulmonary Tuberculosis | -            | -        |
| C       | 47        | Male   | Caucasian | V                    | Pulmonary thromboembolism | +            | -        |
| D       | 90        | Female | Caucasian | V                    | Ruptured abdominal aortic aneurysm | +            | -        |
| E       | 75        | Male   | Caucasian | VII                  | Perforated chronic duodenal ulcer | -            | -        |

Tissue Sample – Five human abdominal aortas were obtained en bloc during routine autopsies within 12 hours after death. Subsequently, the tissue samples were submitted to a careful dissection. Adjacent specimens were then excised circumferentially with parallel blades into rectangular segments (20 mm × 4 mm), fixed with 10% buffered formalin, and stored at 4°C until usage. It has been demonstrated that arterial tissue remains feasible for post mortem lipid analysis for up to 4 to 5 days if it is kept at this temperature [19]. All proceedings followed a protocol approved by the institution's research ethics committee (CAPPesq/HCFMUSP).

NMR Experiments and Analysis – The Five samples were dried using a piece of absorbent paper in order to remove the excess of solvent. Subsequently, each specimen was packed into appropriate zirconium rotors. In addition, an extra sample consisting of pure 10% buffered formalin was prepared and its NMR spectra was obtained to distinguish the formalin and the zirconium rotor spectra. High resolution 13C and 1H NMR experiments were performed at room temperature using a VARIAN INOVA spectrometer operating at 100.5 and 400.0 MHz, respectively, with a 7 mm MAS double-resonance probe. The spinning speed was adjusted at 5 kHz using a pneumatic system in manual mode, which ensures a rotation stability of ±100 Hz. MRS spectra were obtained using a proton-decoupling field of 60 kHz, recycle delays varying between 7 and 10 s and a single Direct Polarization sequence (DP). Typical π/2 pulses lengths of 3.0 and 3.5 µs were applied to both nuclei. Special care was taken to ensure an optimal signal to noise ratio (S/N) and an approximate number of >2000 and 16 accumulations were used to acquire the 13C and 1H signals, respectively. Adamantane (29.2 and 38.3 ppm) and water of tissues (4.8 ppm, approximately) were used as reference for the chemical shift axis. To obtain the areas from resonances of UFA, PUFA, (CH2)n and CE, lorentzian line shapes were used to fit the peaks. The chemical shift positions used in this process are 130.0 ppm for UFA, 128.2 ppm for PUFA, 30.0 ppm for (CH2)n and 19.5 ppm for CE.

3 Results

Assignments of 13C and 1H NMR Spectra - Prior to the investigation of the pathological process of atherosclerosis by NMR, the spectral assignment of different chemical group in the 1H and 13C spectra is required. This was done based on typical chemical shift values in the spectra simulated. The ACD/1H-NMR Predictor and ACD/13C-NMR Predictor (ACD/Labs) were used to generate the theoretical spectra of the molecules, which contribute to the total 1H and 13C spectra.
The dominant pattern of arteriosclerosis is atherosclerosis, normally characterized by the accumulation of lipids, mainly the cholesterol and cholesteryl esters, inside the intima resulting in thickening and loss of elasticity of arterial walls. Cholesterol (Chol) is a steroid macromolecule derivative from cyclopentane peridrofenantrene, which is composed by four non-planar fused rings, and with an OH group in carbon C19 (see Figure 1a). The Chol macromolecule can be esterificated to long chains of fatty acids (chains of 14, 16 or 18 carbon atoms, without ramification, saturated or non-saturated), such as COOH\[(CH₂)₃CH₃, linoleic, oleic and linolenic acids (see Figures 1b, c, d and e), in order to compose esters of cholesterol, i.e., cholesteryl esters (CE's). Other lipids, such as Triglycerides (TG) and Phosphatidyl-choline (Ph-cho), can also be present in atheromatous plaques. Besides the necrotic center formed by the extra cellular lipids mentioned above, the lesions are covered by fibrous cap which contains connective tissue including mainly collagen formed by Proline (Pro). Therefore, using the Chol, CE’s, TG, Ph-cho and Pro molecules as an input in ACD programs, some simulated chemical shift values matched with the observed in experimental 13C and 1H spectra, proving the existence of these molecules in the atheromatous plaque. There have been articles showing that the calcification of the different tissues strongly depends on the environment where these tissues are found [20]. Based on this result, the assignment of chemical shift values in experimental data could be done with relative success. Figure 1a-e shows the Chol and CE’s molecular structure, while figure 2 shows the 13C (a) and 1H (b) high resolution spectra.

The spectra shown in figure 2a are arranged in a sequence of the atherosclerotic lesion classification, as follows: A (control sample), B, C, D and E. The last one, F, presents the 13C spectrum associated with the solvent (buffered formalin, narrow line) used to keep the samples fresh, and a broad line associated to the NMR sample holders. All the spectra presented in figure 2a are the result of subtraction of this broad baseline from the original spectra. As can be seen, all the spectra contain at least two peaks in the interval of 90.0 to 80.0 ppm, which are associated with traces of solvent in tissues. In region of 71.0 to 10.0 ppm (aliphatic carbons), the spectra shows a complex set of peaks of different intensities, mainly associated with the molecules of Chol and CE’s. Due to the semi-solid state of the samples the spectra is not well resolved, making it difficult to associate the chemical group of the lipid molecules to the absorptive lines in the experimental spectra. In addition, coincidences of chemical shift values were noted between the simulated 13C spectra of all lipid molecules, which is reasonable once all those molecules have the same basic chemical structure of Chol, as shown before in figure 1a. Even so, some differences also could be noted in the chemical shift values, mainly due to the presence of different radicals of fatty acids in cholesterol molecule. Based on the chemical structure of each lipid molecule separately, the 13C high resolution spectra were simulated and their respective chemical shift values were compared to experimental ones in order to identify the presence of these molecules in the atheromatous plaques. The Table 2 summarizes these results of assignments based on 13C spectra and Chol/CE’s structures.

The remnant peaks at the aliphatic region are either not well resolved or the intensities are too low to ascribe them with any other chemical group in the lipids. A remarkable characteristic in all 13C spectra is the presence of a broad line occurring at \(\sim 110.0 \text{ ppm}\), which was ascribed to Teflon® ribbon used as filler material into NMR rotors to maintain stable the rotation in MAS measurements.

Simulations of 13C high resolution spectra to TG, Ph-cho and Pro show the presence of peaks centered at 30.0 and 60.0 ppm, approximately. As can be seen in figure 2a, there are peaks with expressive intensity in these regions for all samples (including the low classification sample), which suggests that these molecules are present in all tissues independent of the existence of atheromatous plaques or not.

Figure 2b shows the 1H high resolution spectra in the same arrangement of the 13C case. Again, the last one, F, show the 1H spectrum associated with the solvent used to keep the samples fresh. For proton resonance it is not necessary to subtract any baseline from the spectra. However, it is clear that the peak at \(\sim 3.6 \text{ ppm}\), present in all samples, are associated with traces of solvent in the tissues, whereas the broad and high peak at \(\sim 4.8 \text{ ppm}\) present in all samples, can be assigned to the water molecules.
Figure 1. Molecular structure of (a) Chol, (b) fatty acids COOH(CH₂)₂CH₃, (c) linoleic, (d) oleic and (e) linolenic used to calculate the theoretical spectra.

Figure 2 (a) $^{13}$C and (b) $^1$H MAS NMR spectra of arterial tissues. The relative concentrations of fatty acids shown on table 3 represent the ratio between the spectral area of the specified fatty acid and the spectral area of (CH₂)n corresponding to the “lipid pool”.
Due the semi-solid nature of tissue sample, the homonuclear coupling among protons is very effective in $^1$H measurement leading to line broadening and consequently loss of resolution in spectra. To overcome this situation, special techniques including high spinning speed (up to 30 kHz), must be used in order to minimize this coupling, and consequently, to achieve a good resolution in the $^1$H spectra. Unfortunately, the MAS probe used in this work has a maximum velocity of approximately 6 kHz. Another factor of equal importance is that due the nature of tissues, high speed is prohibitive. A possible alternative for this case is to perform measurements in a liquid state where the high resolution of $^1$H lines is naturally achieved and the lipidic components are kept unchanged. Anyway, the simulated $^1$H spectrum by de ACD program of Cho molecule shows a pattern of multiple lines characteristic of liquid system, with chemical shift values between 0.70 and 5.27 ppm, approximately. As can be seen in figure 2b, there are also peaks in this same range of chemical shift, which could be associated with the Chol and CE’s molecules present in the tissues. This hypothesis is supported by the $^{13}$C data that confirm the presence of this kind of molecule.

| Assignment                                   | $^{13}$C Chemical Shift (ppm) |
|----------------------------------------------|------------------------------|
| CE-COOH[CH$_2$]$_2$CH$_3$, C=O (C48)         | 173.9                        |
| Linoleic/Oleic/Linolenic, acyl C=C (C2-5)    | 128.2 to 130.0               |
| Chol and CE-COOH[CH$_2$]$_2$CH$_3$, CH (C4,C5)| 57.1, 50.3                   |
| Chol and CE-COOH[CH$_2$]$_2$CH$_3$, quaternary C (C1,C2) | 42.7, 36.8               |
| Chol and CE-COOH[CH$_2$]$_2$CH$_3$, CH$_2$ (C14,C22,C10) | 40.0, 36.8, 32.2             |
| Linoleic, CH$_2$ (C14, C11, C16 and 17)     | 32.2, 30.0, 28.3             |
| Oleic, CH$_2$ (C11, C8-9 and 15-16, C13 and 14) | 32.2, 30.0, 28.3             |
| Linolenic, CH$_2$ (C14, C16 and 17)         | 30.0, 28.3                   |
| CE-COOH[CH$_2$]$_2$CH$_3$, CH$_2$ (C29-31 and 42-45, C18,25,41) | 30.0, 28.3               |
| Chol, CH$_2$ (C9,25)                        | 28.3                         |
| Linoleic/Oleic/Linolenic, terminal CH$_3$ (C18) | 14.3                        |
| Chol, terminal CH$_3$ (C17,C20,C26 and 27)  | 12.2, 19.5, 22.8             |
| CE-COOH[CH$_2$]$_2$CH$_3$, terminal CH$_3$ (C17,C50,C20) | 12.2, 14.3, 19.5             |

4 Discussion

To establish a quantitative relationship between the resonance spectra and evolution of lipidic components of atheromatous plaques, we examined the UFA/PUFA, PUFA/(CH$_2$)$_n$, UFA/(CH$_2$)$_n$ and CE/(CH$_2$)$_n$ ratios corresponding to the relation between the area under the corresponding spectral lines in the fatty acids. The UFA/PUFA, PUFA/(CH$_2$)$_n$ and UFA/(CH$_2$)$_n$ ratios are considered markers of the degree of saturation in fatty acids whereas CE/(CH$_2$)$_n$ ratio tells us the relative proportion of CE in the tissues. We can use the methylene carbons, (CH$_2$)$_n$, as a reference present in all fatty acids (see Table 1) to obtain the desired ratios. Indeed, these methylene carbons are usually considered as a “lipid pool” [21]. To highlight the differences in lipidic composition of the plaques, the calculated fatty acid ratios of the samples are organized as follows: i) patient with low classification atherosclerotic lesion (I – II), ii) average values (and standard deviation) from patients with classification V and iii) patient with classification VII. These results are summarized in figure 3 which shows the ratios and respectively bar errors.

As we can see in figure 3, the UFA/PUFA ratio of subject I - II is low as compared with subjects V and VII (approximately 2 times lower). As lesion classification increases, the UFA/PUFA ratio also increases until it achieves an apparent plateau for subjects V onward up to VII. On the other hand, PUFA/(CH$_2$)$_n$ and UFA/(CH$_2$)$_n$ ratios are higher for subject I – II and decrease when the lesion classification increases until achieving a new apparent plateau (~ 0.2) for subjects V onward. The PUFA/(CH$_2$)$_n$ and UFA/(CH$_2$)$_n$ increase approximately 6 and 4 times respectively from lesion classification VII to I - II. Therefore, based on these observations we can suggest that patients with low lesion classification (apparently healthier)
have higher levels of PUFA and UFA than subjects with higher classification. At the same time, it seems that the subjects classified as V and VII do not show significant differences among themselves. Additionally, when the lesion classification increases from I – II to V and VII, the conversion rate of PUFA into UFA, by breaking of two or three double bonds, is duplicated as can be seen in the UFA/PUFA ratio. The final step in this cycle would be the conversion of UFA in saturated fatty acids. Unfortunately, it can not be seen clearly in the spectra, most probably due to a lower mobility of these molecules. We believe that this apparent cycle of events where polyunsaturated are converted to unsaturated and subsequently to saturated fatty acid, is associated with oxidative process\[21\] of chemical compounds present in the plaques with higher classification of atherosclerotic lesion (i.e., V – VII).

**Figure 3.** Evolution of lipidic ratios of all samples shown according to increase of classification Atherosclerotic lesion. For subject V, average values and standard deviations were calculated using samples B, C and D.

Regarding CE/(CH\(_2\))\(_n\), we observed in figure 3 a similar behavior for PUFA/(CH\(_2\))\(_n\) and UFA/(CH\(_2\))\(_n\), i.e., the levels of CE relative to the total concentration of methine carbons (“lipid pool”) is higher for low lesion classification subjects (I – II) and decreases approximately three times until stabilizing in a new plateau observed for those subjects with higher classification. This finding is partly in agreement with Toussant et al\[21\] who found values of approximately two times the CE when compared in the same fashion. These authors relate the decrease of CE in more stenotic lesion with increase of OR (Obstruction Ratio). Similarly, Brown and Goldstein\[22\] have associated the loss of CE in more stenotic lesion with a possible inhibition of the re-esterification cycle caused by the cytotoxic effect of oxidized lipoproteins which leads to the accumulation of free cholesterol. Indeed, the nature of lipid’s mobility (liquid, mixing of liquid-crystalline and crystalline) and their respective phase transitions, is considered another key factor that can contribute to destabilization of the plaques when subjected, for example, to external physical effort\[23\]. Other NMR approach, such as Cross Polarization (CP) pulse sequences, can be adopted to acquire signals from more crystalline lipids in order to provide more quantitative parameters to elucidate this matter. Further works are planned in this sense.

5 Conclusions

Based on the classification of atherosclerotic lesion and our NMR results, we found that the lipidic composition of atheromatous plaque evolves from a state with high levels of PUFA and UFA for a subject with lower classification (I – II) to a new state containing, respectively, 6 and 4 times less unsaturated fatty acids that those subjects with more stenotic lesions (V – VII). In particular, the average rate (or speed) of conversion of PUFA to UFA is, approximately, two times higher in more stenotic lesion. Besides, less mobile CE (“soft” lipids) is present in these plaques with progress of the disease. We consider that our preliminary NMR spectroscopy in vitro results of \(^1\)H and, mainly \(^{13}\)C, gives us some important clues about the evolution of lipidic components of atheromatous plaque that could be related to vulnerability to rupture, demonstrating the importance of this method to study the disease.
Competing interests
The author(s) declare that they have no competing interests.

Acknowledgments
We thank to Fundação de Amparo à Pesquisa do Estado de São Paulo by the financial support.

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