Preservative solution for skeletal muscle biopsy samples

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Abstract

Context: Muscle biopsy samples must be frozen with liquid nitrogen immediately after excision and maintained at -80°C until analysis. Because of this requirement for tissue processing, patients with neuromuscular diseases often have to travel to centers with on-site muscle pathology laboratories for muscle biopsy sample excision to ensure that samples are properly preserved. **Aim:** Here, we developed a preservative solution and examined its protectiveness on striated muscle tissues for a minimum of the length of time that would be required to reach a specific muscle pathology laboratory. **Materials and Methods:** A preservative solution called Kurt-Ozcan (KO) solution was prepared. Eight healthy Sprague-Dawley rats were sacrificed; striated muscle tissue samples were collected and divided into six different groups. Muscle tissue samples were separated into groups for morphological, enzyme histochemical, molecular, and biochemical analysis. **Statistical method used:** Chi-square and Kruskal Wallis tests. **Results:** Samples kept in the KO and University of Wisconsin (UW) solutions exhibited very good morphological scores at 3, 6, and 18 hours, but artificial changes were observed at 24 hours. Similar findings were observed for the evaluated enzyme activities. There were no differences between the control group and the samples kept in the KO or UW solution at 3, 6, and 18 hours for morphological, enzyme histochemical, and biochemical features. The messenger ribonucleic acid (mRNA) of β-actin gene was protected up to 6 hours in the KO and UW solutions. **Conclusion:** The KO solution protects the morphological, enzyme histochemical, and biochemical features of striated muscle tissue of healthy rats for 18 hours and preserves the mRNA for 6 hours.

Key Words

Enzyme activity, morphology, mRNA, preservative solution, skeletal muscle tissue

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Introduction

Muscle biopsy samples must be frozen with liquid nitrogen as soon as possible following excision and maintained at -80°C until the specific pathological, enzyme histochemical, biochemical, or molecular evaluation is performed;[1] else, the tissues might lose some of their diagnostic characteristics.[2] This need often causes difficulty in the transport of muscle biopsy tissue samples and increases transport cost because samples have to be transferred under special conditions. Often, sample transfer is impossible because of the unavailability of these special conditions for transport. For patients with neuromuscular diseases, the lack of efficient transportation methods creates the need for them to travel to other locations with on-site muscle pathology laboratories for biopsies. Unfortunately, these patients generally face a great deal of difficulty traveling to these sites.[1]

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There is a great need for an appropriate preservative solution, in particular for tissue-organ transplantation. Several preservatives have been developed, including the histidine-tryptophan-ketoglutarate (HTK) solution,[3] the University of Wisconsin (UW) solution,[4] the Stanford solution,[5] the St. Thomas solution,[6] and the Euro-Collins solution.[7] The UW solution is the most widely used among these solutions; however, there is not enough information about whether the UW solution protects the morphological, enzyme histochemical, biochemical, or molecular features of the striated muscle tissue. Thus, an alternative preservative solution that is easy-to-prepare, stable, and inexpensive is needed for muscle biopsies.

In this study, we developed a preservative solution and determined whether this solution is protective of the

morphological, enzyme histochemical, biochemical, and molecular characteristics of striated muscle tissues for a period of time sufficient for the samples to be delivered to a muscle pathology laboratory.

Materials and Methods

Here, we developed a solution and evaluated its protectiveness on rat striated muscle tissue.

Preparation of solutions

The preservative solution named Kurt-Ozcan (KO) solution was prepared using the following chemicals: MgSO4 (5 mmol/L), raffinose pentahydrate (30 mmol/L), lactobionate (100 mmol/L), KOH (25 mmol/L), KH2PO4 (25 mmol/L), adenosine (5 mmol/L), glutathione (3 mmol/L), and allopurinol (1 mmol/L). The pH value of the KO solution was 7.4. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). While preparing the solution, we aimed to support energy for cell viability and to constitute a medium containing certain pH, electrolyte, and polymeric molecules for muscle cells until molecular, biochemical, and histophatological analysis were done.

Experimental samples

Ethics approval of this study was received from the Gulhane Military Medical Academy and Medical School, Animal Research Ethics Committee. Striated muscle tissue samples were obtained from eight healthy Sprague-Dawley rats that were sacrificed prior to tissue retrieval. The muscle tissue samples obtained from these rats were divided into six groups, defined as follows: Group 1: Tissue frozen in liquid nitrogen and kept in the freezer at -80°C for 3, 6, 18, and 24 hours (control group); Group 2: Tissue placed into 10 ml KO solution and kept at room temperature for 3, 6, 18, and 24 hours; Group 3: Tissue placed into 10 ml KO solution and kept at +4°C for 3, 6, 18, and 24 hours; Group 4: Tissue placed into 10 ml UW solution and kept at +4°C for 3, 6, 18, and 24 hours; Group 5: Tissue kept at room temperature for 3, 6, 18, and 24 hours without any solution; and Group 6: Tissue kept at +4°C for 3, 6, 18, and 24 hours without any solution.

A total of 72 pieces of the striated muscle tissue were collected from each rat and divided into three sets of 24 pieces, designated as follows: Approximately 0.4 cm in diameter, for morphological and enzyme histochemical examination; 0.1 cm in diameter for molecular examination; and 0.2 cm in diameter for biochemical analysis. It was intended that using the same muscle group of rats at least for same analyses at the beginning of the study, but it was going to cause to sacrifice much more rats. So, different muscle groups were used. This may be thought as a limitation for this study.

Morphological and enzyme histochemical evaluation

Tissue sections (thickness, 8 μm) were prepared from the muscle biopsy samples and stained with modified Gomori trichrome (mGT) and hematoxylin and eosin (H&E) stains. Semi-quantitative morphological evaluation was conducted with scores assigned based on the following criteria:

1. Organization of muscle fascicles-compared to the control group, scored as normal: 0, slight disorganization: 1, moderate disorganization: 2, and apparent disorganization: 3
2. Edema-scored as normal: 0, slight: 1, moderate: 2, and significant: 3
3. Artificial changes-scored as normal: 0, slight artificial changes: 1, moderate artificial changes: 2, and significant artificial changes: 3

Thus, the total possible score was 9. If the score was closer to 0, that meant the tissue closer to normal, but if the score was closer to 9 that meant it has many abnormalities..

Additional histochemical and enzyme histochemical staining, using periodic acid Schiff (PAS), Oil red O (ORO), cytochrome oxidase (COX), nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR), and succinate dehydrogenase (SDH) stains, were performed, and their differences compared to the normal muscle tissue were evaluated. Histochemical and enzyme histochemical staings were done according to reference 8.

Biochemical evaluation

Muscle tissues were homogenized in 0.05M Tris-HCI and 0.15M KCI (pH 7.5) with Silent Crusher M homogenizer (Heidolph Instruments GmbH & Co KG, Schwabach, Germany) three times for 15 sec at 14000 rpm. Homogenate were centrifuged at 600 g for 10 min at 4°C. Supernatant was centrifuged again at 600 g for 10 min at 4°C and enzyme measurements were performed in second supernatant.

Citrate synthase (CS) activity was measured by following the increase in absorbance due to the formation of 5-thio-2-nitrobenzoate anion at 412 nm (Extinction coefficient: 13.6 $mM^{-1}cm^{-1}$) using a spectrophotometer (Shimadzu, Japan) at 30°C. Reaction medium contained 1mM DTNB 0.3 mM acetyl-CoA and 0.5 mM oxaloacetate. The assay was initiated by addition of oxaloacetate. CS activity was expressed as μmol/min/mg protein.

Succinate dehydrogenase activity was measured by following the decrease in absorbance due to the reduction of DCPIP at 600 nm (Extinction coefficient: 19.1 mM-1cm-1) at 30°C. Reaction medium contained 25 mM potassium phosphate buffer (pH 7.2), 5 mM MgCl2, 20 mM sodium succinate, 0.05 mM DCPIP, 2 mM KCN, 2 μg/ml antimycin A, 2 μg/ml rotenone, and 65 μM ubiquinone. The assay was initiated by addition of ubiquinone. Succinate dehydrogenase activity was expressed as μmol/min/mg protein

The protein concentration of supernatants was measured according to the method of Lowry *et al*.[9] with bovine serum albumin as the standard. All the chemicals used for enzyme assays were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Molecular evaluation

For molecular evaluation, messenger ribonucleic acid (mRNA) levels were measured to determine the tissue samples in which mRNA degradation occurred at the earliest time. Total mRNA was isolated and the level of the *beta-actin* (β-actin) gene, which is expressed in striated muscle tissue, was analyzed. mRNA levels

were determined using the following procedure: Briefly, tissue samples were homogenized by placing the tissue into 500 µL of denaturation solution (guanidine thiocyanate, EZ-RNA Total RNA isolation kit; Biological Industries, Israel) and then isolating total RNA according to the manufacturer's instructions provided in the kit. The obtained RNA was treated with RNAse- and deoxyribonucleic acid (DNA)se-free diethylpyrocarbonate (DEPC)-distilled water to prevent DNA contamination. OligoYap 5.0 software was used for design of primer sequences, polymerase chain reaction (PCR) optimization, calculating primer concentration, and controlling primer-dimer formations and amplification efficiency. The primers and probes used were as follows: β-*actin* Forward 5′- catgtttgagaccttcaac-3′; β-*actin* Reverse 5′-atcacaatgccagtggtacga-3′; β-*actin*-Probe-FAM-5′-cccagccatgtacgtagccatccagg-3′-BHQ-1. The amplicons were checked by gel electrophoresis. Real-time PCR (RT-PCR) was performed and the C_T values of the samples were determined with relative mRNA quantification using a TaqMan-based assay with a 7500RT-PCR System (Applied Biosystems, USA).

Statistical evaluation

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) for Windows V. 17.0. Chi-square and Kruskal Wallis tests were used for comparison of sample groups. If the *P*-value was less than 0.05, the difference was considered significant.

Results

Morphological and enzyme histochemical evaluation

As summarized in Table 1, group 1 (gold-standard method where tissue samples are routinely frozen with liquid nitrogen and stored at –80°C) was shown to preserve the morphological characteristics of skeletal muscle tissue even after 24 hours.

As indicated in Table 1, samples kept in KO solution exhibited very good morphological scores after 3, 6, and 18 hours of storage, but artificial changes that rendered the evaluation difficult (above acceptable limits) were observed at 24 hours

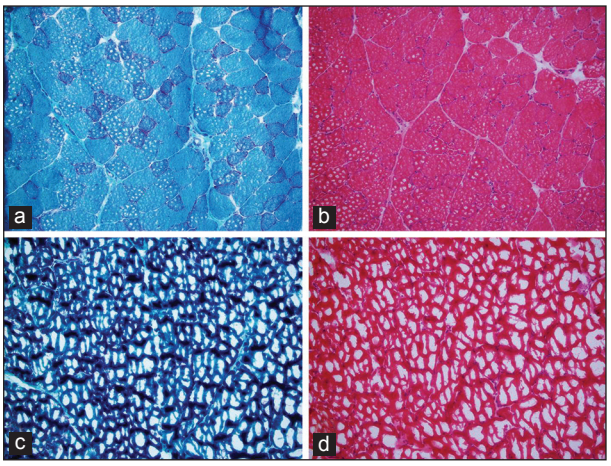

Figure 1: (a) Sample from Group 3 (samples in KO solution at +4°C) at 18 hours (mGT, ×200); (b) Sample from Group 3 at 18 hours (H and E, ×200) (c) Sample from Group 3 at 24 hours (mGT, ×200) (d) Sample from Group 3 at 24 hours (H and E, ×200), mGT = Modified gomori trichrome, H&E = Hematoxylin and eosin

Table 1: Mean scores for morphological evaluation (mGT vs. H and E stains)

Hours and parameters	Group 1			Group 2			Group 3			Group 4			Group 5			Group 6		
Hours	3	6	18	24	3	6	18	24	3	6	18	24	3	6	18	24	3	6	18	24
Parameters																				
Organization	0	0	0	0	0.12	0.75	1	1	0	0	0.25	0.75	0.12	0.12	0.12	0.87	0.25	1	1	2	0.12	0.25	0.62	1
Edema	0	0	0	0	0.75	1.87	2	2.75	0	0.12	0.37	1.75	0	0	0.12	2	1.62	2	2.75	3	0	0.12	1.37	2.87
Artificial changes	0	0	0	0	0.12	2	2.87	2.87	0.12	0.12	0.37	2.75	0	0.12	0.12	2	1.37	3	2.75	3	0.12	0.12	1.75	2.5
Total score	0	0	0	0	1	4.62	5.87	6.62	0.12	0.24	1	5.25	0.12	0.24	0.36	4.87	3.24	6	6.5	8	0.24	1	3.74	6.37
P value*					>0.05	<0.05	<0.05	<0.05	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05	>0.05	<0.05	<0.05

**P* values obtained by comparison of groups with Group 1 (for same hours), mGT = Modified gomori trichrome, H and E = Hematoxylin and eosin

[Figure 1]. Similar findings were observed for samples in the UW solution. When samples in KO and UW solutions at +4^{0}C were compared to the control group, there was no statistically significant difference among the samples stored for 3, 6, or 18 hours. However, at 24 hours, statistically significant artificial changes were observed for samples in both KO and UW solutions [Table 1].

The protectiveness of the treatments used for Group 2, Group 5, and Group 6 was worse than the KO (Group 3) and UW (Group 4) solution groups. Artificial changes were observed in Group 5 at 3 hours. In addition, we observed that the morphology of the tissues in Group 2 was protected for 3 hours, while that of the tissues in Group 6 was protected for 6 hours [Table 1].

The enzyme histochemical findings were parallel to the morphological findings. In Group 1 (control group), very good results were obtained at all-time points [Figure 2]. The KO (Group 3) and UW (Group 4) solutions also exhibited good results for 3, 6, and 18 hours at +4^{o}C [Figure 2], but artificial changes were observed in the samples kept in both solutions for 24 hours. In other groups, the quality of enzyme histochemical staining was worse. In fact, for all groups, it is important to note that the enzyme histochemical stains did work, even for the 24-hour samples; however, since the muscle tissue contained many artificial changes, it was difficult to evaluate.

Biochemical evaluation

SDH and CS activities were measured to assess the protectiveness of the specified conditions on tissue biochemical activity. The mean values of each groups' enzyme activities are shown in Table 2. The enzyme activities were parallel to the morphological findings. The KO and UW solutions preserved SDH and CS enzyme activities until 18 hours just as much as Group 1 (the gold-standard group). At 18 hours, no statistically significant difference was found in the enzyme activities between Group 1 and Group 3 or Group 4. However, a decrease in the activity of SDH andCS was identified at 3 hours for Group 5 and at 6 hours for Group 2 and Group 6.

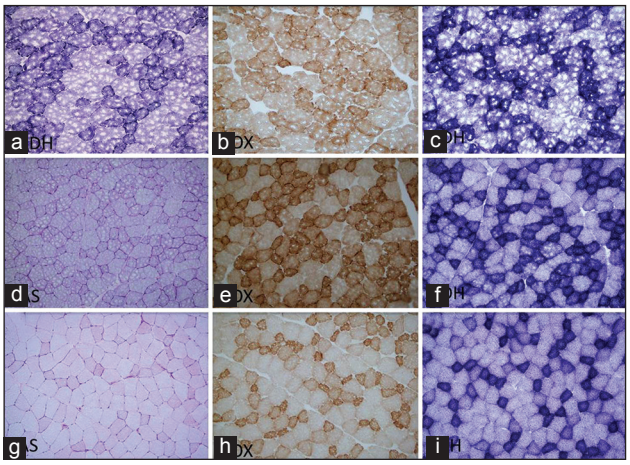

Figure 2: (a, b and c) Group 4 (UW solution, at +4°C), at 18 hours. (d, e and f) Group 3 (samples in KO solution at +4°C) at 18 hours (g, h and i) Group 1 at 24 hours

Table 2: Mean enzyme activities of sample groups

Enzymes	Group 1				Group 2				Group 3				Group 4				Group 5				Group 6			
Hours	3	6	18	24	3	6	18	24	3	6	18	24	3	6	18	24	3	6	18	24	3	6	18	24
Succinic dehydrogenase (μM/min/mg protein)	0,037	0.033	0.038	0.036	0.034	0.023	0.017	0.009	0.034	0.036	0.037	0.015	0.035	0.038	0.036	0.013	0.025	0.020	0.013	0.010	0.033	0.024	0.018	0.010
Citrate synthase (μM/min/mg protein)	0.32	0.35	0.31	0.37	0.31	0.20	0.15	0.10	0.33	0.35	0.32	0.10	0.36	0.36	0.35	0.11	0.22	0.18	0.10	0.06	0.31	0.21	0.17	0.10
P value*					>0.05	<0.05	<0.05	<0.05	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05	<0.05	<0.05	<0.05

**P* values obtained by comparison of groups with Group 1 (for same hours)

Molecular evaluation

The C_T values were obtained to assess the mRNA levels for each sample group and are summarized in Table 3. According to these values, the KO and UW solutions were able to preserve mRNA for the β-actin gene up to 6 hours. In addition, mRNA was protected up to 3 hours in Group 2 and Group 6. A decrease in the mRNA levels of Group 5 was observed as early as within 3 hours of storage.

Briefly, the KO solution (Group 3) was shown to preserve the morphology and enzyme activities of striated muscle tissue for 18 hours and mRNA levels for 6 hours, at +4°C [Table 4]. Similar findings were also observed for UW solution (Group 4). For samples kept at room temperature without any solution (Group 5), abnormalities in all features of muscle were identified within just 3 hours. The KO solution was found to be protective for 3 hours for samples kept at room temperature (Group 2). In addition, it is interesting that although the samples were not kept in any solution (Group 6), the morphological, enzyme histochemical and biochemical features of the muscle tissue were protected for 6 hours at +4°C, and the mRNA was maintained for 3 hours.

Table 3: C_T values of all samples

	No	Group 1			Group 2			Group 3			Group 4			Group 5			Group 6		
		3. h.	6. h.	18. h.	24. h.	3. h.	6. h.	18. h.	24. h.	3. h.	6. h.	18. h.	24. h.	3. h.	6. h.	18. h.	24. h.	3. h.	6. h.	18. h.	24. h.
CT value	1	13	13	13	13	13	14	17	19	13	14	15	19	13	14	13	17	15	17	19	23	13	16	18	23
	2	14	13	13	14	14	15	18	19	13	14	14	16	14	13	15	18	14	18	20	23	13	17	18	23
	3	13	14	14	14	14	15	18	20	13	14	16	17	14	13	16	18	14	18	20	24	13	16	18	22
	4	13	13	14	14	14	16	18	20	14	13	14	18	14	14	13	18	16	18	21	24	14	14	18	22
	5	13	13	13	13	13	16	17	20	14	13	16	18	13	13	13	17	13	17	20	23	14	15	19	23
	6	14	14	14	13	13	16	18	21	14	13	15	19	13	14	14	18	16	17	21	23	13	14	18	23
	7	14	14	14	13	14	17	19	21	14	14	16	19	13	14	15	18	16	18	21	24	13	17	18	22
	8	14	14	13	14	14	17	18	20	13	14	14	19	13	13	16	18	14	18	20	24	14	15	18	23
	P value‡					>0.05	<0.05	<0.05	<0.05	>0.05	>0.05	<0.05	<0.05	>0.05	>0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05	<0.05	<0.05	<0.05

‡*P* values obtained by comparison of groups with Group 1 (for same hours), h = hour

Discussion

Muscle biopsy samples are an important tool in the diagnosis of neuromuscular diseases. The need for muscle biopsy samples often requires patients with these diseases to travel to faraway places for muscle biopsy procedures. But, many of these patients are already in need of care, and therefore, they might not be able to undergo muscle biopsy. In this study, we investigated a solution that could eliminate the necessity of urgent freezing of samples and transfer of muscle biopsy samples at very low temperatures. A solution that protects the morphological, enzyme histochemical, biochemical, and molecular properties of the muscle tissue until it reaches a muscle pathology laboratory was developed and assessed.

In current clinical practice, the striated muscle tissues excised for morphological evaluation are immersed in liquid nitrogen in cooled isopentane and maintained in liquid nitrogen for approximately 10 min.[8] After this immediate refrigeration treatment, the muscle tissue must be kept frozen at -80°C until samples are cut and processed for morphological, enzyme histochemical, biochemical, and molecular evaluation of the muscle tissue. Fixation with formaldehyde may eliminate features required for the diagnosis of several muscular diseases, including congenital myopathies, mitochondrial myopathies, or storage diseases.

Previous reports have shown that samples of myofibrils and inter-myofibrils, evaluated in a series of autopsy procedures, exhibited weak mGT and H&E staining in sections of striated muscle tissue, just 6–10 hours after death.[8] The activities of phosphorylase and oxidative enzymes also started to disappear after the first hour. Based on our results, morphological and enzyme histochemical abnormalities were observed within the first 3 hours for samples kept at room temperature. In another study reported by Eriksson *et al*., muscle fiber type separation could be done with NADH-TR staining up to 10

Table 4: Brief information about protection time of solution and conditions

Parameters	Liquid nitrogen (Group 1)	KO solution at RT * (Group 2)	KO solution at +4°C (Group 3)	UW solution at +4°C (Group 4)	RT (Group 5)	+4°C (Group 6)
Morphology and enzyme histochemistry	24 hour**	3 hour	18 hour	18 hour	Morphological changes at 3. hour	6 hour
Enzyme activities	24 hour**	3 hour	18 hour	18 hour	Decrement at 3 hour	3 hour
mRNA levels	24 hour**	3 hour	6 hour	6 hour	Decrement at 3 hour	3 hour

*Room temperature, **The longest period for evaluation was 24 hours in this study (It is known that liquid nitrogen is protective for years), KO = Kurt-Ozcan, RT = Real time, UW = University of Wisconsin, mRNA = Messenger ribonucleic acid

days if the muscle tissue was kept at +4°C.[11] However, in our study, difficulties in evaluating enzyme histochemical staining were observed in the samples kept at +4°C after just 24 hours, although this difficulty was not due to staining, but due to tissue artifacts.

Degradation in muscle tissue begins shortly after tissue perfusion with oxygen and nutrients ends. Degradation generally occurs because of the degradative enzymes of the cell itself,[12] a process known as autolysis. Degradative enzymes may be either the enzymes in the cell's own lysozymes or enzymes released from leukocytes that migrate to the scene. Because of this degradative effect, denaturing of intracellular proteins is observed. The number of studies conducted on cardiac muscles has increased over the last few years because the damage to cardiac muscle largely contributes to patient death. Research has shown that myocardial necrosis begins within 30 min after death. However, macroscopic findings are apparent at 12 hours,[12] whereas morphological changes may be observed at 4 hours with microscopic examination.[12] Biochemical evidence of cardiac muscle damage has been shown to appear at earlier hours. Consistent with these findings, we observed biochemical changes in our study before morphological changes appeared.

Tissue preservation solutions have improved, particularly as the number of organ transplantation procedures has increased. While numerous solutions such as Celsior, Euro Collins, or HTK exist, currently the most commonly used preservation solution is the UW solution. However, since these solutions are specifically used in organ transplantation, there is not sufficient data describing their protectiveness potential for striated muscle tissue in the literature. In 1999, Mohara *et al.* evaluated the protectiveness of the Celsior and UW solutions for transplanted heart muscles.[13] In their study, tissue samples from 14 mongrel dogs were evaluated. Seven hearts were transplanted after being kept in UW and the remaining seven hearts were transplanted after being kept in Celsior solution at +4°C for 12 hours. The muscle tissue was evaluated using electron microscopy and they showed that the samples kept in the UW solution exhibited more mitochondrial degeneration than the samples kept in the Celsior solution. They concluded that the UW solution protects transplanted muscle tissue for up to 12 hours and the Celsior solution is just as protective as the UW solution, and more protective in some instances. While electron microscopy methods were not used in this study, from our evaluation, we were able to determine that the samples kept in the UW solution at +4°C were preserved for up to 18 hours. The KO solution was similarly shown to protect the morphological and enzyme histochemical properties of the samples for up to 18 hours.

In a 2003 study, Van Der Heiden *et al.* compared six perfused and six non-perfused muscle tissues to determine whether perfusion before storage at +4°C for 16 hours improves the properties of muscle tissues.[14] UW and HTK solutions were used for perfusion with both groups of muscle tissues incubated at +4°C for 16 hours. Comparison was conducted by measuring the function of the muscle tissue, and it was shown that perfusion did not yield any improvement of muscle tissue function.

Although the protectiveness of UW solution on muscle tissue has been highlighted in many studies, there are some studies that emphasize the idea that perfusion may also cause musculoskeletal injury. In a 2002 study conducted by Tsuchida *et al*., the hindlimb muscles of rats were collected.[15] One group of tissues were kept at 25°C for 5 hours and the another group was perfused for 24 hours with UW solution and then kept at 25°C for 5 hours. Transplantation was performed, and at the end of 24 hours of reperfusion, it was found that the muscle ATP levels were lower in the group perfused with the UW solution, indicating that perfusion with UW solution may have resulted in increased musculoskeletal injury.

Based on our results, our KO solution protects the morphological, histochemical, and biochemical features of the striated muscle tissue of healthy rats for 18 hours at +4°C, and mRNA was shown to be protected for about 6 hours. Similar results have been observed with use of the UW solution. If only morphological and enzyme histochemical examinations will be conducted on excised striated muscle tissue samples, then the tissue may be transferred within 6 hours, at +4°C without the use of any preservative solution. Nevertheless, if biochemical or molecular examination is to be conducted, this time has to be much shorter.

Here, we have developed a solution to preserve muscle biopsy samples for a period of time at least long enough to reach an advanced muscle pathology laboratory. The KO solution was shown to preserve the morphological, enzyme histochemical, and biochemical characteristics of muscle tissue at +4°C for 18 hours, for which in most countries, an 18-hour period would be sufficient for the transport of a sample from the site to a muscle pathology laboratory.

Acknowledgements

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