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Impact of Devascularization and Tissue Procurement on Cell Number and RNA Integrity in Prostatectomy Tissue

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Abstract

BACKGROUND—Minimizing the time between tissue devascularization in robot-assisted laparoscopic radical prostatectomy (RALP) and tissue procurement should produce the highest quality tissue for research study. This study examines the relationship between intra-operative time and 2 indicators of tissue integrity: number of epithelial cells per gram of tissue and RNA integrity numbers (RINs). The study also compares the RIN values of tissue obtained intra-operatively by biopsy, before and after devascularization, to those from RALP specimen tissue, obtained through the routine research tissue procurement process.

METHODS—Prostate tissues from two series of patients were analyzed. In the first, tissue from 18 patients undergoing RALP was analyzed for number of epithelial cells per gram of tissue. In the second, RIN values of tissue from 46 patients involved in a clinical study were analyzed. RIN values were assessed from RALP specimen tissue as well as tissue removed intra-operatively by biopsy, before and after devascularization.

RESULTS—Time from RALP to tissue procurement was not significantly associated with number of epithelial cells per gram of tissue or with RIN values. RINs of biopsy tissue obtained intra-operatively before and after devascularization were similar. However, the RIN values of tissue from RALP specimens were significantly higher than those of biopsy tissue obtained either before or after devascularization.

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Disclosure statement

The authors have no conflict of interest to disclose.

CONCLUSIONS—Tissue quality, defined by number of epithelial cells or RIN values, was not affected by time between devascularization and procurement. Obtaining tissue from intra-operative biopsies, either before or after devascularization, is not necessary and actually produced lower RINs than found in tissue from RALP specimens, obtained through the routine research tissue procurement process.

INTRODUCTION

The increasing frequency of RALPs raised concern that the quality of prostatectomy tissue is diminished by the increased time the prostate tissue sits in the body after devascularization compared to open radical prostatectomy (ORP). Several groups (1–5) have studied the effect of this increased time with regard to quality of gene expression. Initial studies (1,2) raised concern that gene expression degraded over time. More recent studies (3–5) have reported no difference in quality between tissue from RALP and ORP.

This study investigated the relationship between two measures of tissue quality--number of epithelial cells per gram and RIN values--and time between devascularization and tissue procurement using tissue from two series of RALP patients. In addition, RIN analysis of biopsy samples taken before and after devascularization was compared to RIN values from RALP specimen tissue, obtained through the routine research tissue procurement process.

MATERIALS AND METHODS

Patients

Prostatectomy tissue was collected and analyzed from two series of patients. The first series comprised fresh human prostate specimens from 18 RALP patients, requested and obtained from the Pathology Resources Network (PRN) at Roswell Park Cancer Institute (RPCI). Participants underwent RALP between March 2011 and May 2012. All specimens were obtained according to a protocol approved by the IRB at RPCI and according to guidelines specified by the National Institutes of Health (NIH) for the use of human subjects. Once specimens were processed by PRN, study personnel retrieved the specimens for analysis of cell number. Time between tissue devascularization and tissue retrieval by study personnel was recorded for each patient.

The second series comprised 46 patients who participated in a clinical trial studying the effects of selenium and finasteride prior to RALP. The study was approved by the IRB at RPCI and all 46 patients were consented and randomized to the study. Participants underwent RALP between March 2009 and March 2012. Tissue from RALP surgical specimens was retrieved from RPCI's PRN according to the study protocol. Detailed information on the time between devascularization and PRN tissue procurement, available for 17 of the 46 patients, was used to compare RIN values and lag time between devascularization and PRN tissue procurement. In addition to the surgical specimens, four core needle samples- two from the right and two from the left - were removed by the surgeon from the peripheral zone, prior to the devascularization of the prostate as part of the protocol for the study. This tissue was immediately frozen in liquid nitrogen. Two additional

cores were extracted and frozen immediately after the prostate was removed from the patient.

Cell Number Analysis

Tissue specimens were collected among a series of 18 patients following a tissue procurement process previously described (6). These specimens were enzymatically digested according to a procedure outlined by Gangavarapu and Huss (7). The total number of epithelial cells isolated from the specimen was obtained by a Beckman Coulter (Brea, CA, USA) Vi-Cell-XR cell viability analyzer.

RNA Integrity Number

Prostate tissues were snap frozen in liquid nitrogen intra-operatively (for before and after devascularization specimens) or obtained from the PRN tissue procurement service (for surgical specimens) under approved institutional protocols. Cryosections were 12 microns thick and mounted on polyethylene naphthalate (PEN) membrane coated slides (Leica, Germany) that had been irradiated for 30 minutes. The entire workspace for the project was cleaned prior to sectioning and staining with RNase AWAY (Invitrogen) according to the manufacturer's instructions. Fresh and previously unused cryostat blades were irradiated and used for sectioning each sample to prevent cross-contamination from previous samples. Sections were stained with Mayer's hematoxylin for 10 seconds, rinsed with distilled water, dehydrated with graded alcohols and allowed to air dry. A pathologist identified the presence of malignant and benign tissues from hematoxylin stained images on the computer display prior to dissection. Laser microdissection was performed using an ultraviolet laser at 50 to 200× magnification with the following laser control setting parameters: power (98), speed (2) and specimen balance (11) using a Leica LMD6000 (Leica, Germany). The desiccates were collected on the lid of a 0.5-ml microcentrifuge tube and frozen in -80C until RNA extraction. Total processing time for each sample was less than 30 minutes to minimize RNA degradation. RNA was isolated from each patient's benign tissue using a kit from Qiagen (Valencia, CA, USA). These RNA samples were sent to RPCI's Genome Core Facility.

RNA was quantitated at the Genome Core Facility using the RNA HS assay kit (Invitrogen, Grand Island, NY, USA) on the Qubit fluorometer (Invitrogen) per the manufacturer's instructions. The quality of the RNA was assessed using the RNA 6000 Pico Bioanalyzer Kit (Agilent, Santa Clara, CA, USA) on an Agilent Bioanalyzer system per the manufacturer's instructions.

Classification of RIN uses a numbering system from 1 to 10, with 1 being the most degraded profile and 10 the most intact (8). RIN values were assessed for each patient from each of the following: biopsy tissue removed before devascularization and after devascularization, and from the RALP specimen removed during surgery.

When a patient's RIN value was < 6, or < 20–30 ng of RNA was collected, additional RNA was obtained and analysis was repeated. If a patient's RIN value remained < 6 after 3 attempts, no additional attempt was made. For patients with multiple RINs, the highest RIN was used in subsequent analyses.

Statistical Analysis

Linear regression was used to test for associations between RIN values or number of cells and the time lag between devascularization and tissue procurement. Analysis of variance (ANOVA) and student t-tests were used to test for associations between RIN values from tissues removed at the 3 time points.

RESULTS

Cell Number

A best fit linear regression ($\text{cell number} = -1943.6 \times \text{time in minutes} + 2 \times 10^6$, $R^2 = 0.0249$) showed a negative slope (Figure 1), which suggested fewer cell numbers after longer periods of time; the relationship was not statistically significant. All patients had benign samples used for cell number analysis, and five patients also had malignant samples used for cell number analysis. Plots (not shown) of number of cells per patient, adjusted for malignant status, and a plot of average number of cells per patient (since some patients had more than one sample) with time yielded similar results; both best fit linear regression lines showed a slight downward trend, but neither revealed a statistically significant relationship between number of cells and time. The number of cells in malignant and benign tissue was similar (not shown). If the two outlier values (one time value is 1200 minutes, or 430% of the average, and one cell number value is 1.23×10^7 , or 780% of the average number of cells) were dropped from the regression analysis, the slope became positive, but remained statistically not significant (not shown).

RIN Values

A best fit linear regression ($\text{RIN} = 0.0027 \times \text{time in minutes} + 7.44$, $R^2 = 0.0068$) showed no significant relationship between RIN values and time (Figure 2). A plot (not shown) of average RIN value instead of highest RIN value per patient with time yielded similar results; the best fit linear regression showed no significant relationship between RIN value and time.

ANOVA analysis of RIN values at 3 time points (Table 1) showed a statistically significant difference among the RIN values at each time point ($F=4.03$, $p=0.0204$). T-tests revealed no significant difference between RIN values ($t=-0.40$, $p=0.6950$) from tissue collected intra-operatively by biopsy, before or after devascularization. RIN values from RALP specimen tissue were significantly higher compared to intra-operative prostate biopsies performed either before or after devascularization (before devascularization vs. RALP surgical tissue: $t=-2.51$, $p=0.014$, after devascularization vs. RALP surgical tissue: $t=-2.31$, $p=0.0234$). A similar analysis (not shown) using average RIN value instead of highest RIN value per patient showed similar results: there is no significant difference between before and after devascularization RIN values. This analysis showed a trend toward improved RALP specimen tissue RIN values compared to RIN values from tissue samples obtained by biopsy before and after devascularization, though neither of the differences was significant.

DISCUSSION

Analysis of tissue from RALP patients showed no association between tissue quality, defined by RIN values and the time from devascularization to tissue procurement. Most of the lag times studied herein are typical of the length of time researchers often wait for “fresh” tissue to be procured: 1 to 4 hours following devascularization. This study also shows that there is no advantage to analyzing tissue removed by intra-operative core biopsy either before or after devascularization compared to tissue from the RALP specimen obtained through the routine research tissue procurement process. In fact, the RIN values of tissue from RALP specimens are significantly higher on average than those of tissue removed before or after devascularization. The tissue collected before and after devascularization is from core biopsies; the larger amount of tissue available from the RALP specimen appears more important than time between devascularization and tissue procurement.

Analysis of tissue from RALP patients also showed no association between tissue quality as defined by number of epithelial cells per gram of tissue and time. Cell number can be affected by many factors; many of these are unknown, but they likely have little to do with cell or RNA quality. In previous studies cell viability was analyzed by flow cytometry in 39 specimens. (7,9,10) When time of devascularization was between 5–6 hours, the percentage of cell viability ranged from 5%–86% and the number of viable cells/gram of tissue ranged from 1.4×10^3 – 1.4×10^6 . The cell number/gram of tissue was even more variable, 6.4×10^3 – 6.5×10^6 . Unfortunately, time of devascularization was not available for these specimens. Cell number/gram was used as a measure of tissue quality in this study as it was the most variable and allowed for the fastest analysis of cell recovery following devascularization. Later viability data was available on 4 of the specimens, averaging 77% viability. Surprisingly, time to devascularization had no effect on the quality of tissue as measured by how many cells/gram were obtained following digestion. The stromal content of the tissue seems to correlate best with the number of epithelial cells/gram (high stromal content = low epithelial cells). Stromal content was not quantitated but observed during processing as how firm the tissue was.

The lengths of time studied herein do not establish how long tissue may wait before its usefulness for research declines; this study only shows that typical wait times do not present a problem for tissue degradation. Further study is needed to address how long tissue may sit before its quality has degraded to the point that it is no longer useful for research. RIN values are an indicator of overall RNA quality, but few of the determinants affecting RIN are known. These study results are limited to two measures of tissue integrity: cell number and RIN. Whether some genes, messenger RNAs or proteins are affected by a lag between tissue removal and processing is not known. Nonetheless, the overall cellular and molecular integrity of prostate tissues appears robust.

Conclusion

Cell numbers and RIN values in prostate tissue used for research are not adversely affected by a wait of as many as 4 hours between devascularization and tissue procurement.

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Figure 1.
Epithelial cells/gram of tissue vs. time between devascularization and tissue procurement.

Figure 2.
Highest RIN and time between devascularization and tissue procurement.

Table 1

Student t-test comparison between highest RIN values from prostate core biopsies obtained before and after devascularization, and tissue procured from RALP specimen

	Before Devascularization Mean: 6.87 ± 0.98	After Devascularization Mean: 6.96 ± 0.82
After Devascularization Mean: 6.96 ± 0.82	t=-0.40 (p=0.6950)	
Tissue Procured from RALP Specimen Mean: 7.38 ± 0.83	t=-2.51 (p=0.0140)	t=-2.31 (p=0.0234)