Original Article

Pretreatment optimization of tissue metabolomics in colorectal cancer

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Background and Objectives: To optimize the pretreatment method of colorectal cancer tissue samples for metabolomics research based on solid-phase nuclear magnetic resonance (NMR). **Methods and Study Design:** The mucosal tissues of colorectal cancer were classified into five groups with a volume of 0.2 cm*0.2 cm*0.2 cm. The pretreatment methods for each group were as follows: I. Preservation with liquid nitrogen alone. Samples were also treated with liquid nitrogen for 10 (II), 20 (III), and 30 min (IV), respectively, immediately after isolation and then transferred to a -80°C refrigerator; V. Only -80°C refrigerator storage. No more than 30 minutes should pass between isolation and pretreatment of tumor samples. The tissue sample testing process was carried out on Bruker AVII-600 NMR Spectrometer. NMR signals were collected and analysed using partial least-squares discrimination analysis (PLS-DA) to explore the effects of different pretreatment methods on the metabolic changes of samples. **Results:** The levels of pelargonic acid, stearic acid, D-Ribose, heptadecanoic acid, pyruvic acid, succinate, sarcosine, glycine, creatine, and L-lactate in the group I (only liquid nitrogen) were significantly lower than the other groups (p<0.05); the content of glycerophosphocholine in the group I (only liquid nitrogen) was lower than that in the other groups (p=0.055). These indicated that the glucose and choline phospholipid metabolism levels of the liquid nitrogen group were significantly lower than those of the other four groups. **Conclusions:** Liquid nitrogen storage can stop the metabolic process of glucose and choline phospholipid in colorectal cancer tissue samples *in vitro*, thus maintaining the metabolic state of tissue samples *in vivo* as much as possible.

Key Words: NMR, magic angle rotation nuclear magnetic resonance, metabolomics, colorectal cancer, pretreatment protocol

INTRODUCTION

Metabolomics is a high-throughput technique for investigating metabolites in human tissues and fluids. The metabolic characteristics of the tissue/fluid at a specific time point are called "metabolic fingerprint". Pattern recognition can be used for processing metabolic fingerprints and selecting biomolecules for disease diagnostics or treatment. These biomolecule sets are also called "metabolome".[1] Metabolome plays an important role in the diagnosis and treatment of many diseases, such as cancer, critical illness, etc. Much cancer research has been conducted based on metabolomics, such as the prediction of cancer stage, the monitoring of metastasis, the prediction of chemotherapy sensitivity, the assessment of drug efficacy and the evaluation of toxicity.[2-19] Common detection techniques in metabolomics include nuclear magnetic resonance (NMR) and mass spectrometry (MS). The advantages of NMR are that the pretreatment of samples is

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very simple and the testing is non-destructive,[20-21] making it more suitable for clinical research. In addition, NMR equipped with a high resolution-magic angle spinning probe (HR-MAS)[22] can be used for the analysis of intact tissue samples, which can be met by small samples obtained via surgery[23-25] or needle aspiration biopsy.[26-27]

Colorectal cancer is the third most common cancer in the world and the second most common cause of death from cancer.[28-29] It is of great significance to establish a metabolic model of colorectal cancer for precision treatment.[30-33] It is well known that the metabolic rate of tumor cells is significantly higher than the normal ones. Ideally, tissue metabolic research should provide a real-time "snap shot" of tissues when they were resected. In the current pretreatment protocol of resected tissue, there is a time interval between sample harvest and detection. The metabolic processes would not stop in resected cancer tissue until its deep frozen. This kind of delay in pretreatment may lead to changes in metabolic state, and the information obtained from detection cannot represent its "*in vivo*" status. This is because various enzymatic reactions in cells lead to changes in metabolic characteristics of tissues.[34] Therefore, the key to ensure the quality of samples is to avoid the freezing delay and use a reasonable sample pretreatment method. Some researchers have discussed and analyzed the concept of freezing delay time. They proposed that delayed freezing leads to metabolic changes, and the effect on the metabolic activity of breast cancer within 60 minutes after being isolated from the body is acceptable.[34] In terms of sample pretreatment methods, most researchers choose liquid nitrogen storage, -80°C storage, or both based on their experience.[35-41] The metabolic characteristics of colorectal cancer are different from those of breast cancer, and the biological behaviors between them are also distinctive. In this study, we aim to explore the comparison of various pretreatment methods based on HR-MAS-MRS technology for colorectal cancer.

METHODS

This was a prospective clinical cohort investigation, and the research protocol included in this was approved by the Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital [2019-202]. The clinical trial's registration number is ChiCTR1900024640. All enrolled patients were required to sign an informed consent form.

Inclusion and exclusion criteria

All patients underwent planned surgery at Sichuan Provincial People's Hospital, and the primary cancer nest was completely removed.

The inclusion criteria were as follows: 1) colorectal cancer; 2) clinical stage cT3NxMx-cT4NxMx; and 3) age 18-80 years.

Exclusion criteria were: 1) severe heart, liver, kidney, and hematopoietic diseases; 2) history of metabolic and endocrine diseases such as diabetes and hyperthyroidism; 3) pregnancy or lactation; 4) mental illness leading to inability to cooperate with treatment, psychotic illness, lack of self-control, and inability to express clearly; 5) participation in other clinical trials; and 6) not signing informed consent.

Sample collection

The mucosal tissues of colorectal cancer were divided into five groups with a volume of 0.2 cm*0.2 cm*0.2 cm and put in cryotubes numbered as (A1, A2, A3, A4, A5) (B1, B2, B3, B4, B5), etc... The tissue samples were processed in the following ways:

I. Stored in liquid nitrogen immediately after they were resected;

II. Transferred to the -80°C refrigerator after storage in liquid nitrogen for 10 minutes;

III. Transferred to the -80°C refrigerator after storage in liquid nitrogen for 20 minutes;

IV. Transferred to the -80°C refrigerator after storage in liquid nitrogen for 30 minutes;

V. Stored in the -80°C refrigerator immediately after they were resected.

NMR experiment

All samples were clipped with a sterile blade and put into a 4-mm zirconium OD rotor (the entire sample volume is 50 µl, and the average weight is 8.8mg); then, 10 µl D_2O was added to the vessel for locking and shimming.[42-45]

The testing process was carried out on a Bruker AVII-600 spectrometer at 20°C, which was equipped with a 1H/13C magic-angle spinning high-resolution probe (Bruker Company, Switzerland). The rotor, which contained the tumor tissue sample, was inserted into the NMR instrument, and the instrument ran at the speed of 5000 Hz for 10 minutes at room temperature. This instrument collected the NMR signals in the form of a spectrum and generated a document to record it. The detailed testing parameters were as follows: center frequency was 600.11 MHz, sampling spectrum width was 20 parts per million (ppm), accumulation times was 64 times, pre-saturation excitation pulse zgpr, excitation intensity was 5 microseconds (18 watts), and the pre-saturation power was 1.8*10-5 watts for water signal. In this testing process, the sampling interval was 5 seconds.

NMR data analysis

The collected NMR signal data were imported into MestReNova software (version 6.1.0, Mestrelab Research SL, Spain), which can show a one-dimensional NMR spectrum through Fourier transform; the ppms from 0.13 to 8.99 was segmented in units of 0.01 ppm to obtain 896 chemical shift segments. Finally, we integrated each chemical shift segment to obtain the corresponding integral value for the purpose of converting the spectrum signal to a digital signal.

Subsequently, we imported the data matrix into MATLAB R2013b (Mathworks, USA), and the partial least-squares discriminant analysis (PLS-DA) was used to reduce data dimensionality.

The variable importance in the projection (VIP) of the PLS-DA model, with corresponding chemical shifts, was calculated. Chemical shifts with VIP values >1 and $p<0.05$ were selected, and corresponding metabolites were investigated using the human metabolome database (HMDB, https://hmdb.ca/). Finally, the up or down of regulated metabolites and disturbed pathways were visualized.[49] All data were not normalized and scaled.

Statistical analysis

Continuous variables are presented as either mean (standard deviation) or median (interquartile range), whereas categorical variables are presented as frequencies and percentages.

For continuous variables that were normally distributed, we used Student t-tests; whereas, for skewed data, we used the Kruskal-Wallis test. Categorical data were analyzed using the chi-squared and Fisher exact probability tests. For evaluation of the consistency between different diagnostic criteria, the kappa test was used. The significance level was set at 0.05. All analyses were conducted using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp, Armonk, NY, USA).

RESULTS

Patients and clinical assessments

The basic characteristics of patients are shown in Table 1. Of 20 patients were enrolled in the study, 8 was diagnosed with ascending colon cancer, 6 had sigmoid colon cancer, and 6 had rectal cancer. Most of the patients were elderly, and their BMI fluctuated within the normal range.

Differences in metabolomics among five groups

As shown in Figure 1, the effect of different pretreatment methods on the metabolic status of samples was significant.

Further analysis demonstrated that there were 60 ppms with VIP>1, and 11 characteristic metabolites related to different metabolic pathways were identified (Table 2). As shown in Figure 2, the relative amounts of nonanoic acid, octadecanoic acid, ribose, heptadecanoic acid, pyruvate, succinic acid, sarcosine, glycine, creatine, and lactic acid in the group I (only liquid nitrogen group) were significantly lower than those in the other four groups (p<0.05). Although statistically insignificant, the content of glycerophosphocholine in the liquid nitrogen group was lower than in the other four groups (p=0.055). There was no significant difference among the group II, III, IV and V.

Metabolic network

To investigate the relationships among differentially represented metabolites, a correlation network diagram was constructed (Figure 3). The glucose and choline phospholipid metabolism levels of the liquid nitrogen group were significantly decreased compared to those in the other four groups.

Table 1. Demographic and baseline characteristics of the study population

Cancer species	Male/Female (n/n)	Age (years)	BMI (kg/m$^{2)}$
Ascending colon cancer (n=8)	4/4	64.6±10.6	22.2±2.63
Sigmoid colon cancer (n=6)	4/2	76.8±6.08	21.25±4.7
Rectal cancer (n=6)	4/2	59.2±13.2	23.6±2.15

BMI: body mass index.

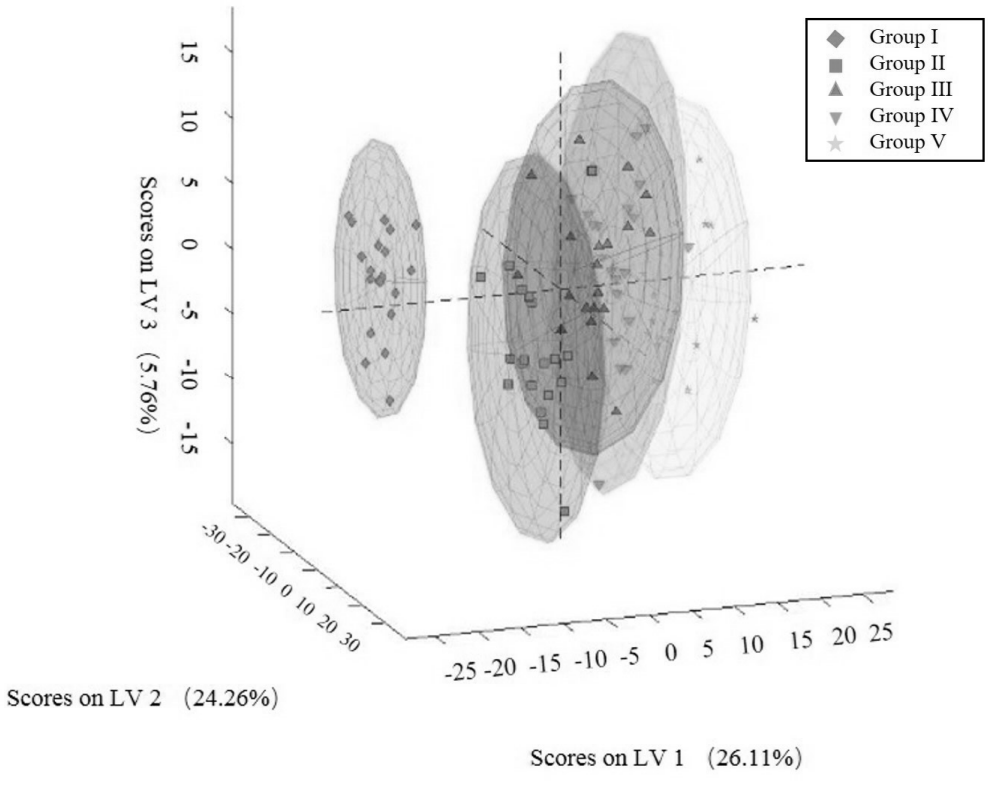

Figure 1. PLS-DA of different pretreatment methods.

Table 2. The relative content of metabolites

PPM	Metabolites	I	II	III	IV	V	*p*
1.28	Pelargonic acid	68.8±9.85	80.9±10.9	78.4±10.3	79.6±10.4	83.6±8.90	0.018
1.8	Stearic acid	33.4±1.86	35.5±3.17	37.6±2.91	36.3±2.10	37.3±4.29	0.005
2.21	D-Ribose	34.6±2.27	37.7±2.76	38.1±2.84	37.6±2.24	36.6±2.84	0
2.3	Heptadecanoic acid	30.5±1.92	33.4±2.93	34.4±1.78	33.4±2.39	34.6±1.73	0
2.46	Pyruvic acid	25.0±1.24	26.00±1.29	26.4±0.83	26.0±0.94	26.5±0.85	0.009
2.6	Succinate	26.3±1.00	27.2±1.15	27.6±1.29	27.4±0.97	28.1±1.48	0.002
3.2	Glycerophosphocholine	61.4±5.86	71.9±14.5	72.8±4.53	74.7±14.9	71.6±3.87	0.055
3.6	Sarcosine	34.0±4.12	40.4±9.64	44.1±17.6	42.2±17.8	51.6±30.9	0.049
3.54	Glycine	34.5±4.38	37.5±7.08	40.0±7.21	37.9±5.56	42.2±7.76	0.042
3.92	Creatine	33.7±3.14	37.7±4.31	39.4±4.79	38.1±5.22	40.8±5.40	0.005
4.08	L-Lactate	28.0±2.81	30.6±3.50	31.9±2.69	30.8±3.88	32.4±3.34	0.003

PPM: Parts Per Million.

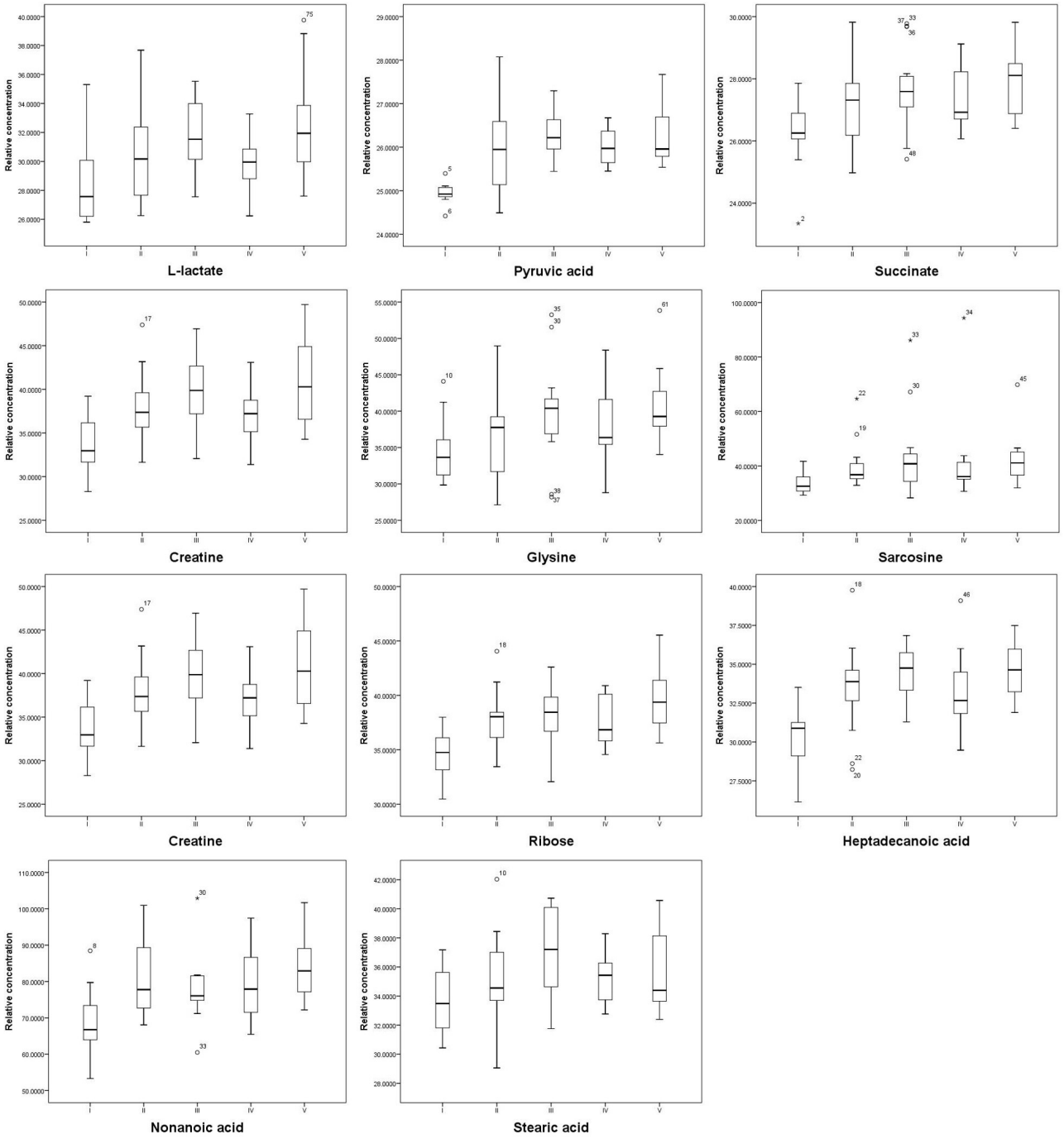

Figure 2. Metabolite levels in different groups.

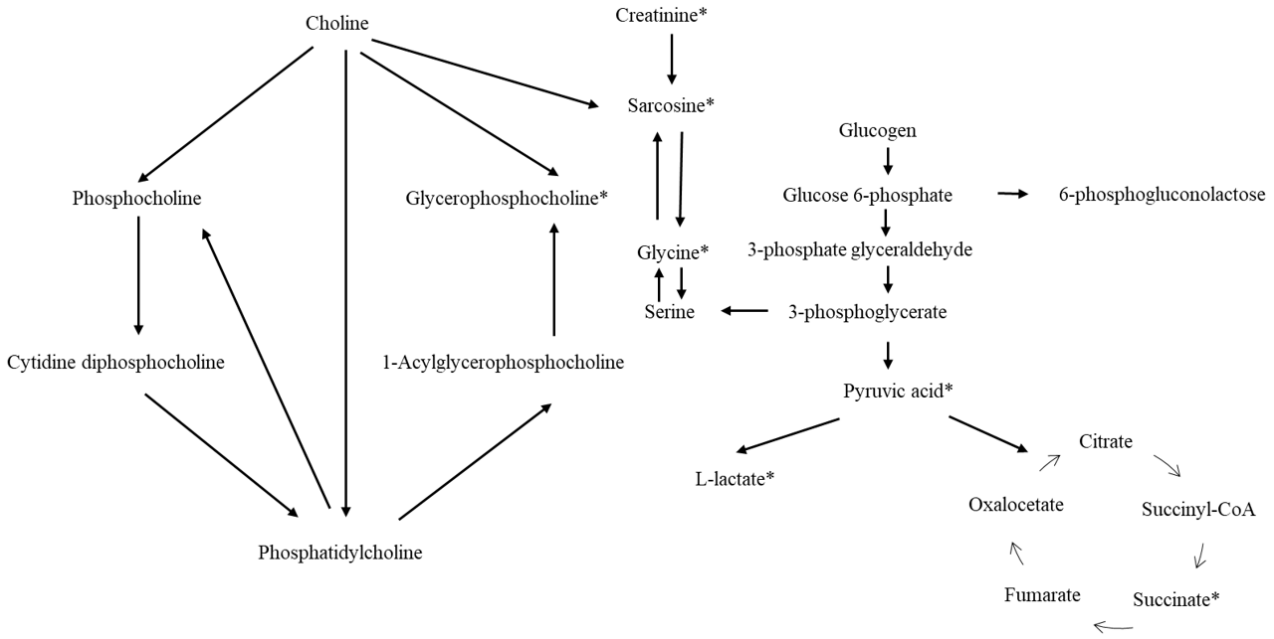

Figure 3. Metabolic network of significantly altered metabolites. *Significantly different metabolic pathways.

DISCUSSION

After decades of development, metabolomics is now widely used in clinical research. In particular, solid-phase NMR has become a common research tool used by clinical researchers to obtain accurate information from tissue samples.

Collecting possible metabolic information from tissue samples *in vivo* is the key to good metabolomics research. However, due to the sample pre-processing and transit required, it is usually impossible to perform NMR testing immediately after the tissue sample separation. Generally, the metabolic processes continue after the tissue samples are removed from the body, and metabolic levels are affected by the sample storage mode. Therefore, tissue samples must be temporarily stored under conditions that minimize the loss of tissue metabolic information and diminish the changes in metabolic state. Inappropriate pretreatment methods of tissue samples may change the metabolic information in vivo, resulting in the acquisition of wrong metabolic information by NMR testing, which would greatly affect the results and even cause trial failure. Therefore, we tried to identify the optimal pretreatment method for colorectal tissue samples in this research.

Glucose is the main source of energy for cell metabolism. It is mainly supplied via three metabolic pathways: glycogen synthesis, glycolysis, and pentose phosphate (PPP).[50] The carbohydrate metabolism process of cancer cells is different from that of normal tissue cells, and their rapid division and proliferation make cancer cells prefer glucose as energy supply material. Cancer cells have extremely fewer organelles and are unable to carry out complex biochemical reactions. In the process of carbohydrate metabolism in cancer cells, the main metabolic mode is called Warburg effect, also known as aerobic glycolysis.[51-53] The level of pyruvic acid and L-lactate, as the downstream metabolites of glucose metabolism, could reflect the speed of glucose metabolism. When the tissue samples are in vivo, glucose metabolism is underway all the time. But after removal from the body, this metabolic process continues or changes to produce some metabolites, which are irrelevant to metabolic information in vivo. In this research, we found that the levels of pyruvic acid and L-lactate were lower in the liquid nitrogen group than those in the other groups. As can be seen in Figure 3, pyruvic acid and L-lactate are downstream metabolites of glucose metabolism, indicating that pyruvic acid and L-lactate are produced continuously along with glucose metabolism after *in vivo*, and the speed of pyruvic acid and L-lactate production in those samples stored in liquid nitrogen was slower than that of those in the other groups. We can therefore conclude that the speed of glucose metabolism in liquid nitrogen is slower than that of those seen in the other groups.

As the core metabolite of choline phospholipid metabolism, choline also had a higher level in the liquid nitrogen than the other groups. It is known that choline continues to decrease as choline phospholipid metabolism progresses. We found that the level of glycerophosphocholine in the liquid nitrogen group is the lowest among all groups, implying that the speed of choline consumption in liquid nitrogen is the slowest. We can conclude that liquid nitrogen storage could slow down the rate or even stop choline phospholipid metabolism.[54-56]

In addition, we also found that the levels of nonanoic acid, octadecanoic acid, heptadecanoic acid, and ribose in the liquid nitrogen group were significantly different from those of the other four groups. Relevant literature findings showed that these compounds are related to the metabolic pathway of putrefaction. However, the specific pathway has not been described in the literature. Based on the existing research data, we can make the following speculations: nonanoic acid, octadecanoic acid, heptadecanoic acid, and ribose may be related to the corruption process of the tissue samples after in vivo. Liquid nitrogen can stop not only the internal metabolic process of the cell but also bacterial-related corruption. Specific metabolic processes, specific mechanisms, and pathways need to be studied after the availability of relevant research.[57-59]

We manually screened 11 compounds with significant differences. However, there are 60 compounds with a VIP value greater than 1, and other compounds too may play important roles in different pathways.

Although we obtained much useful information from this research, the following limitations are noted. The sample size of our study was not large; thus, the results can only be used as a catalyst for methodological innovation. There was no statistical difference in the changes of most metabolites. Therefore, only if the sample size is expanded in the future can we continue to explore the metabolic discipline of related metabolites and consequently explain the corruption of metabolic pathways.

Conclusion

Storing tissue samples in liquid nitrogen immediately after removal can stop their glucose metabolism and choline phospholipid metabolism. Among the pretreatment protocols we tested in this research, the loss of metabolic information from tissue samples stored in liquid nitrogen was the minimal, and the impact was also minimal. In conclusion, storing resected colorectal cancer tissue samples directly into liquid nitrogen can preserve in vivo metabolomic information. We recommend it as a standardized pretreatment procedure for clinical tissue samples for HR-MAS-MRS research.

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AUTHOR DISCLOSURES

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature or kind in any product, service or company that could be construed as influencing the position presented in the manuscript entitled. This manuscript has not been published or submitted elsewhere.

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