Localized *In Vivo* ^{31}P NMR Studies on Rabbit Skeletal Muscle Tissue from Premortem to Postmortem Period

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Abstract: Localized *in vivo* ^{31}P NMR spectroscopy was applied to evaluate the postmortem catabolism of high energy phosphates in rabbit skeletal muscle tissue. In the premortem processes all of the important high energy phosphate metabolites were characterized, and particularly phosphocreatine (PCr) resonance signal was the strongest. In the immediate phases of the postmortem processes the signal intensities of PCr, phosphomonoesters (PME), phosphodiesters (PDE), α-, β- and γ-adenosine triphosphate (ATP) resonance began to decrease while the signal intensity of inorganic phosphorus (Pi) resonance began to increase. The present study suggests that localized *in vivo* ^{31}P NMR spectroscopy may provide more precise biochemical information of the early postmortem period based on the metabolic alterations of phosphate. The unique ability of localized *in vivo* ^{31}P NMR spectroscopy to offer noninvasive information about tissue biochemistry in animals as well as human may have an impact on thanatochronology and medicolegal science.

Key words: legal medicine; ^{31}P NMR; skeletal muscle

INTRODUCTION

Since Hoult et al.[1] reported that it is possible to obtain ^{31}P NMR spectra of muscle tissue, ^{31}P NMR spectroscopy has been successfully employed in the study of living systems ranging from bacteria to entire animals and the human. The ^{31}P NMR spectroscopy modality has been increasingly applied to identify and quantify the levels of biochemical compounds as well as to investigate the metabolism of a variety of diseases and disorders since it is a noninvasive and potentially risk-free technique. The energy metabolism bounded with the

presence of high energy phosphates [phosphocreatine (PCr), ATP], inorganic phosphate (Pi) was reported by ^{31}P MRS in cardiac muscle,[2] cat,[3] cod,[4] chicken and pigeon,[5] guinea pig,[6] rabbit,[7] rat,[8] and human.[9] ^{31}P NMR spectroscopy is also able to estimate intracellular pH, metabolic activities of the glucogenolytic muscle fibers, enzyme activities of citric cycle, oxidative phosphorylation, pentose cycle, lipids metabolism and ATPases activity.[10-11]

Before the advent of NMR spectroscopy biochemical analysis was the only means of investigation to provide diagnostic aids in practical medical jurisprudence. Thanatochronological characteristics have been sought by several experimental procedures [12-15] which had been devised to estimate the time of death. The procedures of chemical thanatochronology had evolved as a chemical indicator to define the postmortem change within first 24 hours after physical death.[16] The recent development of spatially localized *in vivo* NMR pulse sequence, image selected *in vivo* spectroscopy (ISIS),[17] was developed for improving localization and optimization of the volume of interest (VOI) in MR images. Hence, spatially localized *in vivo* ^{31}P MRS could provide a better quality of information on the postmortem biochemical changes in the muscle for evaluation of the time of death in medicolegal science.

The purpose of this study is to evaluate the postmortem catabolic process which affect the phosphate metabolites in the skeletal muscles to provide diagnostic aids in legal medicine as well as to establish a protocol of *in vivo* forensic experiments. Employing spatially localized *in vivo* ^{31}P NMR spectroscopy with the ISIS pulse sequence, we have investigated the sequential alterations of the ^{31}P NMR spectra of the postmortem skeletal muscle in a homogeneous group of rabbits.

MATERIALS AND METHODS

Subjects

Adult ten male rabbits (New Zealand White) weighting 3.5 to 4 kg in body weight fed on standard laboratory diet were used. For the premortem examination they were injected under intraperitoneal narcosis with 8 ml/kg of 20 % urethane solution. For the postmortem examination they were then sacrificed by intravenous air embolism with 20 ml. Since it took approximately 25 minutes for *in vivo* ^{31}P NMR spectrum per case, the earliest measurement in the postmortem period was obtained at 30 min. The time sequence in the postmortem period was designed as follows: eight rabbits were examined at 30, 60, 90, 120, 150, 180 min and 24 hours respectively.

NMR Spectroscopy

All localized *in vivo* ^{31}P NMR experiments were performed on 1.5 T whole-body

magnetic resonance imaging (MRI)/magnetic resonance spectroscopy (MRS) system (Signa Advantage 4.8 Version, GE Medical System, Milwaukee, Wisconsin) with use of a ^{1}H and ^{31}P dual 6 inch surface coil (Hospital of the University of Pennsylvania, Philadelphia, PA) that was tuned with two excitation frequencies (63.86 MHz for ^{1}H and 25.85 MHz for ^{31}P). All localized *in vivo* ^{31}P NMR spectra were obtained from the rabbit left tight muscle tissue at ambient temperature (22 °C). The localization was guided by T_1-weighted, axial MR images obtained with 30 cm FOV and 5 mm thickness as the first step in the NMR spectroscopic examination. T_1-weighted MR image was selected for short time performance. Using point resolved spectroscopy (PRESS) pulse sequence, the external magnetic field B_0 was shimmed to the sensitive volume of the surface coil positioned on the rabbit left thigh muscle by means of the proton resonance from tissue water. The water line width (full width half maximum) was typically 6-7 Hz.

ISIS pulse sequence was employed for localized volumes of 60 cm^3 (2 × 5 × 6 cm^3). A hard pulse was used for the excitation of a homogeneous region of interest. Spectral parameters were as follows: 256 × 128 of acquisition matrix; 8 NEX of acquisition; 30 msec of echo time; 3 sec of repetition time; 256 of number of averaging; 2500 Hz of spectral width; 2048 of number of data points; 8 of phase cycle. All of the *in vivo* ^{31}P NMR spectroscopic data were transferred to a Sun SPARC station *IPC* (Sun Microsystems, Mountain View, California, U.S.A.) and processed by the SAGE data analysis package (GE Medical Systems, Milwaukee, Wisconsin, U.S.A.). The raw data of free induction decay (FID) were obtained from completion of scan averages per each examination. An exponential multiplication of line broadening 3 - 5 Hz was applied for apodization of suppressing the noise level. After one-dimensional Fourier transformation, the spectra were phased manually by zero and first order phase correction. All localized *in vivo* ^{31}P NMR spectra were plotted and analyzed in the pure absorption mode. For quantitative measurement of area, all resonance peaks were simultaneously fitted to Lorentzian lineshapes using Marquart algorithm.

Chemical shifts in the localized *in vivo* ^{31}P NMR spectra were referenced to the position of PCr (δ = 0.0 ppm). *In vivo* ^{31}P NMR resonances in the spectra obtained from rabbit left tight muscle tissue were tentatively assigned on the basis of prior assignment.[18]

The pH was calculated from the difference (δ_{obs}) of the fitted line positions of PCr and Pi with use of the following formula:[19]

$$pH = 6.803 - \log[(5.33 - \delta_{obs})/(\delta_{obs} - 3.22)]$$

Statistics

Statistical analysis was performed using SPSS (SPSS for Windows, Version 6.0,

SPSS Inc., Chicago, IL).   The data were analyzed with two-tailed t-tests, where $p < 0.05$ was considered significant to account for multiple comparisons.

RESULTS

Fig. 1. shows the T_1-weighted axial MR image of rabbit skeletal muscle tissue defining the volume of interest (VOI) selected for localized *in vivo* ^{31}P NMR spectroscopy. The size of VOI located within left thigh muscle tissue is $2 \times 5 \times 6$ cm^3 corresponding to a volume of 60 ml.

A typical localized *in vivo* ^{31}P NMR spectrum obtained from rabbit skeletal muscle tissue in the premortem period is shown in Fig. 2. In the premortem period the phosphate components of skeletal muscle tissue consist of phosphomonoesters (PME), PCr, phosphodiesters (PDE), Pi, α-, β- and γ-phosphorus of ATP. Resonance peaks of phosphate metabolites were assigned as PME, 7.1 ppm; Pi, 5.3 ppm; PDE, 3.6 ppm; PCr, 0.0 ppm; γ-phosphorus of ATP, -2.4 ppm; α-phosphorus of ATP, -7.7 ppm; β-phosphorus of ATP, -16.1 ppm in Fig. 2.


Fig. 1. T1-weighted axial MR image of rabbit skeletal muscle tissue defining the Volume Of Interest (VOI) selected for localized *in vivo* ^{31}P MRS.

PCr

PME PDE | γ   α   β
Pi

20   0   -20

ppm
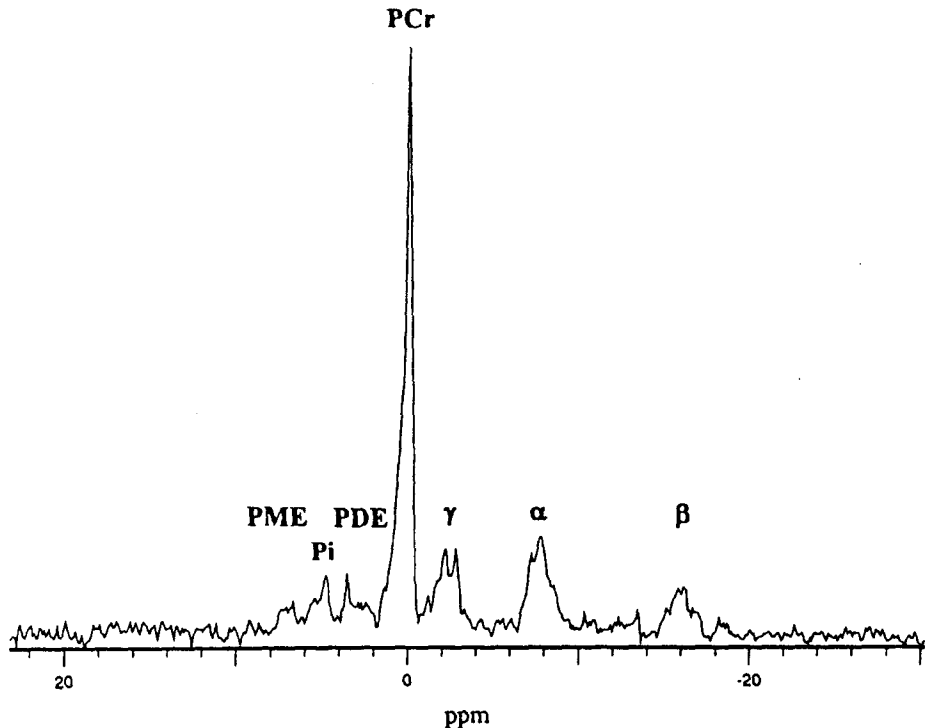
Fig. 2. A typical image-guided *in vivo* [31]P MR spectrum obtained from rabbit skeletal muscle tissue in the premortem period. Chemical shifts are indicated in parts per million (ppm).

Signal intensities of PCr, Pi, α-, β-, and γ-phosphorus of ATP corresponding to the time period at the premortem and the postmortem (30, 60, 90, 120, 180 min and 24 hours) are shown in Table 1. Values given in Table 1 are mean ± standard deviation. Fig. 3. shows the spectral intensity changes of major phosphate metabolites of skeletal muscle tissue from the premortem and the early postmortem period. A typical localized *in vivo* ^{31}P NMR spectrum obtained from the skeletal muscle tissue in the 24 hours postmortem period is shown in Fig. 4.

In vivo ^{31}P NMR spectrum in the 30 min postmortem period was characterized by decreasing PCr, α-, β- and γ-phosphorus of ATP, increasing Pi signal, immediately disappearing PME and PDE signals. Conversion of Pi to PCr with remarkable increase of Pi content but significant depletion of PCr as well as ATP was shown *in vivo* ^{31}P NMR spectrum in the 60 min postmortem period. Accumulation of a large amount of Pi
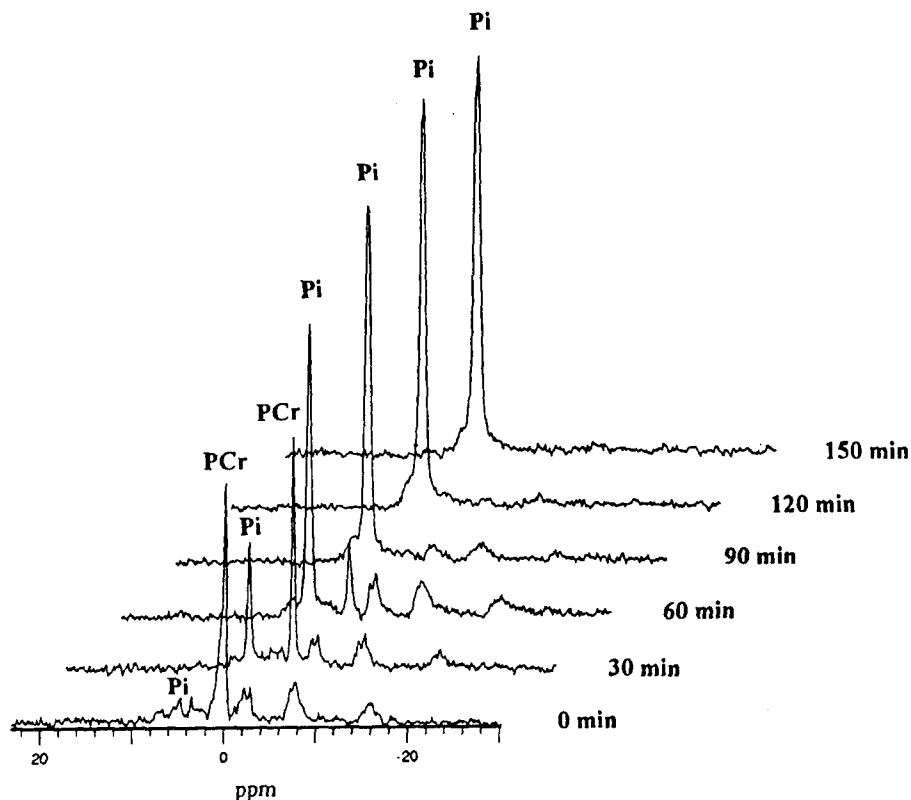
Fig. 3. *In vivo* ^{31}P MR spectra showing the intensity changes of major phosphate metabolites of rabbit skeletal muscle tissue from premortem and postmortem period.

and almost disappearance of PCr, α-, β- and γ-phosphorus of ATP were demonstrated in the 150 min postmortem spectrum.   Only a large Pi signal and complete disappearance of PCr, α-, β- and γ-phosphorus of ATP were shown in the 180 min postmortem spectrum which was similar to the 24 hours postmortem spectrum in Fig. 4. Among α-, β- and γ-ATP, β-ATP signal was disappeared first. Only a significantly higher content of Pi was observed in the 24 hours postmortem period. Fig. 5. shows the graphic representation of intensity variations of phosphate metabolites of skeletal muscle tissue from premortem to postmortem period.

The pH of 7.29 ± 0.10 measured in the premortem period was continuously decreased

Table 1. Statistical Evaluation of *In Vivo* ^{31}P MRS Signal Intensity and pH Changes of Major Phosphate Metabolites from Premortem to Postmortem Period (mean ± SD) ($p < 0.005$).

Time (min)	Pi	PCr	γ-ATP	α-ATP	β-ATP	PH
0	43.6 ± 11.3	554.8 ± 88.0	132.9 ± 30.0	143.4 ± 24.1	73.9 ± 13.1	7.29 ± 0.10
30	287.5 ± 20.0	333.3 ± 67.5	124.8 ± 29.0	136.7 ± 23.0	51.0 ± 28.2	7.12 ± 0.11
60	545.8 ± 43.5	127.5 ± 18.0	121.3 ± 10.6	135.0 ± 33.7	38.6 ± 17.0	6.89 ± 0.08
90	686.0 ± 64.0	33.5 ± 16.8	54.2 ± 28.0	56.1 ± 29.0	23.1 ± 7.4	6.70 ± 0.06
120	812.5 ± 82.0	19.1 ± 2.1	10.3 ± 2.9	11.3 ± 2.4	4.6 ± 1.0	6.57 ± 0.05
150	987.3 ± 95.5	8.7 ± 1.5	2.2 ± 1.5	2.5 ± 1.8	1.3 ± 0.7	6.41 ± 0.08
180	1113 ± 140	0	0	0	0	A
24 hours	1991 ± 218	0	0	0	0	A

[a] Not available.

in the postmortem period. The pH was measured to be 6.57 ± 0.05 in the 20 min postmortem period. The pH change of phosphate metabolites from the premortem to postmortem period is shown in Table 1.

DISCUSSION

We have demonstrated the ability of a noninvasive technique, localized *in vivo* ^{31}P NMR spectroscopy at 1.5 T system to monitor phosphate metabolic alterations of rabbit intact skeletal muscle tissues in postmortem period. A high degree of spatially localized *in vivo* ^{31}P NMR spectra obtained by ISIS pulse sequences revealed the well characterized high energy phosphate (PCr and α-, β- and γ-ATP) and Pi. Alterations of phosphate metabolite content in the postmortem period can be detected by localized *in vivo* ^{31}P NMR spectroscopy which allows accurate kinetic measurements in the postmortem period. As can be seen in Fig. 3., PCr and Pi were the most easily monitored among the various phosphate metabolites. The area of PCr resonance was decreased and that of Pi resonance was increased as a
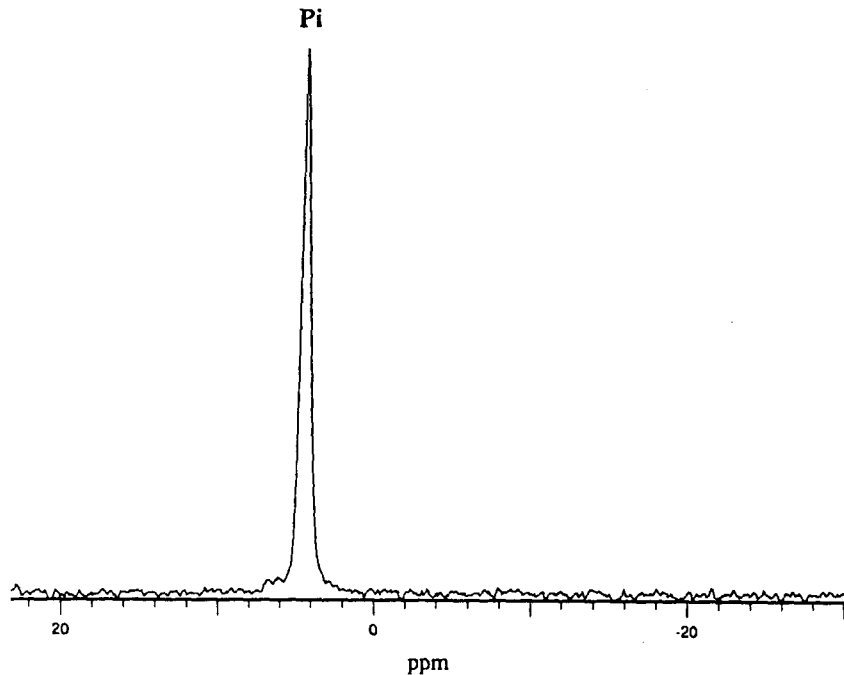
Fig. 4. A typical image-guided *in vivo* [31]P   MR spectrum obtained from rabbit skeletal muscle tissue in 24 hour postmortem.

function of time. It was appeared that the first 150 min postmortem process was the most active period for phosphate metabolites.

Time dependent postmortem variations of ATP and PCr metabolites in the skeletal muscle tissue were slightly different from those by Renou et al.[7] who reported the postmortem catabolism of high energy phosphate compounds and the associated intracellular pH variation on the various muscle tissues of rabbits. The ATP content was remained constant up to 240 min and disappeared at 360 min in their study, while those contents remained comparatively constant at 60 min and disappeared at 120 min in our study. The PCr content completely disappeared at 3 hours in our study, and at 6 hours in their study. The retardation of disappearing ATP and PCr contents in their study may be illustrated by the difference of muscle preparation and magnet field strength. The intact *in vivo* samples of the whole body and 1.5 T were used in our study while dissected and excised samples and 4.7 T were used in their study. It was, however, consistent with Renou et al. that time dependent postmortem variation of PCr and Pi showed exponential decay and
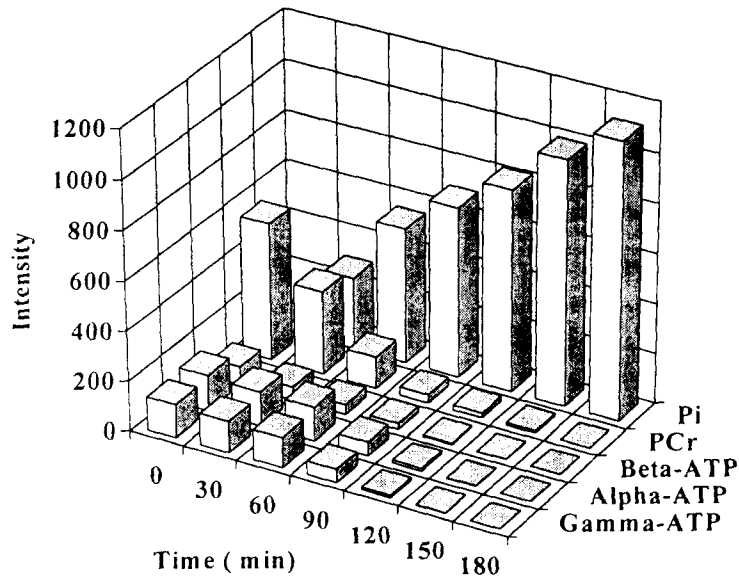
Fig. 5. Graphic representation of intensity alterations of phosphate metabolites of rabbit skeletal muscle tissue from premortem to postmortem.

linear increment, respectively as a function time. Our observation and their findings suggest that the ATPase for metabolite breakdown is activated in the early (2-6 hours) postmortem period.

It was reported that the relative content of phosphate compounds in various muscles were different depending on the animal species and the different composition of the muscle fibers [5,7]. A common characteristic of the two group[5,7] is that sampling was from the dissected muscle tissues. Since slaughtering of the animal and cutting out the muscle during sampling results in decrease of a part of PCr and activation of glycolytic process in contraction state,[20-21] it seems that it is important to investigate corpse without damage. Although localized *in vivo* ^{31}P NMR spectroscopy showed that it is not easy to identify the various muscle fibers on MR image, *in vivo* technique could prevent the metabolite disturbances due to twitching and damage to the tissue during the dissection and excision process, and frozen procedure. To our knowledge, the results presented here constitute the first *in vivo* ^{31}P NMR postmortem study using ISIS pulse sequence for preservation of the intact *in vivo* samples of the whole body.

The time dependent postmortem variation of the pH in rabbit intact skeletal muscle

tissue is in good agreement with Renou et al..[7] The pH values measured in the premortem period were 7.29 ± 0.10 in our study and 7.14 in their study, repectively. Both studies showed that the pH values were continuously decreased in the postmortem period. In Table 1. the pH was measured from the premortem to the 150 min postmortem period. The pH could not be determined from the 180 min postmortem period since the PCr signal was disappeared. The decrease of pH indicated that the skeletal muscle tissue was acidifying in the postmortem period.

On the qualitative view point, ^{31}P NMR spectra of the skeletal muscle tissue in the low and high magnetic fields are most affected by the postmortem catabolic changes, and it is necessary to make spectrum corresponding to further prolonged postmortem tissue. In order to ascertain the precision in determining the thanatochronology, the immediate postmortem skeletal muscle should be examined to estimate the time dependence of peak intensities of phosphate metabolites.   However, at present *in vivo* NMR technique in 1.5 T, at least 256 transients accumulated in a total time of 25 min are required for qualitative data.

In conclusion, using an ISIS pulse sequence at 1.5 T, localized *in vivo* ^{31}P NMR spectroscopy was employed to investigate the postmortem catabolic processes of the metabolites in the rabbit skeletal muscle for diagnostic aids in medicolegal science and establishment of practical model in thanatochronology. The present results suggest that localized *in vivo* ^{31}P NMR spectroscopy could yield a good estimate of the thanatochronology by simultaneous qualitative and quantitative analysis of the biochemical change of metabolites in muscle tissue during the postmortem period. Localized *in vivo* ^{31}P NMR spectroscopy can be a useful modality in the field of thanatochronology and medicolegal science in the near future.

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