Effects of Cold Ischemia on Gene Expression: A Review and Commentary

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Frequently investigators request that tissues be collected and processed in less than one hour following removal from a patient. Some biorepositories expend significant personnel time and other resources in trying to meet such goals; however, it is unclear whether the perceived benefits of relatively short cold ischemia times warrant these added costs. The literature of human surgical tissues prospectively exposed to cold ischemia at several time points was reviewed to compare the changes in transcripts/genes and microRNA with time of cold ischemia. Also, reports of protein changes in response to cold ischemia were correlated to changes in genes. The literature is limited; however, for most tissues, only a small proportion of transcripts/genes (<1%) changes up to 3 hours following surgery and most transcripts increase rather than decrease in less than 2 hours of cold ischemia. Biorepositories and investigators must consider the literature for evidence of significant changes in molecular results from tissues before spending significant resources on relatively rapid collection of tissues to meet cold ischemia times of less than 3 hours. Instead, those using human tissues in research must consider if the cold ischemia times affect their use in specific research; hence are these tissues “fit for purpose?”

Keywords: cold, ischemia, human, tissue, collection, research

Introduction

FREQUENTLY, INVESTIGATORS REQUEST that tissues be collected and processed in less than one hour following removal of the tissues from a patient (i.e., less than 1 hour of cold ischemia time—defined as the time from removal of tissue from a patient until the tissue is stabilized e.g., fixed, frozen). Such requests are based on the perception that when cold ischemia times exceed 1 hour, tissues undergo molecular degradation significant enough that the tissues no longer reflect the original physiological state of the tissues before surgery. Some biorepositories expend significant resources in attempting to obtain such tissues for investigators.

It is very important that evidence-based standards for cold ischemia rather than “pseudostandards” based on opinions are utilized for tissues collected both for research and clinical use. Very rapid collection of tissue is expensive; biorepositories and investigators must know if there is evidence in the literature to justify these expenses! Of note, the literature is not extensive in any area of preanalytical variables, including cold ischemia; however, standards must be based on the literature that exists.¹

To collect and process specimens in ≤1 hour may require biorepository personnel to be present in the operating room, to transfer tissues rapidly to surgical pathology and to dissect the tissue immediately. These steps may be very difficult when there are numerous operating rooms and multiple concurrently processed specimens, as is typical for large volume tissue biorepositories.

While the collection of one aliquot from a large tissue resection (e.g., radical mastectomy) within 30 minutes sometimes is possible, careful identification and protection of surgical margins require a longer time just to mark and sample the margins to ensure their diagnostic accuracy. This is one reason why specimens collected within 1 hour of warm plus cold ischemia for The Cancer Genome Atlas (TCGA) project were much more expensive (×10) than tissues collected routinely by biorepositories. Are such added costs justified based upon evidence in the literature? Our view is that the answer in most cases is no!

Of concern and independent of standards are the biases that may affect the experimental results if differences in pre-analytical variables (e.g., time and temperature of specimen processing) are not considered in evaluating experimental results² as well as the false discovery rate (FDR), which is likely to be high when over 10,000 transcripts are evaluated.³

The objectives of this review and commentary are to present available information on one preanalytical variable, cold ischemia, in the context of the proportion of transcripts, genes, and proteins that change while a tissue is maintained for various times at cold ischemia.

The phrase “cold ischemia time,” has been used in various combinations with “warm ischemia time,” “RNA integrity number (RIN),” “mRNA,” “changes in transcripts,” and “delayed fixation” to search the literature, including PubMed. All articles were reviewed in which the research focused on tissues maintained at cold ischemia temperatures

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for various time points. In most cases, the times of cold ischemia were compared with the global proportion of changes in transcripts, genes, proteins, and microRNA. Only prospective studies were considered to be informative due to questions as to the accuracy of biorepository information not controlled by the investigators.

In most cases, results of changes in specific genes had to be estimated based on graphs in articles, to perform calculations of the extent of individual genes that changed, because usually specific results were not reported in the articles. For each transcript, for example, JUND, values of the 0 or equivalent time points, for example, 5–15 minutes, were used to normalize the cold ischemia time points of each specific study. These results are tabulated to permit comparison.

This commentary mainly focusses on cold ischemia and whether cold ischemia times >1 hour significantly affect the molecular signatures of tissues used in research; but some other preanalytical variables, including warm ischemia, defined as the time a surgical sample is still within the body, but with a compromised blood supply, are briefly discussed to put cold ischemia into a more global perspective.

Preanalytical Variables

Preanalytical variables are defined as variables that occur before analysis; they comprise a wide range of variables that affect tissues,² including variables secondary to the patients who are the source of the tissue (e.g., patient age, race, sex, diet, comorbidities, prior medical interventions [e.g., neoadjuvant therapy, tissue biopsy, surgery], and tissue handling [e.g., collection, processing, storage, and distribution of research tissues]).

Preanalytical variables may alter the molecular composition of tissues resulting in variation in biomolecules, subsequently affecting downstream analyses. For example, cancers typically are very heterogeneous⁴ such that different areas of the same cancer will likely produce different results. In addition, the possible time for removal of tissue from human subjects will vary depending upon the location of tissue and nature of surgery. A detailed analysis of preanalytical variables other than cold ischemia is beyond the scope of this article; readers are referred elsewhere for such discussions.^{2,5}

When the literature on biorepository sciences is reviewed, it becomes evident that each molecule within a tissue or an organ responds differently to warm and cold ischemia,⁶ and tissue responses among individuals (i.e., biological differences) are more important.^{6,7} In some cases, warm ischemia cannot be separated from cold ischemia, thus this is only briefly discussed below.

Warm Ischemia

Controlling the effects of surgery poses significant challenges to the collection of biospecimens because the effects of a patient's surgery cannot be controlled without very extensive resources; also, attempts at such control may affect patient care. Potential variables during surgery include the type of surgery, the effects of anesthesia, fluid and blood administration, duration of surgery, and intraoperative tissue damage; however, warm ischemia time may be one of the more important variables affecting changes in biomolecules.^{6,8}

A study by Gündisch et al.⁶ found that 74% of the proteins in the liver were decreased in the samples due to warm ischemia compared to respective biopsy specimens obtained before surgical resection. In contrast, for prostate, Schlomm et al.⁸ reported increased expression of genes during about 90 minutes of warm ischemia followed by a slight increase in expression when radical prostatectomies were maintained for various times of cold ischemia. During both warm and cold ischemia, only 9% of genes of the prostate increased with statistical significance.⁸

This complicates evaluation of operative plus postoperative ischemia time (OPIT), so that studies of OPIT affecting a specific molecule in the pancreas, an organ with a substantial enzymatic component, may not mirror the effects of OPIT on the same molecule in the bone marrow. This is, however, speculative with little support as to the extent of molecular changes that differ among organs in cold ischemia actually described in the literature.

At autopsy, macroscopic autolytic changes in the pancreas and spleen exceed those in many other tissues (personal observations of WEG and DSA). We propose that it is too costly to treat every molecule–tissue–organ combination as if the tissues being studied were from the pancreas. In contrast, epithelium from the liver, kidney, and stomach also has extensive enzymatic components. Studies of liver, however, support the stability of proteins during varying periods of cold ischemia for up to 6 hours,⁶ and the retrieval and culture of viable cells with a stem cell marker (i.e., Ep-CAM) from the liver were successful before 48 hours of cold ischemia.⁹

Cold Ischemia

Standards of cold ischemia for various tissues may have to be considered separately until more extensive data as to the effects of cold ischemia are collected. Nevertheless, based on the literature, the major tumors of breast, colorectum, liver, and prostate are unlikely to have major molecular damage secondary to typical times of cold ischemia.^{3,6,8–18}

Effects of Cold Ischemia of RNA Stability

RNA integrity number (RIN) is a surrogate measure of mRNA stability. RIN specifically is an indication of the stability of ribosomal RNA (rRNA) based on the two major peaks of rRNA, 18S and 28S. Alternatively, 18S and 28S peaks or their ratio can be measured separately as a related indication of rRNA stability. Of note, the stability of mRNA is assumed to be very similar to RIN or the 28S:18S rRNA ratio, but this has not been studied adequately and likely varies with a specific transcript and organ system.^{3,10–17}

Importantly, most studies of RIN or 28S:18S ratios are performed with specimens held at room temperature (usually assumed to be 22°C–25°C) and some studies of the effects of cold ischemia focus only on the cold ischemia period after specimens leave the operating room and/or arrive in surgical pathology and do not consider the period of cold ischemia in the operating room or during transport.¹⁹ More importantly, most studies do not evaluate the effects of warm ischemia, which is likely to interact with the effects of cold ischemia.^{10–13} Nevertheless, the following question remains: *How are RIN and/or 28S:18S ratios affected by different times of cold ischemia?* Multiple studies have

reported that there is no statistical difference in RIN and/or there are minimal changes in RIN ($\leq 10\%$) or 28S:18S ratios during cold ischemia times of 1–6 hours.^{3,10–17,19,20} Few studies report large and/or statistically significant changes in RIN, except studies that held specimens at 37°C or at 60°C where decreases in RIN of about 50% were reported at 3.15 hours at 60°C.^{3,13} An outlier is a study in which colorectal cancer specimens were held at 4°C, but RIN decreased 44% (statistically significant) between 10 minutes and 1.5 hours; however, the changes in 28S:18S ratios were not statistically different.²¹

A study using robot-assisted laparoscopic prostatectomy reported that cell type classifications, for example, stromal/epithelial, benign/malignant, and prostate volume, are significant predictors of RIN.²² This suggests that there are variations in RIN among cell types and hence among organs due to cold ischemia.

A direct measure of the stability of mRNA is the evaluation of changes in RNA transcripts measured by “gene chips,” RNA sequencing, or analysis by real time, reverse transcriptase–quantitative polymerase chain reactions (RT-Q-PCR).

Studies of the Effects of Cold Ischemia on RNA Transcripts

Typical gene chips may measure up to 48,000 transcripts. Some of these transcripts are associated with specific genes and their proteins, but other transcripts are to unknown genes and/or genes with no clearly identified functions. In most reported studies of changes in transcripts during cold ischemia usually at RT, typically less than 1%–3% of transcripts statistically change over the initial 2–3 hours of cold ischemia,^{3,7,10–12,18,23} but up to 10% of transcripts have been reported to change after 6 hours.

Nine studies of cold ischemia at different time points in several types of tissues are reported in the literature, and changes in transcripts in five specific studies when more than two tissues of the same diagnosis were studied, are listed in Table 1. In Table 1, there are only three studies that address changes in transcripts during cold ischemia of 2–4 hours, although the study of Musella et al.¹¹ evaluates two different diagnoses at these conditions (i.e., colorectal cancer and uninvolved colon). For these studies, only a small proportion of transcripts/genes changed ($\leq 1\%$ at 3 hours, 2% at 4 hours) following surgery.

In evaluating the literature, specific studies are based on the use of one or two specimens or in some cases one diagnosis (e.g., one specimen of colon cancer, one specimen of normal or uninvolved colon). Thus, the statistical significance discussed is based on the method of analysis rather than comparing changes among multiple specimens with the same diagnosis. Also, in these studies, problems with multiple comparisons and the related FDR are seldom addressed and individual data are not presented.

Therefore, the results from multiple specimens of the same diagnostic group, Tables 1, 3, 5, and 7, should be given greater weight because they average changes among multiple specimens than the results of one or two samples listed in Tables 2, 4, and 6. We view changes in transcripts and genes during cold ischemia, which are based on only 1 or 2 specimens, as being mainly useful for estimating how fast some changes can occur in a single specimen and the

most common direction of these changes. Thus, in Tables 2 and 4, some changes in transcripts and genes have occurred within 15 minutes and the usual direction of changes is an increase in transcripts or specific genes. Although the results are both limited and variable when only 1 or 2 specimens are analyzed, these reports are included because there is such limited data on the effects of cold ischemia.

Table 3 lists the changes in specific genes when three or more cases are evaluated. The main correlation between the time of cold ischemia and changes in specific genes based on analysis of more than three specimens with the same diagnosis (breast cancer, prostate cancer, or noncancer prostate) is an increase in most genes analyzed at 1, 3, and 5 or 6 hours of cold ischemia at RT. For renal cell carcinoma, the results of cold ischemia are more variable. At 4°C, *Bax*, *Vimentin*, and *CA9* decreased at 2 hour and *FOS* increased at 2 hour. In contrast, at 22°C, *Vimentin* and *FOS* increased at 2 hour and *Bax* and *CA9* decreased.

Table 4 lists changes in genes when only 1 or 2 samples were analyzed for changes in gene expression. Most genes of the lung were noted to be unchanged at RT for 1 hour. For an immature teratoma of the ovary, three of four genes increased at 15, 30, 60, and 120 minutes at RT. For a tonsil at 4°C, 4 of 6 genes increased at 30 minutes and five of six genes increased at 6 hours. In contrast, at RT for 30 min, three of the same six genes increased, one of six was unchanged, and two genes decreased, and at 6 hours of RT, three genes increased and three genes decreased.

Effects of Cold Ischemia on Proteins

There have been few quantitative, statistically analyzed studies of the effects of cold ischemia on proteins except for semiquantitative immunohistochemical and *in situ* hybridization studies, which focus primarily on evaluating clinically important biomarkers. The study of Gündisch et al.⁶ evaluated 11 nonmalignant intestinal specimens with reverse phase protein arrays using 23 antibodies, including phosphoproteins; only one protein, phosphorylated p44/p42 MAPK, significantly changed (increased) at 30 and 60 minutes, but was unchanged at 3 hours (i.e., perhaps it had returned to baseline).

The causes of such changes have not been studied; however, it is our view that the initial increased levels were due to the effects of cold ischemic stress and stress of hypoxia. We would speculate that this gene was no longer responsive to these changes at 3 hours.

Similarly, in this same report, when 17 liver specimens were evaluated using 30 antibodies, including several phosphoproteins, 4 proteins (13%) increased significantly with 3/30 of these increased at 30 minutes, 1/30 increased at 60 minutes, and 4/30 increased at both 180 minutes and 360 minutes. As presented in Table 5, the 3 proteins that increased at 30 minutes were immunologically the same as 3 of the 4 proteins that increased at 180 and 360 minutes. In this same study, mass spectrometry analysis of four of the liver specimens revealed that one protein (of the 1254 proteins detected) increased significantly after 1 hour of cold ischemia, and 18 proteins (1.4%) were significantly altered after 6 hours (9 proteins increased and 9 proteins decreased).

The study of Espina et al.²⁴ evaluated 11 different clinical specimens collected from various organs and disease states (i.e., normal/benign, different conditions, and malignancies) and maintained them at different times of cold ischemia.

TABLE 1. REFERENCES SHOWING PERCENTAGE OF TRANSCRIPTS/GENES CHANGED IN SURGICAL TISSUES DURING VARIOUS TIME POINTS OF COLD ISCHEMIA

Reference	Tissue and (n) screened	Temperature	Time point (hours) after removal of tissue from body					Comments
			Number of transcripts changed/Total transcripts evaluated (% of transcripts changed)					
Aktas ¹⁸ et al.	Breast cancers (11)	RT	0.33 hr 83.2/16588 (0.5)	0.67 hr 88.3/16588 (0.5)	1 hr 129.9/16588 (0.8)	2 hr 161/16588 (1)	3 hr 128.8/16588 (0.8)	Specimens kept in RNA later, but compared to snap frozen changes were about equivalent at 40 min
Borgan ⁷ et al.	Breast cancer (10)	RT	1 hr 494/24712 (2)	Up to 6 hr 1788/24712 < 10% of genes change				At 1 hr, transcript changes during the time from 0 to 0.5 hr were added to those at 0.5 to 1 hr. To be conservative, 10% was chosen as a value in that the starting point of analysis was uncertain.
De Cecco et al. ¹⁰	Breast cancers (8)	RT	2 hr 121/15921 (0.8)	6 hr 157/15921 (1)	24 hr 657/15921 (4)			Most transcripts were upregulated; confirmed by RT-PCR
Musella et al. ¹¹	Colorectum uninvolved (13)	RT	1 hr 32/16698 (0.2)	3 hr 32/16698 (0.2)	6 hr 32/16698 (0.2)			
	Colorectal carcinoma (14)	RT	1 hr 31/16698 (0.2)	3 hr 31/16698 (0.2)	6 hr 31/16698 (0.2)			
Liu et al. ³	Renal cell carcinoma (10)	4°C	0.08 hr 30/38500 (0.08)	0.5 hr 6/38500 (0.02)	1 hr 29/38500 (0.08)	2 hr 32/38500 (0.08)	4 hr 633/38500 (2)	The changes at 37°C would mirror changes secondary to warm ischemia
		22°C	15/38500 (0.04)	164/38500 (0.4)	469/38500 (1)	352/38500 (0.9)	NR	
		37°C	387/38500 (1)	375/38500 (1)	1048/38500 (3)	3945/38500 (10)	NR	

NS, not specified; RT, room temperature; NR, not reported.

TABLE 2. REFERENCES SHOWING PERCENTAGE OF TRANSCRIPTS/GENES CHANGED IN SURGICAL TISSUES ($N \leq 3$) DURING VARIOUS TIME POINTS OF COLD ISCHEMIA

Reference	Tissue and (n) screened	Temperature	Time point (hours) after removal of tissue from body				Comments
			Number of transcripts changed/total transcripts evaluated (% of transcripts changed)				
Bray et al. ¹² *Huang ²⁰ et al.	Rectal–Rectosigmoid cancer (3) Uninvolved colorectal (1)	NS	0.25 hr	0.5 hr	2 hr	Of the 3 specimens analyzed for changes in transcripts, one was a tubulovillous adenoma and 27% average relative change; 64% genes increased and 36% decreased relative to 5 min and 12% average relative change; 33% increased and 67% decreased relative to 5 min and 50% average relative change; 50% genes increased and 50% decreased relative to 5 min. Most genes were increased and most increases occurred within the first 15 min. No statistical analysis.	
			47/15921 (0.3)	49/15921 (0.3)	200/15921 (1)		
		RT	1 hr				
			1636/2400 (68.2)				
			1 hr				
Spruessel et al. ⁴⁰	Uninvolved colorectal (2)	NS	427/2400 (17.8)				
			1 hr				
		321/2400 (13.4)					
		0.25 hr	0.5 hr				
		1797/11984 (15)	2396/11984 (20)				
Dumur et al. ²³	Papillary serous carcinoma of the ovary (1) Immature teratoma of ovary (1)	RT	0.25 hr	0.5 hr	1 hr	2 hr	
			0/22283 (0)	0/22283 (0)	0/22283 (0)	0/22283 (0)	
		RT	0.25 hr	0.5 hr	1 hr	2 hr	
			4/22283 (0.01)	4/22283 (0.01)	4/22283 (0.01)	4/22283 (0.01)	

*Three categories of genes reported on the same specimen.

TABLE 3. REFERENCES OF SPECIFIC GENES EXPRESSED IN SURGICAL TISSUES AT VARIOUS TIME POINTS OF COLD ISCHEMIA

Reference	Tissue and (n) screened	Temperature	Genes	Time point (hours) after removal of tissue from body (relative expression level to "0")				Comments
Borgan et al. ⁷	Breast cancer (10)	RT	<i>JUND</i>	0.5 hr (1.8)	1 hr (1.4)	3 hr (2.8)	6 hr (2.8)	Although title contains "warm ischemia," the article discusses cold ischemia as defined in this article.
			<i>FOSB</i>	(0.5)	(1.0)	(5.6)	(8.0)	
			<i>GADD45B</i>	(0.8)	(1.4)	(1.4)	(2.8)	
			<i>LIM3</i>	(0.8)	(2.0)	(2.0)	(3.2)	
			<i>THC2271582</i>	(O)	(O)	(O)	(O)	
			<i>BG950086</i>	(O)	(O)	(O)	(O)	
			<i>BC032118</i>	(O)	(O)	(O)	(O)	
			<i>ZFP36</i>	(O)	(O)	(O)	(O)	
			<i>FEM1B</i>	(O)	(O)	(O)	(O)	
			<i>DFNB31</i>	(O)	(O)	(O)	(O)	
Dash et al. ³⁹	Prostate cancer (4)	RT	<i>EGr-1</i>	1 hr (1.8)	3 hr (1.8)	5 hr (1.9)		Results are from prostate tissues not suspected to have cancer, which were then pooled for analysis. Thus an <i>n</i> =1.
			<i>JUNB</i>	(1.8)	(1.8)	(1.8)		
			<i>JUND</i>	(O)	(O)	(O)		
			<i>ATF</i>	(O)	(O)	(O)		
			<i>Hepsin</i>	(C)	(C)	(C)		
			<i>EGR-1</i>	1 hr (1.4)				
			<i>JUNB</i>	(1.2)				
Schlomm et al. ⁸	Prostate (9)	RT	<i>SP100</i>	(2.2)				Minimal changes in expression of <i>CD44</i> and <i>HSPA1B</i>
			<i>KRT17</i>	(1.5)				
			<i>p21</i>	(1.4)				
			<i>PIM1</i>	(1.4)				
			<i>DUSP</i>	(1.3)				
			<i>Vimentin</i>	0.08 hr (1.0)	0.5 hr (1.0)	1 hr (0.8)	2 hr (0.8)	
			<i>Bax</i>	(0.8)	(0.8)	(0.8)	(0.7)	
			<i>FOS</i>	(1.1)	(0.9)	(0.9)	(1.1)	
			<i>CA9</i>	(1.0)	(0.9)	(0.9)	(0.9)	
			<i>Vimentin</i>	0.08 hr (0.8)	0.5 hr (1.0)	1 hr (1.4)	2 hr (1.6)	
Liu et al. ³	Renal cell carcinoma (10)	4°C	<i>Bax</i>	(0.8)	(0.8)	(0.7)	(0.6)	<i>CD44</i> and <i>HSPA1B</i> overexpressed
			<i>FOS</i>	(1.1)	(0.9)	(0.9)	(2.8)	
			<i>CA9</i>	(1.0)	(0.9)	(0.9)	(0.8)	
			<i>Vimentin</i>	0.08 hr (0.8)	0.5 hr (1.0)	1 hr (1.4)	2 hr (1.6)	
			<i>Bax</i>	(0.8)	(0.8)	(0.7)	(0.6)	
		22°C	<i>FOS</i>	(1.1)	(1.6)	(2.3)	(2.8)	<i>CD44</i> and <i>HSPA1B</i> overexpressed
			<i>CA9</i>	(0.9)	(0.9)	(0.8)	(0.8)	
			<i>Vimentin</i>	0.08 hr (1.3)	0.5 hr (2.1)	1 hr (2.3)	2 hr (3.0)	
			<i>Bax</i>	(0.7)	(0.7)	(0.7)	(0.5)	
			<i>CA9</i>	(1.1)	(1.6)	(2.3)	(2.8)	

O, overexpressed - extent not analyzed in article; C, constant-extent not specified.

TABLE 4. REFERENCES OF SPECIFIC GENES EXPRESSED IN SURGICAL TISSUES ($N \leq 3$) AT VARIOUS TIME POINTS OF COLD ISCHEMIA

Reference	Tissue and (n) screened	Temperature	Genes	Time point (hours) after removal of tissue from body (relative expression level to "0")				Comments
Blackhall et al. ⁴¹	Lung (2)	RT	<i>JNK3</i> <i>CAIX</i> <i>PRSS25</i> <i>HHR6B</i> <i>AP-1</i> <i>JUNB</i>	0.5 hr	1 hr			
				(0.9)	(1.0)			
				(1.0)	(1.0)			
				(1.0)	(1.0)			
				(0.9)	(0.9)			
				(1.0)	(0.9)			
Micke et al. ¹⁴	Tonsil (1)	on ice? 4°C	<i>cfos</i> <i>TGFβ1</i> <i>SMAD7</i> <i>HIFα</i> <i>Bcl2</i> <i>PCNA</i>	0.5 hr	6 hr	16 hr		
				(1.1)	(1.4)	(1.0)		
				(1.1)	(1.4)	(0.6)		
				(1.3)	(1.4)	(1.2)		
				(0.8)	(0.7)	(0.7)		
				(1.6)	(1.3)	(0.6)		
		RT	<i>PCNA</i> <i>cfos</i> <i>TGFβ1</i> <i>SMAD7</i> <i>HIFα</i> <i>Bcl2</i> <i>PCNA</i>	(0.8)	(1.1)	(1.0)		
				0.5 hr	6 hr	16 hr		
				(0.8)	(0.08)	(0.3)		
				(1.4)	(1.7)	(1.1)		
				(1.8)	(1.5)	(1.7)		
				(1.0)	(0.8)	(0.7)		
Dumur et al. ²³	Immature teratoma of ovary (1)	RT	<i>POMC</i> <i>XIST</i> <i>FOXG1B</i> <i>CDH10</i>	0.25 hr	0.5 hr	1 hr	2 hr	
				(0.2)	(0.5)	(0.4)	(0.3)	
				(8.0)	(5.6)	(5.6)	(4.0)	
				(46.0)	(23.0)	(53.0)	(32.0)	
				(4.0)	(2.2)	(3.7)	(8.0)	

None of these specimens had the same diagnosis. Table 6 shows normal uterine endometrial tissue maintained at 4°C and RT at various times of cold ischemia. Of the 20 proteins/phosphoproteins evaluated, at 4°C, 3 of 4 proteins/phosphoproteins were increased after 45, 60, 90, and 120 minutes and 2 of 3 of these increased proteins were also increased at RT at the same times. HIF-1α was not significantly changed at 4°C, (not in Table); however, at RT, it was significantly decreased at 45 and 60 minutes, but was essentially unchanged at 1.5 hours (0.9) and 2 hours (1.2).

Espina et al. report variation in protein levels over time between patient matched squamous cell carcinoma and normal lung tissue during cold ischemia.²⁴ Squamous cell carcinoma tissue showed a decrease in apoptotic proteins (CC3 Asp-175), with increases in proliferation/survival (AKT Thr-308, EGFR Tyr-1148, GSK3 αβ Ser-21/9, ERK Thr-202/Tyr-204, and IRS-1 Ser-612), stress/inflammation (STAT1 Tyr-701, and IκB Ser-32), and hypoxia/ischemia (p38 Thr-

180/Tyr-182).²⁴ Normal tissues showed increases in proliferation/survival (EGFR Tyr-1148 and GSK3 αβ Ser-21/9), hypoxia/ischemia (p38 Thr-180/Tyr-182), and stress/inflammation (STAT1 Tyr-701) with a decrease in IκB Ser-32.²⁴

For clinical biomarkers, most studies have used immunohistochemistry and *in situ* hybridization, and have concentrated on delay to formalin fixation (DFF) after specimens are received in pathology.²⁵ At the protein level, the following markers have been reported not to change within 8 hours or more of DFF: Ki67, PCNA, AE1/AE3, CK7, CK14, CAM 5.2, EMA, GCDFFP-15, and mammaglobin.^{25–27}

As would be expected, the main controversies involve the important clinical markers in breast cancer, ER, PR, and HER2. The usual controversies involve the following question: Does a relatively small change in one of these biomarkers cause clinically important changes, that is, changes in the classification of a tumor (e.g., HER+ to HER2-) and hence the medical care of a patient?

TABLE 5. STATISTICALLY DIFFERENT RPPA RESULTS OF CRYOPRESERVED LIVER SPECIMENS (TOTAL PROTEINS EVALUATED=30; 26 DID NOT STATISTICALLY CHANGE)

Time of cold ischemia (n=number of specimens)	0 min (n=15)	30 min (n=15)	60 min (n=15)	180 min (n=15)	360 min (n=11)	Range (min-max)
PRAS40	1.00	1.34	1.50	2.21**	2.08**	0.51–6.70
p44/42MAPK(Erk1/2)	1.00	1.25*	1.51*	1.81**	1.65*	0.47–3.18
Stat3	1.00	1.32*	1.49	1.56**	1.59*	0.44–3.06
Caldesmon-1	1.00	1.22*	1.42	1.68**	1.53*	0.47–3.06

Results are compared to the "0" time point.

* $p < 0.05$.

** $p < 0.01$.

Modified from Gündisch et al.⁶

TABLE 6. PROTEINS/PHOSPHOPROTEINS

Reference	Tissue and (n) screened	Temperature	Proteins/ phosphoproteins	Time point (hours) after removal of tissue from body (relative change to "0" time point)				Comments
				0.75 hr	1 hr	1.5 hr	2 hr	
Espina et al. ²⁴	Uterine tissue normal Endometrium/ myometrium (1)	4°C	CC3 D-175	(2.4)	(4.0)	(3.0)	(3.2)	0 time = 30 min postexcision
			CC9 D-330	(1.6)	(2.1)	(1.5)	(2.0)	
			ASK1 S-83	(1.4)	(2.1)	(2.4)	(2.0)	
			AKT S-473	(0.40)	(0.45)	(0.43)	(0.40)	
		RT	CC3 D-175	(2.2)	(3.4)	(3.8)	(1.9)	
			CC9 D-330	(2.0)	(1.5)	(2.0)	(2.4)	
			ASK1 S-83	(0.65)	(0.7)	(0.65)	(0.7)	
			HIF-1 α	(0.45)	(0.7)	(0.9)	(1.2)	

For example, Portier et al.²⁷ found that up to 3 hours of DFF had no practicable effects on clinical interpretation of HER2 by IHC or FISH in 84 patients, and Pekmezci et al.²⁸ found that in 164 cases of breast carcinoma, the HER2 status did not change when needle biopsies were compared with lumpectomies or mastectomies on the same patients.

Of importance, there were effects of both warm ischemia plus cold ischemia in lumpectomies and mastectomies in this study. In contrast, Khoury et al.²⁹ identified a statistically significant decrease in HER2 in breast cancer (6 of 10 cases) at 2 hours; however, these changes were not interpreted as to their clinical importance (i.e., it was not stated as to whether or not the reported decreases in HER2, based on clinical standards, would have changed the classification of a tumor from HER2+ to HER2-).

Similarly, a change secondary to delay to fixation in which a tumor's classification changed from ER+ to ER- would have been clinically significant. Yildiz-Aktas et al.³⁰ also investigated ER, PR, and HER2 expression in breast cancer resections maintained at 4°C and RT at variable cold ischemia times. Of the samples maintained at 4°C, 8 of 25, 6 of 25, and 6 of 25 showed a reduction in staining for ER, PR, and HER2, respectively, but the changes did not occur until 24–48 hours of cold ischemia. Of the room temperature specimens, 11 of 23, 10 of 23, and 11 of 23 showed a reduction in ER, PR, and HER2, respectively, which occurred during 24–48 hours of cold ischemia.

Reported changes during DFF in ER and PR also are controversial. Khoury et al.²⁹ noted a decline in immunostaining score for ER of 3% at 2 hours at RT, 9% at 4 hours, and 20% at 8 hours, and in PR of 11% at 1, 2, and 4 hours and 15% at 8 hours. While these changes were not statistically significant, these studies had a relatively low statistical power; also, these changes were not evaluated as to clinical importance and, subsequently, were reported to vary based on the antibody used.^{31,8,32}

In a report by Yildiz-Aktas et al.³⁰ previously discussed, of the samples held at 4°C, 2 of 25 samples (8%) converted from ER+ to ER-, 1 sample at 24 hours, and another at 48 hours, 3 of 25 samples (12%) converted from PR+ to PR-, 1 at 24 hours, and 2 at 48 hours. Of the samples held at RT, 1 of 23 (4%) converted from ER+ to ER- at 24 hours, 3 of 23 (13%) converted from PR+ to PR-, 1 at 4 hours, 1 at 24 hours, and 1 at 48 hours.

Neumeister et al.³³ reported no statistically significant loss of ER, PR, HER2, or Ki67 in 25 formalin-fixed paraffin-embedded breast cancer specimens within a 4 hour window of delayed fixation.³³ In contrast, Pekmezci et al.²⁸ noted a loss of ER+ classification in 5/149 cases (3.4%) and PR classification in 9/126 cases (7.1%), when needle biopsies were compared with lumpectomies or mastectomies in the same patients.

Of importance, lumpectomies and mastectomies have periods of warm ischemia plus cold ischemia in contrast to

TABLE 7. MICRORNA EXPRESSION AT VARIOUS TIMES OF COLD ISCHEMIA

Reference	Tissue and (n) screened	Temperature	miRNA	Time point (hours) after removal of tissue from body (relative expression level to "0")				Comments
				0.5 hr	1 hr	3 hr	6 hr	
Borgan et al. ⁷	Breast cancer (10)	RT	hsa-miR-1224-5p	(1.0)	(1.5)	(2.0)	(2.0)	
			hsa-miR-1225-5p	(1.4)	(1.4)	(4.0)	(4.3)	
			kshv-miR-K12-3	(1.3)	(2.5)	(3.8)	(5.3)	
			hsa-miR-125a-3p	(1.1)	(1.4)	(2.8)	(3.5)	
			hsa-miR-371-5p	(O)	(O)	(O)	(O)	
			hcmv-miR-US4	(O)	(O)	(O)	(O)	
			hsa-miR483-5p	(O)	(O)	(O)	(O)	
			hsa-miR-663	(O)	(O)	(O)	(O)	
			hsa-miR-557	(O)	(O)	(O)	(O)	
			hsa-miR-765	(O)	(O)	(O)	(O)	

The top most significantly changed miRNAs are listed above; those with (O) represent other overexpressed miRNAs in the top 10, whose specific changes as to extent are not clearly presented in the article.

needle biopsies. There is a possibility that these changes in biomarker interpretation are due to warm ischemia rather than cold ischemia.

Effects of Cold Ischemia on miRNA

microRNAs (miRNA) are 18–24 single-stranded non-coding nucleotides, which can modulate gene expression at the post transcriptional level.³⁴ The main report of the effects of cold ischemia on miRNAs studied 10 cases of breast cancer, in which 507 unique miRNAs were evaluated; of these, 56 (11%) showed an increased expression with an increase in the time of cold ischemia.⁷ Table 7 presents data concerning changes in miRNA in response to cold ischemia. Data from the top 4 statistically significant miRNAs were derived from graphs and show clear increases at 0.5, 1, 3, and 6 hours. Six other miRNAs also were overexpressed, but the exact extent was not clearly specified in the article.⁷

Effects of Temperature on Clinical Specimens in Cold Ischemia

The temperature in which the surgical specimens are maintained may affect the extent of changes in transcripts. Specimens held at 4°C have a lower percentage of change in transcripts with time of cold ischemia than those maintained at RT or at 37°C.³

Setting of a Standard for a Maximum Time of Cold Ischemia

This subsection has been added to address one common theme from the original reviewers of this commentary, that is, the need for a standard to control the time of cold ischemia. Establishing a standard for the maximum time of cold ischemia is not without its problems and risks. What would such a standard mean? Suppose the standards were set at less than 3 hours, do biorepositories not collect or discard specimens collected at 4 hours, 6 hours, and/or 24 hours? Should investigators reject all specimens exceeding 3 hours of cold ischemia time? What happens to “The Standard” when technology advances, which is happening rapidly.

Initially, establishing a standard for cold ischemia time was thought to be important to minimize the degradation of mRNA; however, when one evaluates those relatively few genes that change over 3 hours, most of these genes and more importantly, their associated proteins increase rather than decrease. For some tissue, the increase in genes begins during warm ischemia⁸ or within minutes of cold ischemia at room temperature,^{7,16} but this may vary with organs.⁶

Therefore, how does one establish a standard for cold ischemia time if the great majority of the few genes that change during cold ischemia actually increase and the time course of this increase varies with specific genes and tissues? Also, it is obvious that there are currently not enough studies to support any strong standard for cold ischemia. We would argue that a standard for cold ischemia cannot be selected until a majority of the few genes that change begin to decrease. This probably eliminates standards of cold ischemia that are less than 3 hours; however, we still would be reluctant to establish a standard for cold ischemia because so few genes actually change.

Also, most studies of cold ischemia clearly note that there is more variability among specimens than variability caused by cold ischemia. Those who work in biorepositories should consider the concept of “fit for purpose,” meaning that any tissue which fits the needs of the research of an investigator is a useful specimen independent of the time of cold ischemia, RIN, or percentage of change in transcripts, microRNA, or proteins.

A clear example of a standard being left behind by advancing technology is the relatively arbitrary concept that for mRNA to be useful, the RIN of the total RNA extracted from tissue should be >7. When one reviews the literature, the early studies of Fleige et al.,³⁵ Fleige and Pfaffl³⁶ indicated that total RNAs with RINs of 5 or greater were useful in short amplicon RT-Q-PCR in which appropriate house-keeping genes are used. Also, short amplicon RT-Q-PCR now is used for mRNA analysis of archival paraffin blocks to identify clinically more or less aggressive tumors³⁷ and direct clinical care.³⁸ This utilization of paraffin blocks is independent of RIN, which is typically low for all paraffin-embedded tissue. Of importance, the effects of the time of cold ischemia should be experimental, random differences (e.g., noise) however, such effects could result in bias; so the time of cold ischemia should be recorded.

Author Views and Comments

It appears to be clear from the literature that cold ischemia is less important than it is currently viewed; thus there still are misconceptions about the importance of short times (<1 hour) of cold ischemia. This review demonstrates the importance of reviewing the literature for effects of pre-analytical variables before a standard is established, leading to potential decisions to select/reject tissues for analyses of biomolecules. The literature is woefully inadequate as to effects on the molecular characteristics of tissues of pre-analytical variables because funding agencies have chosen to underfund biospecimen research, resulting in little understanding of the most important component used in translational research, human tissues.

The literature must be evaluated carefully as to the molecular effects of cold ischemia. It is our view that sometimes when investigators study cold ischemia, they cannot accept their own results and in the abstract recommend shorter times for cold ischemia than are supported by the results of their own studies (personal observations). This is a reflection of authors wanting to support the common concept that tissues must be processed very rapidly and their effort to report positive results. Also, there is sometimes poor editorial review of the complete article, low statistical power of most studies, absent or poor statistical analysis, or review and inappropriate presentation and evaluation of experimental data.

Of critical importance is a consideration of an FDR, which is very high when only a few transcripts are noted to change when thousands of transcripts have been evaluated. Practically, unless more than 0.5% of genes were identified to change in response to cold ischemia, many genes identified to change would be falsely identified.³

Establishing a standard for cold ischemia is difficult because of the small changes in levels of RIN and/or 28S:18S rRNA ratios and low numbers of transcripts that change during cold ischemia. Of note, based on the changes in

transcripts, which are low and tend to be increased within 3 hours of cold ischemia, the question should be evaluated as to whether RIN actually is a good surrogate measure of the usefulness of mRNA.

Actual increases in proteins, in response to increased times of cold ischemia of up to 6 hours, do not justify increased costs of selecting a standard for cold ischemia of less than 3 hours. In addition, specimens with cold ischemia times of up to 12 hours are still useful for many studies. Of interest is that Ep-CAM-positive hepatocytes can be cultured within 48 hours of cold ischemia.⁹

Most mRNAs and proteins do not degrade during 3–6 hours of cold ischemia at room temperature, in that, most of the transcripts and proteins that have been evaluated during these times of cold ischemia actually increase. These increases occur in specific types of genes, whose changes may be induced by ischemia, acidosis, and/or stress.¹⁸ Thus, in some cases, a specimen with a longer time of cold ischemia may be more representative of the immediate preoperative state than a more rapidly processed specimen.^{6,24} When transcripts and proteins are increasing, it is almost impossible to select a good standard for cold ischemia; one can only try to study matching times of cold ischemia; even then, warm ischemia time may be the more important variable. Warm ischemia is not easily controlled and hence, data on warm ischemia frequently are not available.^{8,32}

As an investigator, even when the number of proteins and transcripts that change are few, what should you do if the molecule that is changed is the gene/protein that you are studying? First, you must be aware that many changes occur during warm ischemia, which practicably cannot be controlled. Then you must accept noise (caused by such changes), but be aware of the potential for bias in your results. It is important to note that many studies report that there is more molecular variability observed among individual patients than secondary to differences in times of cold ischemia.^{6,7} In general, rather than emphasizing a need for rapid processing of tissue, it might be more important to maintain records as to times of cold ischemia.

Conclusions

In summary, it is important that biomarker standards are based on evidence and not opinions or anecdotal reports.^{1,2} Thus, the view that one has to start “somewhere” in establishing standards is not without potential problems to biorepositories because of the tendency to select arbitrarily short times for standards without fully understanding the costs and effects of such standards. Also, over time, these standards become established and are difficult to change in that, some investigators still believe that a RIN of 7 is critical to any research focused on mRNA.

Importantly, efforts to obtain solid tissue samples of cancer within 1 hour, while attempting to protect surgical margins, may be expensive and, more worrisome, may compromise diagnostic specimens, while not contributing to an improved quality of research samples. Thus, if there is a need for standard times for stabilizing tissues to support research, the time should not exceed 3 hours; however, specimens not stabilized within 3 hours may still be very useful for many studies.^{3,9,24,39}

Maintenance of specimens at 4°C after removal at surgery and during transportation to the pathology laboratory is

somewhat beneficial in stabilizing changes in some biomolecules.^{3,24} Variability in molecular results depends more on specimen and patient characteristics than on time of cold ischemia. Investigators and biorepositories currently should adopt the concept of “fit for purpose” rather than selecting arbitrary standards for times of cold ischemia.

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