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Comparison of three methods for collecting interstitial fluid from subcutaneous tissue in mini pigs ☆

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ABSTRACT

Interstitial fluid, owing to its similarity to blood components and higher sensitivity and specificity, finds widespread application in disease diagnosis and tumor marker detection. However, collecting interstitial fluid, particularly from the deep subcutaneous connective tissue, remains challenging.

- This study aimed to compare three different collection methods - push-pull perfusion, multi-filament nylon thread implantation, and tissue centrifugation - for collecting interstitial fluid from the subcutaneous connective tissue layer of mini-pigs. High-performance ion chromatography was employed to analyze the conventional cation components in the samples and compare ion composition analysis between the different methods.
- Results indicated that while the distribution of conventional cations in the interstitial fluid collected by the three methods was generally consistent, there were slight variations in the detection rates and concentrations of different ions. Hence, suitable collection methods should be selected based on the ions or collection sites of interest.

Specifications table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	interstitial fluid physiology
Name of your method:	extraction of subcutaneous connective tissue fluid
Name and reference of original method:	Brekke H K, Oveland E, Kolmannskog O,et al.Isolation of interstitial fluid in skin during volume expansion: evaluation of a method in pigs.J.American Journal of Physiology Heart & Circulatory Physiology, 2010, 299(5):H1546–53.DOI:10.1152/ajpheart.01142.2009.
Resource availability:	sodium pentobarbital (0.5 mL/kg)(Abcam, American) xylazine promethazine solution (0.1 mL/kg)(Jilin Shengda Animal Medicine, China) peristaltic pump (ISMATEC, Switzerland) deionized water nylon thread (Kaiping Sheyaguo Electronic Commerce,China)

(*continued on next page*)

☆ **Related research article:** Unpublished articles, in the uploaded files, are called Related Research papers.

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	acetone solution
	75% ethanol
	Ringer's solution
	straight surgical needle (Ningbo Medical Stitches, China)
	4 ml centrifugal filter device (Amicon Ultra-4 ml 100 Kda, Merck-Millipore, American)
	15 ml centrifugal filtration device (Amicon Ultra-15 ml 100 Kda, Merck-Millipore, American)
	5810R Benchtop High-Speed Centrifuge (Eppendorf, Germany)
	ion chromatography method (DionexTM ICS-5000+ HPIC system, Thermo Fisher, American)
	Dionex IonPac CS12A cationic chromatography column(Thermo Fisher, American)
	Dionex IonPac CG12A cationic chromatography guard column(Thermo Fisher, American)

Method details

In recent years, numerous techniques for collecting interstitial fluid (ISF) have been applied for analysis, such as Microneedle [1–4], Implanted wick [5,6], Micropipettes [7], Catheter [8], Implanted capsules [9], Tissue centrifugation [10,11] (TC), Microdialysis [12,13], Capillary ultrafiltration [14,15], and so forth. Most of these methods are primarily utilized for collecting ISF from the dermis layer of the skin, as they are primarily intended for human and small animal samples. There is relatively less research on ISF collection methods from deeper subcutaneous connective tissues, such as samples from muscular inflammatory edema, where the aforementioned methods are mostly unsuitable. Here, we have chosen three methods, among which the multi-filament nylon thread implantation (MFNTI) and TC are more commonly employed techniques. The MFNTI is one of the commonly used in vivo sampling methods, albeit it may cause trauma and bleeding during implantation; whereas TC mostly involves ex vivo sampling post-mortem. Both of the aforementioned methods have relatively large sampling areas. Additionally, we introduce a push-pull perfusion (PPP) method for in vivo microregional sampling, which enables more precise sampling of the desired ISF sites. By comparing the differences in ion concentrations of ISF collected by these three methods, we evaluate their merits and drawbacks.

Animals

Three male uncastrated BaMa mini-pigs (Beijing Liuliuhe Kexing Experimental Animal Breeding Center) weighing 12 ± 2 kg were utilized for this study, with the license number SCXK (Jing) 2017–0003. The mini-pigs were provided with concentrated feed twice a day, with 150 g given at 8:00 and 100 g given at 20:00, and water was provided ad libitum. The animals were individually housed in cages at a room temperature of 20°C ± 2°C and a humidity of 50% ± 10% RH. The entire experiment was approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (Ethics Approval No.: D2022–04–14).

Anesthesia and preparation of the skin

3% solution of sodium pentobarbital (0.5 mL/kg) and xylazine promethazine solution (0.1 mL/kg) were separately injected into the muscles of both sides of the buttocks of the mini-pigs for anesthesia. The skin where ISF was to be collected was exposed by removing the hair using electric clippers. Any unclean areas were cleaned and dried using 75% alcohol swabs. ISF was collected from each site of each pig using three different methods, as illustrated in Fig. 1A, with a collection interval of one week for each method.

Push-pull perfusion method

After anesthesia of the mini-pigs, the area where ISF needs to be collected was disinfected with iodine and alcohol was used to remove the iodine. After the local area was dried, improvements were made based on the early methods used by our team [16]. A self-made catheter needle (shown in Fig. 1B) was used to puncture the subcutaneous connective tissue, and the needle tip was placed in the desired collection site. The inner needle was connected to the tubing of the catheter needle and then connected to the peristaltic pump. The peristaltic pump extracted deionized water to fill the tubing, and then the inner needle was inserted into the outer needle. After 90 min of insertion, when the local environment stabilizes, the peristaltic pump was activated with a parameter of 0.7 RPM to perform PPP. The outer needle infused deionized water into the tissue space, while the inner needle extracted ISF. When all the deionized water in the tubing had been perfused, the peristaltic pump was stopped. The inner needle was removed, and the pump was run in reverse to collect the ISF in a centrifuge tube, which was stored at −20 ˚C. ISF was collected from 15 points on the trunk and limbs of each pig, and a total of 45 samples were collected from three pigs (shown in Fig. 1C).

Multi-filament nylon thread implantation method

The nylon thread was cut into 12 cm pieces, placed in acetone solution for 30 min, and then in 75% ethanol for 30 min to remove wax and other debris. After drying, the thread was placed in Ringer's solution for 60 min and then dried for later use. After anesthesia of the pigs, folded the nylon thread in half and inserted it into a straight surgical needle. The needle was then inserted into the subcutaneous tissue in the direction shown in Fig. 1A. The nylon thread was implanted 5 cm long, and the site was covered with plastic wrap to prevent evaporation. After 90 min, the implanted thread was removed, and the nylon thread was visually inspected for blood contamination. Only white and light pink threads were accepted. The nylon thread was placed in a 4 ml centrifugal filter

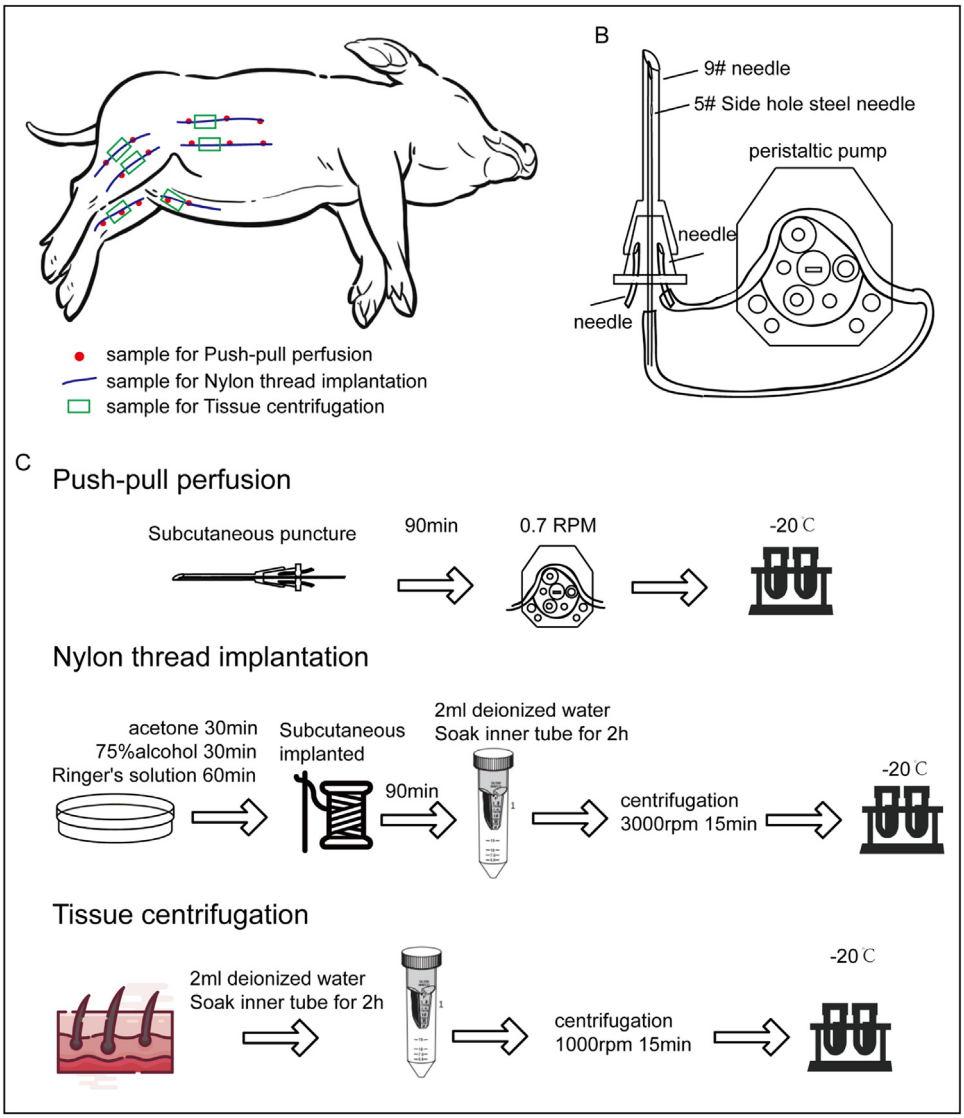

Fig. 1. A: Schematic diagram of the sampling sites for the three ISF collection methods. The red dot indicates the site for PPP, the blue line indicates the site for buried suture method, and the green box indicates the site for TC method. B: A self-made sleeve needle, with an outer needle of 9# gauge and an inner needle of 5# gauge with a side hole, sealed with glue at the bottom. There are two additional needles, one connected to the peristaltic pump hose and the other used for balancing air pressure. C: Schematic diagram of the ISF collection process for the three methods.

device, and 2 ml of deionized water was added for soaking and dilution for 2 h. The device is then centrifuged at 3000 rpm for 15 min, and the collected liquid from the bottom of the outer tube was placed in a −20 ˚C freezer for later use. Three lines were taken from the trunk and limbs of each pig, and a total of 18 samples were collected from three pigs (Fig. 1C).

Tissue centrifugation method

After euthanizing pigs by injecting an excessive amount of sodium pentobarbital into the abdominal cavity, a 2 × 1 cm piece of skin tissue was removed, washed with deionized water to remove surface blood contamination, and dried with filter paper after removing fat. The sample was then placed in a 15 ml centrifugal filtration device, soaked in 2 ml deionized water for 2 h, diluted, and centrifuged at 1000 rpm for 15 min. The liquid was collected from the bottom of the outer tube and stored at −20°C for later use. Three pieces of tissue were taken from the trunk and limbs of the same pig, and a total of 18 samples were collected from three pigs (Fig. 1C).

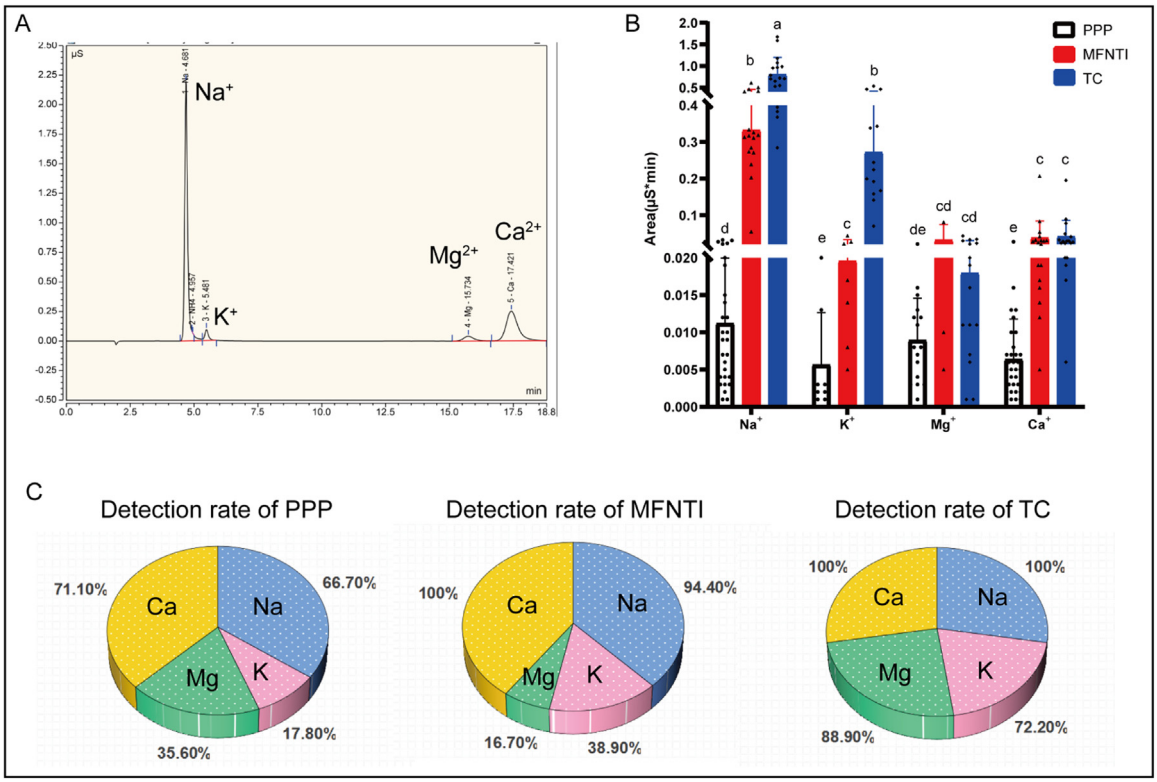

Fig. 2. A: Chromatogram of ion peaks in the sample. The peak area is proportional to the ion content, and the ion content is ranked as follows: Na^+ > Ca^{2+} > Mg^{2+} > K^+. B: Comparison of peak areas of each ion in ISF collected by the three methods. Significant differences are marked using letter labeling. C: Pie chart showing the detection rates of each ion using the three methods.

High performance ion chromatography

This method is simple, selective, sensitive, and has good reproducibility. It can simultaneously determine multiple components. Therefore, the ion chromatography method is feasible for determining the ion components in ISF [17–19].

1) Chromatographic Conditions

Cationic chromatography column: Dionex IonPac CS12A; Cation chromatography guard column: Dionex IonPac CG12A; Detector: Conductivity detector; Eluent: Isocratic elution with 20 mmol/L methylsulfonic acid (MSA) solution; Flow rate: 1.0 mL/min; Injection volume: 25μL; Column temperature: 30°C; Suppressor current: 50 mA.

2) Sample Preparation

100μL of ISF was transferred into a centrifuge tube, followed by the addition of 10 mL of ultrapure water. The mixture was vortexed for 5 min and then centrifuged at 5000 rpm for 5 min. The sample was filtered through a 0.22 μm aqueous phase filter membrane and analyzed under the aforementioned conditions. Duplicate samples were prepared and analyzed, recording retention times, peak heights, or peak areas for quantitative analysis.

Statistics

Data processing was performed using SPSS 22.0 software for statistical analysis. Continuous variables were expressed as mean ± standard deviation, and normality and homogeneity of variance were assessed. For variables meeting both criteria, one-way ANOVA was conducted; if data met only one or neither criteria, the Kruskal-Wallis H test was employed, with $P < 0.05$ indicating statistical significance.

Ion chromatography analysis

The chromatogram of the ISF sample was shown in Fig. 2A, with the elution order of Na^+, K^+, Mg^{2+}, Ca^{2+}. The peak area was proportional to the ion content, and among the four ions, the Na^+ content was the highest. As trace metal ions were also present in the dilution water, the ion peaks from the dilution water needed to be subtracted during data processing. If the result after subtraction was negative, it was considered as undetected.

Table 1
Comparison of ion concentrations in ISF collected by the three methods ($\mu S \cdot min$, $\bar{x} \pm s$).

Method	Conc. of Na+	Conc. of K+	Conc. of Mg2+	Conc. of Ca2+
PPP	0.0112±0.0088	0.0056±0.0070	0.0089±0.0057	0.0064±0.0054
MFNTI	0.3321±0.1251	0.0196±0.0132	0.0320±0.0425	0.0381±0.0458
TC	0.8044±0.3957	0.2715±0.1430	0.0179±0.0123	0.0416±0.0436

Analysis of ion content in ISF collected by three methods

The overall detection rates of the four ions by the PPP method were 66.7% for Na^+, 17.8% for K^+, 35.6% for Mg^{2+}, and 77.1% for Ca^{2+}. The detection rates for Na^+ and Ca^{2+} were higher (Fig. 2C). The Na^+ content in the overall sample was significantly higher than that of K^+ ($P = 0.036$) and Ca^{2+} ($P = 0.013$), but not significantly different from that of Mg^{2+}. There was no significant difference between the other ions (Fig. 2B).

The overall detection rates of the four ions by the multi-filament nylon thread implantation (MFNTI) method were 94.4% for Na^+, 38.9% for K^+, 16.7% for Mg^{2+}, and 100% for Ca^{2+}. The detection rates for Na^+ and Ca^{2+} were higher (Fig. 2C). The Na^+ content in the overall sample was significantly higher than that of K^+ ($P < 0.001$), Mg^{2+} ($P < 0.001$), and Ca^{2+} ($P < 0.001$). There was no significant difference between the other ions (Fig. 2B).

The overall detection rates of the four ions by the TC method were 100% for Na^+, 72.2% for K^+, 88.9% for Mg^{2+}, and 100% for Ca^{2+}. The detection rates of all four ions were relatively high (Fig. 2C). The Na^+ content in the overall sample was significantly higher than that of K^+ ($P < 0.001$), Mg^{2+} ($P < 0.001$), and Ca^{2+} ($P < 0.001$); the K^+ content was significantly higher than that of Mg^{2+} ($P < 0.001$) and Ca^{2+} ($P < 0.001$). There was no significant difference between the other ions (Table 1).

The ion content distributions in the ISF collected by the three methods were similar, with the Na^+ content being the highest and significantly different from the other ions. There was no significant difference among the other three ions. Although the ion content distribution was consistent, there were differences in ion concentrations among the three methods. For Na^+ and K^+, there were significant differences among the three methods, with the TC method having the highest ion concentration, followed by the MFNTI method and the PPP method. There was no significant difference in Mg^{2+} concentration among the three methods. There was no significant difference in Ca^{2+} concentration between the MFNTI and TC methods, but both were significantly higher than the PPP method.

Discussion

Three methods all showed good detection rates for Na^+ and Ca^{2+}, with TC method having the highest detection rate. However, in TC method, the distribution of K^+ among the four ions was somewhat higher compared to the other two methods. This may be due to the diffusion of potassium ions along the concentration gradient during centrifugation, causing the K^+ that are rich in intracellular fluids to leak into the extracellular fluids, leading to this result. This result is consistent with Elham's report [20], and therefore this method is not suitable for research on K^+.

The results of the distribution of ISF ion content in samples collected from different parts of the three methods are basically consistent. Among the four conventional cations detected, Na^+ had the highest content, which is consistent with the fact that the most abundant cation in extracellular fluid is Na^+. Although there are some differences in ion concentration among the three methods, because the ISF collected by the three methods is all diluted, it is not possible to compare them quantitatively at the same dilution concentration. Therefore, the significance of comparing ion concentrations between different directions is not significant, and this issue will be further improved in the future.

From the ion content distribution of the three methods, all three methods can be used to collect ISF, but different methods are suitable for studying different ions or different parts. If you want to study Na^+ and Ca^{2+}, all three methods can be used. However, if the sample size is small, it is not advisable to use the PPP method, as the slightly lower detection rate will reduce the available sample size. The PPP method is weak in detecting K^+, while the MFNTI method is weak in detecting Mg^{2+}. The MFNTI method is suitable for studying K^+, while the study of Mg^{2+} is suitable for using the PPP and TC methods.

Although TC has a high detection rate, it is not suitable for long-term comparative observation of biological samples as it requires material collection. In such cases, the PPP and MFNTI methods can be utilized. While the ion content collected by the PPP method is relatively low, it offers the advantage of more precise and limited collection sites, allowing for more accurate targeting of the desired area. On the other hand, the amount of ISF collected by the MFNTI method depends on the length of the nylon thread, which is typically over 5 cm. This method is suitable for collecting ISFs in the trunk and limbs of large animals, and the other two methods may be preferred when sampling is performed in less flat areas.

This study focused solely on the ion composition of ISF under normal physiological conditions, using the three sampling methods described. Future studies will explore in-depth analysis of ISF under pathological conditions, including protein and metabolic aspects. As noted in the introduction, while numerous methods exist for collecting ISF, few are suitable for the subcutaneous connective tissue layer. The three methods presented in this article offer novel approaches and insights for scientific research on ISF and also provides sample collection methods for analyzing ISF using HPLC.

The PPP method, MFNTI method, and TC method are all effective techniques for collecting ISF from subcutaneous connective tissue in mini-pigs, and they show similar distributions of conventional cations. However, specific collection methods should be chosen based on the research objectives and the location of interest for fluid collection.

Ethics statements

The authors affirm that the animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Feng Xiong: Data curation, Writing – original draft. **Yu Zheng:** Data curation, Writing – original draft. **Yinggen Ouyang:** Validation, Software. **Xiaojing Song:** Validation, Software. **Shuyong Jia:** Validation, Software. **Guangjun Wang:** Visualization, Investigation. **Shuyou Wang:** Visualization, Investigation. **Qi Liu:** Visualization, Investigation. **Jing Zhao:** Conceptualization, Methodology, Writing – review & editing. **Weibo Zhang:** Conceptualization, Methodology, Writing – review & editing.

Data availability

The authors do not have permission to share data.

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