Effects of cryogenic grinding on soft-tissue optical properties

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Optical properties obtained from spectrophotometer measurements of reflectance and transmittance were determined for both frozen-ground and intact soft tissues. The tissues used in these experiments were calf aorta, rat jejunum, and rabbit sciatic nerve. Tissue specimens from each tissue type were frozen in liquid nitrogen and then ground with a pestle and mortar into a fine powder. A tissue paste formed once the powder returned to room temperature. The tissue paste was then sandwiched between glass slides for spectrophotometer measurements. For comparison, the optical properties of the intact specimens were also measured. Total transmission and diffuse reflection were obtained on a Varian Cary 5E spectrophotometer (400–850 nm). Absorption and reduced scattering coefficients of the tissues were determined with the Inverse Adding Doubling method. Our results suggested that within the 400-nm to 850-nm spectrum, optical properties of the ground tissue approximated intact tissue within limits of experimental error.

Key words: Spectrophotometer measurement, integrating sphere, tissue optics, optical properties.

1. Introduction

Tissue optical properties are important parameters for prescribing light dosimetry in laser medicine. Optical properties are usually obtained by placing a tissue specimen on the port of an integrating sphere in a spectrophotometer and then recording reflectance and transmittance of the specimen.[1,2] From these measurements, absorption and reduced scattering coefficients can be calculated by using an Inverse Adding Doubling model.[3] One important criterion for obtaining accurate data is that the specimen must fill the sample port of the integrating sphere, which usually has a diameter greater than 1 cm. Often tissue samples are not sufficiently large to permit integrating sphere measurements. By homogenizing the tissue and spreading the paste over a wider area, one can obtain a sample with an area sufficient to cover the sample port. Homogenized tissue paste has been used for added absorber experiments[4] and for goniometry measurements to determine anisotropy.[5] In this study we investigated the feasibility of estimating the optical properties of intact tissue from spectrophotometer measurements from ground tissue.

2. Methods

A. Tissue Selection

Both intact and paste specimens of rat jejunum and calf aorta were used in the spectrophotometer measurement. Moreover, two of the intact rat jejunum specimens were measured after they were thawed from liquid nitrogen (LN_2) freezing as a control on the effect of freezing the tissue. Because rabbit sciatic nerves were too small to fill the integrating sphere port, only the tissue paste was used in the measurement. As a way to test whether the paste specimen could be a mixture of tissue obtained from different donors, two rabbit sciatic nerve paste specimens were prepared with nerves from two different animals.

B. Tissue Preparation

The following protocol was used to produce ground tissue. Tissue was harvested and used within 12 h after the animals were sacrificed. Prior to usage, tissue specimens were wrapped in gauze soaked with cold phosphate-buffered saline and stored at 4 °C. The tissue was cleaned and trimmed so that a homogenous specimen was obtained. A tissue specimen was cut into two pieces; one piece was reserved as the intact control sample, and the other piece was put into a mortar. LN_2 was slowly poured into the

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Table 1. Thickness of Each Tissue Specimen[a]

Calf aorta		Rat jejunum			Rabbit Sciatic Nerve Paste (n = 5)
Intact (n = 4)	Paste (n = 6)	Intact (n = 3)	Thawed (n = 2)	Paste (n = 5)	
1.00 (i)	0.73 (i)	0.21 (v)	0.35 (vi)	0.17 (v)	0.25[b]
1.03 (ii)	0.63 (i)	0.27 (vi)	0.29 (vii)	0.06 (v)	0.17[b]
1.45 (iii)	0.70 (ii)	0.40 (vii)		0.15 (vi)	0.16
1.39 (iv)	0.43 (ii)			0.16 (vii)	0.17
	0.50 (iii)			0.20 (vii)	0.09
	0.65 (iv)				

[a]Thickness is given in millimeters; parenthetical roman numerals denote animal numbers.
[b]Specimen was a mixture of nerve tissues from two different donors.

mortar until it covered the tissue. As the LN_2 slowly boiled away, the tissue became brittle. LN_2 was poured until it covered the tissue for the second time. After the LN_2 had boiled away, the tissue was gently broken into smaller pieces with a pestle. LN_2 was added again and the tissue was ground with the pestle until it turned into a fine powder. LN_2 was added as necessary to keep the tissue pieces brittle for easy grinding. It usually took three freeze-grind cycles to achieve fine tissue powder. The resulting tissue powder was placed onto a clean glass slide. The diameter of the powder particles was visually estimated to be approximately 0.5 mm. The powder began to thaw when it touched the glass slide, and a tissue paste was formed. The paste was spread evenly over the glass slide. A cover slide was placed over the paste to avoid trapped air bubbles, and the slides were taped together. The time between grinding and optical properties measurements was less than 1 h.

Each intact control sample had a slight variation in thickness over the whole specimen area. When the sample was sandwiched between glass slides, a more uniform thickness was achieved. Precautions were taken to ensure that the thicknesses of both the intact and the paste specimens were uniform over the area that was placed over the sample port of the integrating sphere. Each sample's thickness was measured with a pair of precision calipers, and the thickness variation over the area of interest did not exceed 0.01 mm. Thickness measurements were repeated after the spectrophotometer measurement; no significant difference in thickness was observed. The thickness of each tissue sample is listed in Table 1. The integrating sphere sample port has a diameter of 1.6 cm. Each sample has an overall dimension of approximately 2.5 cm × 2 cm. All slides were kept in cold gauze soaked with phosphate-buffered saline until reflection and transmission were measured with the spectrophotometer.

C. Spectrophotometer Measurements

Total transmission and total reflection were measured on a Varian Cary 5E spectrophotometer equipped with an integrating sphere. The spectral range was 400 nm to 850 nm. The absorption coefficient, μ_a, and the reduced scattering coefficient, $\mu_s' = \mu_s(1 - g)$, were calculated with INVERSE ADDING DOUBLING software,[6] which is suitable for single and multiple scattering.

3. Results

The optical properties of calf aorta and rat jejunum specimens computed from the spectrophotometer measurements with the Inverse Adding Doubling method are listed in Tables 2–4. The paste optical properties in Table 2 are the averages over all paste specimens. Table 3 shows that the large standard deviation of the rat jejunum paste data could be reduced when the thinnest specimen was excluded from

Table 2. Comparison of Optical Properties of Intact, Thawed, and Paste Rat Jejunum

	Intact (n = 3)				Thawed (n = 2)		Paste (n = 5)			
λ (nm)	μ_a (1/mm)	μ_s' (1/mm)	μ_a (SD)	μ_s' (SD)	μ_a (1/mm)	μ_s' (1/mm)	μ_a (1/mm)	μ_s' (1/mm)	μ_a (SD)	μ_s' (SD)
400	1.12	1.74	0.15	0.12	0.85	1.77	1.15	2.27	0.68	0.63
450	0.53	1.40	0.17	0.12	0.33	1.35	0.59	1.82	0.52	0.60
500	0.20	1.13	0.12	0.12	0.10	1.05	0.30	1.48	0.35	0.54
550	0.33	1.02	0.15	0.12	0.18	0.94	0.39	1.39	0.36	0.49
600	0.18	0.87	0.13	0.12	0.08	0.78	0.28	1.21	0.34	0.46
650	0.13	0.78	0.11	0.12	0.05	0.68	0.26	1.13	0.33	0.46
700	0.14	0.72	0.12	0.12	0.05	0.62	0.28	1.08	0.35	0.44
750	0.15	0.68	0.13	0.12	0.07	0.60	0.32	1.06	0.37	0.43
800	0.17	0.66	0.13	0.12	0.08	0.56	0.36	1.04	0.42	0.43
850	0.20	0.64	0.16	0.17	0.09	0.53	0.37	1.04	0.38	0.50

Table 3. Comparison of Optical Properties of Intact and Paste Rat Jejunum, Excluding the Thinnest Paste Specimen

	Intact (n = 3)				Paste (n = 4)			
λ (nm)	μ_a (1/mm)	μ_s' (1/mm)	μ_a (SD)	μ_s' (SD)	μ_a (1/mm)	μ_s' (1/mm)	μ_a (SD)	μ_s' (SD)
400	1.12	1.74	0.15	0.12	0.87	1.99	0.29	0.17
450	0.53	1.40	0.17	0.12	0.38	1.56	0.21	0.15
500	0.20	1.13	0.12	0.12	0.15	1.25	0.12	0.14
550	0.33	1.02	0.15	0.12	0.24	1.17	0.14	0.11
600	0.18	0.87	0.13	0.12	0.14	1.01	0.11	0.11
650	0.13	0.78	0.11	0.12	0.12	0.92	0.10	0.11
700	0.14	0.72	0.12	0.12	0.13	0.88	0.11	0.11
750	0.15	0.68	0.13	0.12	0.16	0.87	0.11	0.10
800	0.17	0.66	0.13	0.12	0.18	0.85	0.12	0.09
850	0.20	0.64	0.16	0.17	0.20	0.82	0.12	0.13

the calculation. In one of the calf aorta specimens, it was possible to obtain one intact sample and two paste samples. The reflectance, transmittance, absorption coefficient, and reduced scattering coefficient as a function of wavelength are presented in Fig. 1. The intact sample had a thickness of 1.03 mm, and the paste samples had thicknesses of 0.43 mm and 0.7 mm. Spectral data for rat jejunum are presented in Fig. 2. Once again, two paste samples were available with thicknesses of 0.17 mm and 0.06 mm. The corresponding intact sample had a thickness of 0.21 mm. Overall, the standard deviations of paste optical properties are larger than those of intact tissue optical properties. The large standard deviations can be explained by the fact that the paste averages were taken from paste specimens of different thicknesses. We have observed that when a tissue paste is made into two different thicknesses, the thickness closer to that of intact tissue consistently gives closer optical property values to the intact tissue. This is illustrated in Figs. 1 and 2. The optical properties as a function of wavelength of nerve tissue paste obtained from multiple donors and paste obtained from single donors are presented in Fig. 3.

4. Discussion

Marchesini *et al.*[7] measured the spectral optical properties of *ex vivo* human colon. Their result of the average absorption coefficient of 13 colon samples has large absorption peaks at 420, 540, and 577 nm, indicating a significant oxygenated blood content. The spectrum of absorption coefficients for rat jejunum in Fig. 2(c) of this paper depicts peaks at 430 and 554 nm, indicating deoxygenated blood. At 700 nm, where the effect of blood is minimal, the absorption of the intact rat jejunum sample is approximately 0.4 mm^{-1}, and Marchesini *et al.* reported an average value of 0.13 mm^{-1} with a standard deviation of 0.04 mm^{-1}. Our reduced scattering coefficient at 700 nm is 0.72 mm^{-1}, and Marchesini reported an average value of 0.65 mm^{-1} with a standard deviation of 0.2 mm^{-1}.

Our intact aorta data had an absorption coefficient of 0.04 mm^{-1} and a reduced scattering coefficient of 2.19 mm^{-1} at 633 nm. Yoon[8] reported values from normal human aorta of $\mu_a = 0.05\ mm^{-1}$ and $\mu_s' = 4.1\ mm^{-1}$ at the same wavelength. These values are certainly lower than the data of Çilesiz and Welch[9] and of Keijzer *et al.*[10]

At the time of our study, we were not able to find any published data on the optical properties of peripheral nerves. This may be because most peripheral nerves are usually too small for a conventional spectrophotometric optical property measurement. In fact, our approximation method was developed to address this difficulty in obtaining intact tissue opti-

Table 4. Comparison of Optical Properties of Intact and Paste Calf Aorta

	Intact Calf Aorta (n = 4)				Paste Calf Aorta (n = 6)			
λ (nm)	μ_a (1/mm)	μ_s' (1/mm)	μ_a (SD)	μ_s' (SD)	μ_a (1/mm)	μ_s' (1/mm)	μ_a (SD)	μ_s' (SD)
400	0.44	4.73	0.04	0.59	0.75	4.35	0.29	1.18
450	0.19	3.60	0.03	0.22	0.23	3.36	0.08	0.84
500	0.08	3.04	0.01	0.25	0.11	2.82	0.04	0.68
550	0.12	2.57	0.03	0.20	0.15	2.50	0.05	0.62
600	0.06	2.31	0.01	0.19	0.07	2.21	0.02	0.52
650	0.04	2.11	0.01	0.20	0.06	2.03	0.02	0.48
700	0.04	1.94	0.01	0.18	0.06	1.88	0.02	0.45
750	0.05	1.81	0.01	0.17	0.08	1.78	0.02	0.42
800	0.05	1.71	0.01	0.16	0.08	1.69	0.03	0.41
850	0.06	1.61	0.01	0.17	0.10	1.62	0.03	0.39

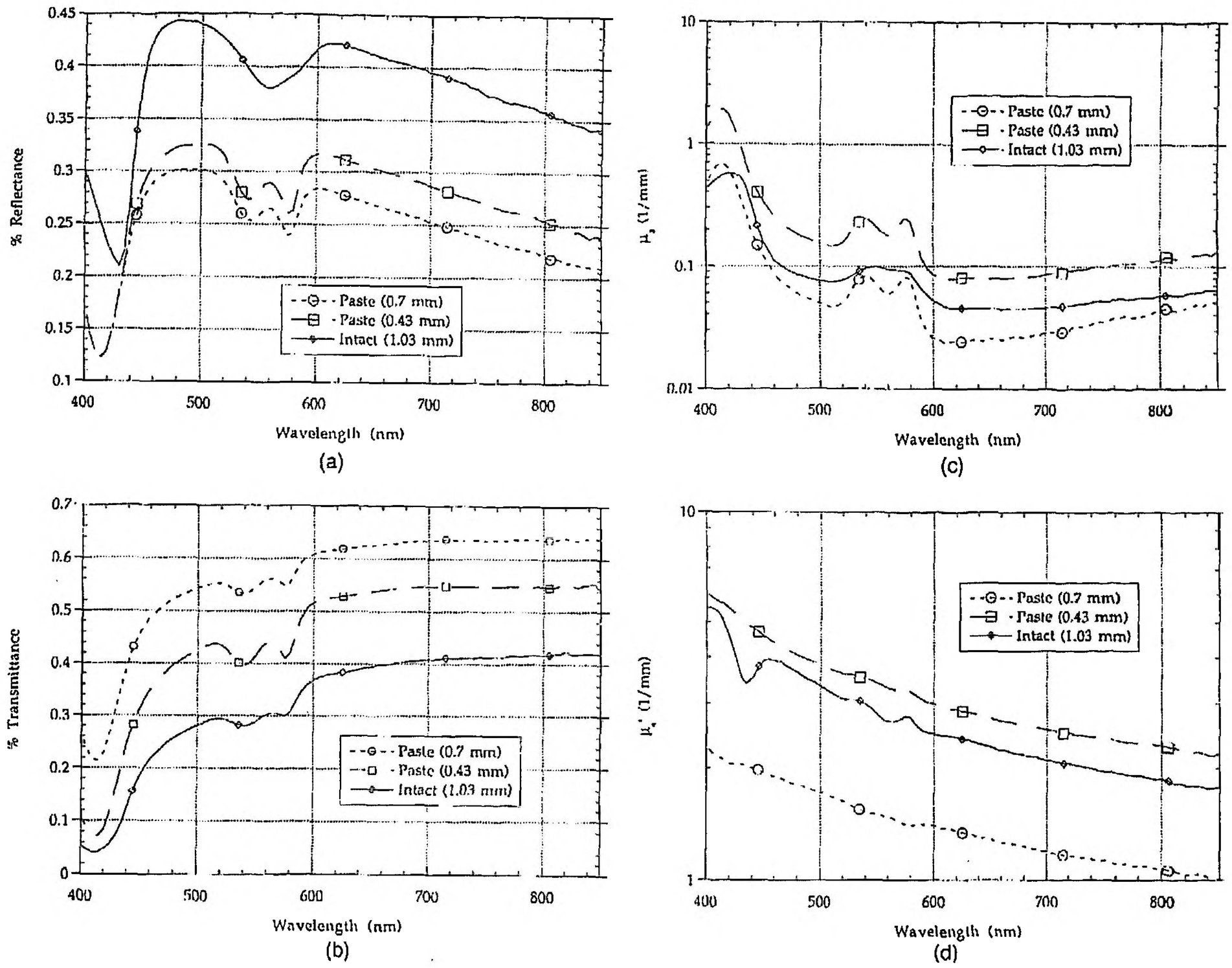

Fig. 1. Calf aorta (a) reflectance, intact versus paste; (b) transmittance, intact versus paste; (c) absorption coefficient, intact versus paste; (d) reduced scattering coefficient, intact versus paste.

cal properties when the surface area of the sample is less than the size of the integrating sphere sample port.

Freezing and grinding certainly cause changes to tissue structure. Depending on the cooling or thawing rates and the final frozen temperature, both extracellular and intracellular ice formation can occur. Extracellular ice formation may cause cellular dehydration as a result of the changes in solute concentration of the cellular constituents. This may cause proteins to lose their tertiary and quartenary structures, resulting in tissue denaturation. Intracellular ice formation may induce mechanical injury as a result of ice crystallization that damages the cell walls.[11,12] Moreover, grinding tissue structures and mixing cellular constituents further elevate the amount of tissue damage. We expect that these factors affect tissue optical properties.

The effects of cryopreservation on human aorta optical properties have been studied by Çilesiz and Welch.[13] Their results suggest that freezing introduces a significant decrease in the absorption coefficient from 300 nm to 800 nm. Our thawed rat jejunum data are consistent with their observation. The absorption of the paste jejunum listed in Table 2 is higher than that of intact jejunum. When we excluded the thinnest rat jejunum paste data from our calculation, Table 3 shows that the absorption of the freeze-ground paste is lower than that of the intact unfrozen tissue. However, the calf aorta freeze-ground paste does not seem to support this observation. We believe the reason for the discrepancy is again due to sample thickness. A number of the calf aorta paste specimens are thin (here we define a thin paste sample as one whose thickness is 50% less than its intact counterpart, and a thick paste has approximately intact tissue thickness; see Table 1). We expect that if the calf aorta paste samples were prepared so that they had a thickness closer to the intact tissue, the absorption would be comparable with or even less than the intact tissue specimens as a result of the freezing process.

Typically, paste samples were not as thick as the intact samples. Although theory predicts that optical properties are independent of thickness, our experience has not shown this to be true. As tissue thickness increases, there is usually a decrease in the measured absorption coefficient.[13] From our re-

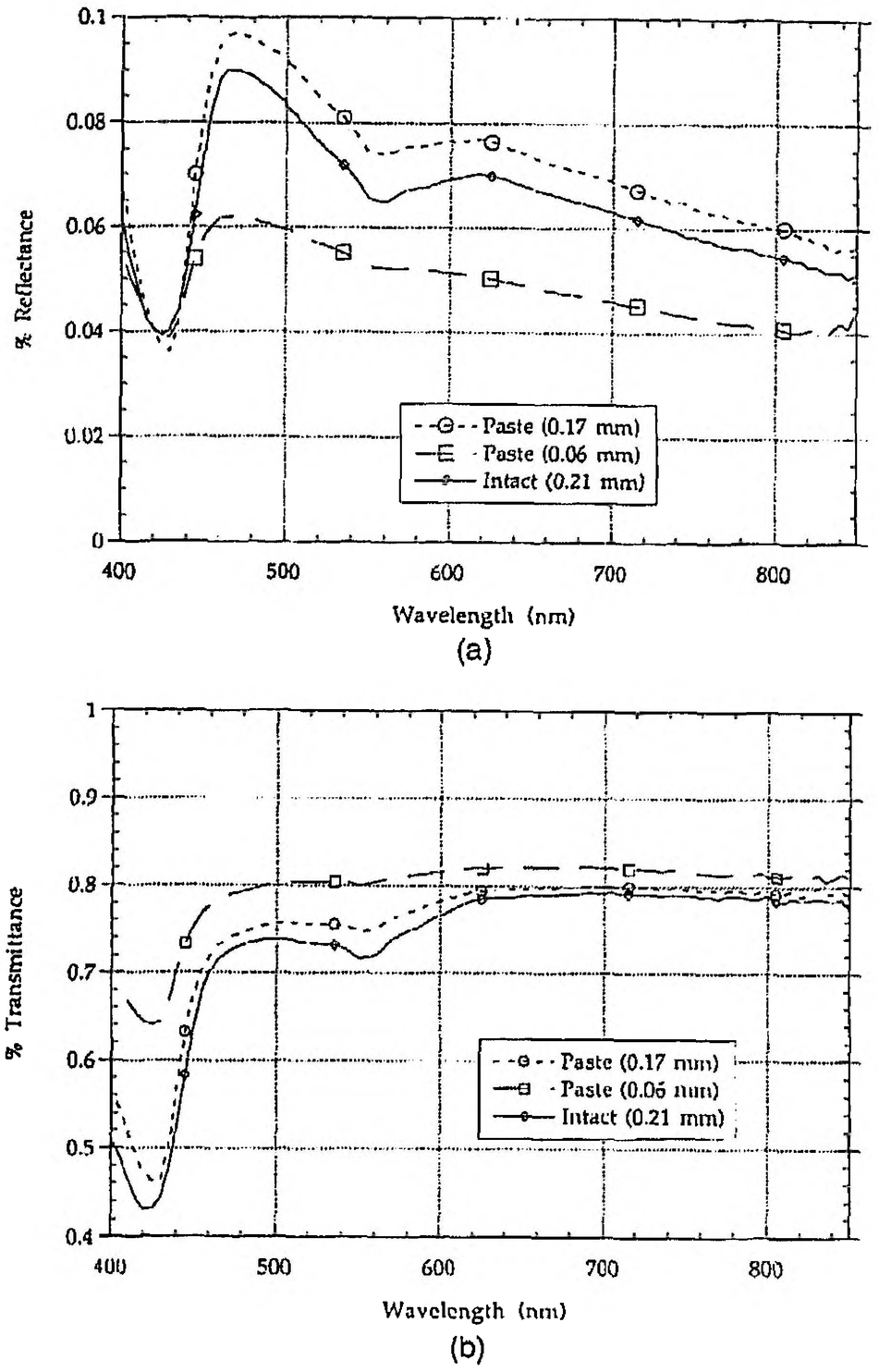

Fig. 2. Rat jejunum (a) reflectance, intact versus paste; (b) transmittance, intact versus paste; (c) absorption coefficient, intact versus paste; (d) reduced scattering coefficient, intact versus paste.

sults, we have noticed that the thin paste specimens tend to have a higher absorption than the thick paste specimens. In Figs. 1(c) and 2(c), the thinner paste samples have higher absorption than the thicker ones. Although the two intact samples are thicker than the thick paste samples, the paste samples have a slightly lower absorption. This is consistent with the observation described above that freezing tissue tends to decrease the absorption.

In our study, the greatest difference between the intact and the paste specimens occurs specifically at approximately 420 nm. The large difference is undoubtedly due to blood absorption. Trace amounts of blood were present in most of the specimens. The ground calf aorta and rabbit sciatic nerve specimens (see Fig. 1 and 3) seemed to have a higher oxyhemoglobin content than the corresponding intact samples. This is depicted by the local absorption peaks at approximately 420, 540, and 577 nm, which are associated with oxyhemoglobin.[14] In contrast, the oxygen level in the intact tissue was lower, as illustrated by the red shift of the 420-nm oxyhemoglobin peak and the local absorption maximum at 555 nm of the deoxyhemoglobin trapped inside the intact specimen vasculature [see Fig. 1(c)]. This increase in the oxygen level of paste specimens is believed to be a result of the grinding process. Oxidation occurred when hemoglobin was exposed to air during grinding. This may provide a closer approximation to tissue *in vivo*, which contains oxygenated blood as illustrated for the calf aorta and rabbit sciatic nerve. It is interesting that neither the intact or paste samples of the rat jejunum appear to contain oxyhemoglobin.

Even though great care was taken when putting a cover slide on the paste, there were still some trapped air bubbles in the slides. These trapped air bubbles can be seen in Fig. 4, which is a micrograph taken from a sample of rat jejunum paste between glass slides enlarged at 25× (original magnification). These trapped air bubbles may have affected the approximation of optical properties, even though air bubbles occupied a small fraction of the tissue paste volume between the glass slides. This may have caused an increase in total transmittance, especially in thin tissue paste specimens in which the air bubbles may have extended through the paste. The effect on the reflectance is not certain. On one hand, the air bubbles may increase the specular reflectance

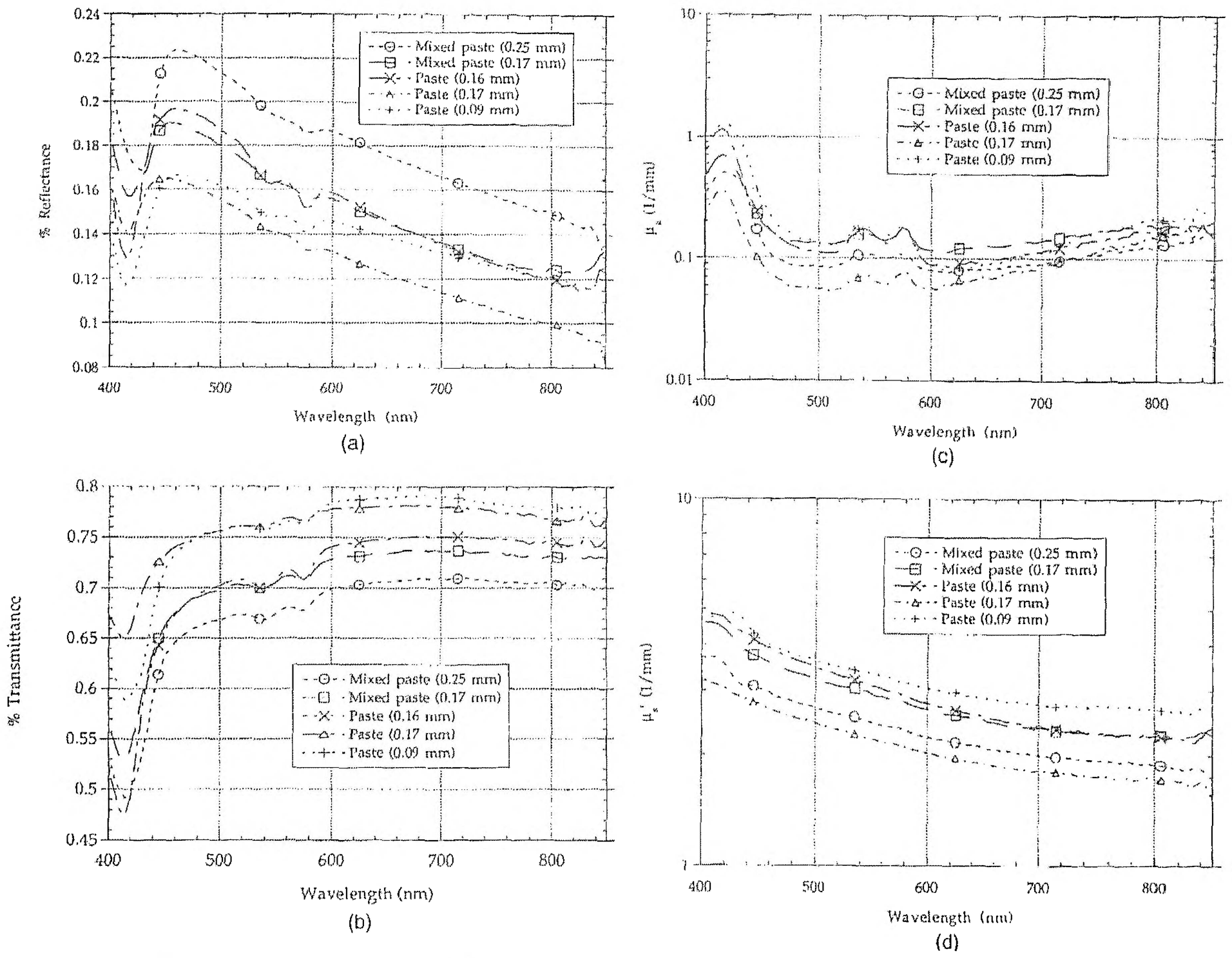

Fig. 3. Rabbit nerve paste (a) reflectance, (b) transmittance, (c) absorption coefficient, (d) reduced scattering coefficient.

as a result of higher index mismatch. On the other hand, the diffuse reflectance may be reduced because the air bubbles had displaced a small amount of the tissue paste volume.

Overall, the data suggest that thick paste specimens better approximate intact tissue. This phenomenon is depicted in the rat jejunum data of Table 3. When the thinnest specimen was excluded from the calculation of average paste optical properties, the paste and intact tissue optical properties were well within experimental error. We believe that the thin paste samples are more susceptible to errors than the thick ones. The changes in water content caused by dehydration and condensation may be more prominent among the thin samples. The trapped air bubbles may occupy a higher percentage of paste volume in a thin sample than in a thick one. Potentially, there is a higher level of thickness measurement uncertainties associated with the thin samples.

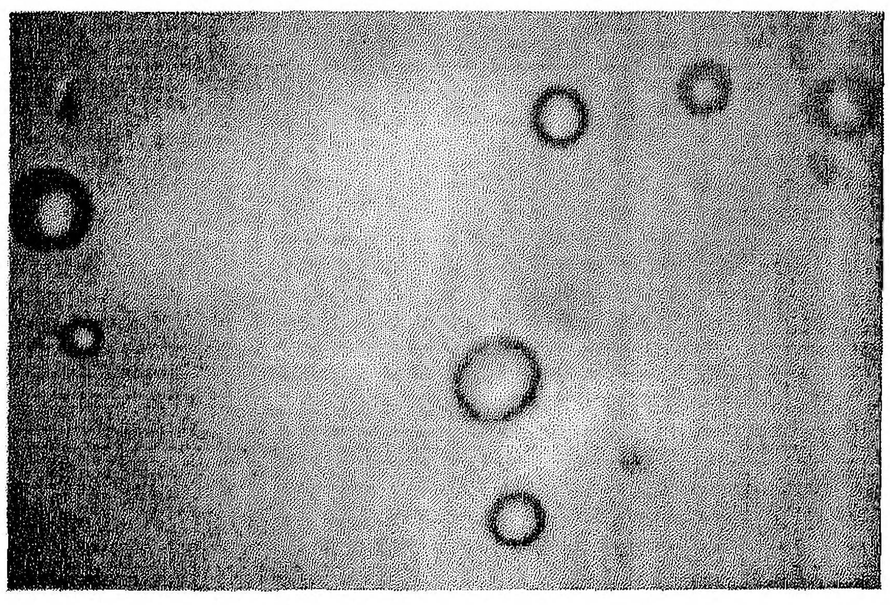

Fig. 4. Micrograph from a rat jejunum paste specimen with air bubbles trapped between glass slides (original magnification, 25×).

From the rabbit nerve specimens, the optical properties obtained from the paste of a single donor are compatible with those obtained from the paste of two donors. This may imply that single tissue types obtained from multiple donors can be ground together to provide sufficient tissue paste for the measurements.

The spectral range that was studied in these experiments covered the visible and part of the near-IR region. However, the agreement is not expected to hold at longer wavelengths. This method will give inaccurate results in the IR range as a result of water absorption, because the water content of ground tis-

sue and that of intact tissue may be different. First, ground tissue has a structure that has been altered by breaking of the cells and the cellular matrix by freezing and grinding. This may give rise to the alteration of both the intracellular and intercellular water distributions. Furthermore, as the frozen tissue powder thaws, it also condenses atmospheric moisture. Because of this increase in the moisture level of the paste specimen, the absorption property is expected to be higher than that of intact tissues in the IR range.

Overall, the shape of the absorption, μ_a, and reduced scattering, μ_s', spectra of the paste samples matched the spectra of the intact tissue. Our results have suggested that the optical properties obtained from the paste specimens approximate values determined from the intact tissues. Although this method is not a substitute for the conventional protocol of obtaining optical properties from intact tissues, the enhancement of the oxyhemoglobin in the paste samples may approximate *in vivo* measurement better than spectrophotometer measurements of intact *in vitro* tissue samples. The goal of this project is to test the feasibility of estimating tissue optical properties from a paste of the tissue. We believe this method can be applied when the tissue of interest is too small or too inhomogeneous for conventional integrating sphere spectrophotometer measurements.

5. Conclusions

The feasibility of estimating tissue optical properties from those of the ground tissue has been demonstrated. We have compared the optical properties obtained from intact and ground rat jejunum and calf aorta. Freezing and grinding enhances the oxygen content of the hemoglobin level in the tissues to better resemble *in vivo* tissue than intact *in vitro* samples. Our results suggest that optical properties of soft tissue can be estimated from that of ground soft tissue in the 400-nm to 850-nm range.

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