Investigation of the physiological response of radiation-induced cystitis patients using hyperbaric oxygen

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ABSTRACT

Introduction: In this pilot study we have taken a novel functional approach to assess whether differences exist in the activity of key genes involved in the response to radiation and oxidative stress between patients with radiation cystitis.  

Materials and methods: Arm 1 consisted of patients who had previously been treated for prostate cancer and who had received definitive radiation treatment and had subsequently developed cystitis and/or proctitis and were being treated by hyperbaric oxygen (HBO). Arm 2 consisted of patients who had never been treated by radiation but who were scheduled for HBO treatment for another pathology. The genes chosen for the study were HMOX1, NOS2, SOD2, TNFα, IL-6 and TGFβ. Blood and urine was collected pre and post HBO treatment.  

Results: Gene expression showed a significant difference in NOS2 (p = 0.0178) and TNFα (p = 0.037) between the control and cystitis patients. The plasma levels of VEGF-A were significantly elevated in cystitis patients and there was a strong trend for significant overexpression in urine. Comparing pre and post-dive samples showed little difference in both groups of patients except for VEGF-A which was reduced after the dive in plasma from cystitis patients.  

Conclusions: This study uncovered some physiological differences in patients with radiation-induced cystitis using HBO treatment as a stimulus to induce mild oxidative stress. Further research is ongoing to assess whether the acute exposure to HBO might be a physiological screening tool to identify patients susceptible to chronic radiation toxicity.

Introduction

Radiation cystitis (RC) of the bladder is common in prostate cancer patients despite the continuing improvements in pelvic radiation delivery using image-guided treatment, intensity modulated treatment, brachytherapy or protons [1]. In a review of the literature, the incidence of radiation cystitis was found to range from 23 to 80% and the incidence of severe hematuria from 5 to 8% [2]. Late RC is a long-term sequela of radiation treatment with a mean duration for developing the condition being 31.8 months [2] whilst severe hematuria can develop up to fourteen years following radiotherapy treatment [3,4].  

RC is a term given to a constellation of symptoms which include hematuria, frequency, urgency, and pelvic pain. RC occurs secondary to obliteratorative endarteritis from hypoxia which causes atrophy and fibrosis of the mucosa. Ulceration of the mucosa leads to the development of fragile telangiectatic blood vessels that easily hemorrhage [5]. The severity of ranges from grade 1 consisting of minor telangiectasia and microscopic hematuria with minimal increase in frequency, urgency, dysuria or nocturia to grade 3 and 4 where there is severe frequency and dysuria, gross hematuria and severe hemorrhagic cystitis.  

Treatments for RC depend on the severity of hematuria and range from conservative management to transfusion-dependent hemorrhage requiring cystectomy. Algorithms have recently been developed to guide treatment options [6]. One approach for patients with severe hematuria refractory to conventional management has been to use hyperbaric oxygen (HBO) [7–9]. A recent randomized phase 2/3 trial of radiation-induced cystitis treated with hyperbaric oxygen therapy (RICH-ART) concluded that HBO treatment relieves the symptoms of late radiation cystitis and is safe and well tolerated [10]. In HBO treatment, 100% O2 is administered in a pressurized chamber (usually ~2 atmospheres) for
5–7 days/week with a daily duration of 60–90 min and in 30–45 sessions. The aim of HBO treatment is to reverse the process of radiation-induced cellular hypoxia in the bladder tissue through better diffusion of oxygen within the tissues and intracellular generation of reactive species of oxygen and nitrogen [11]. This results in activation of intracellular signaling pathways leading to the induction of neovascularization through VEGF expression [12], and therefore angiogenesis and granulation tissue formation, fibroblast proliferation, and optimization of the cellular immune functions. The overall and complete response to HBO treatment have varied from 64.8% to 100% and 20% to 100%, respectively [13].

Accumulated damage from RC may be irreversible and hence early diagnosis or, better still, predicting patients who are susceptible to develop RC is essential to improve the quality of life of cancer survivors. However, the evaluation and prediction of normal tissue radiosensitivity is an ongoing field of study without definitive conclusions. There is no doubt that individual radiation sensitivity is partly determined genetically which has led to studies of whether it can be inferred from the reaction of cells exposed ex vivo to ionizing radiation or from analyses of the genotype [14]. Ex vivo assays have centered on measuring a variety of DNA damage assays (chromosome aberrations, micronuclei, γH2AX foci, apoptosis) in isolated peripheral blood lymphocytes or fibroblasts [14]. Association of genotype to adverse effects of radiotherapy has been studied in blood and saliva using both a targeted and genome-wide approach. Meta-analyses of these studies identified SNPs in ATM and TGF/β associated with late radiation toxicity [15,16]; although these results have not been consistently validated [17–21]. Recently, a deep learning approach was used to validate genetic risk factors for various late toxicities after prostate cancer radiation treatment [22]. This promising approach did identify 9 significant SNPs associated with urinary toxicities.

In this pilot study we have taken a novel functional approach to assess whether differences exist in the activity of key genes involved in the response to radiation and oxidative stress between patients with radiation cystitis compared to those without bladder pathologies. HBO was used as a tool to invoke an oxidative stress response in patients undergoing their first “dive” and downstream genes involved in increased growth factor response and diminished inflammatory response were assessed before and after the “dive” in plasma and urine.

**Materials and methods**

**Patient population and HBO treatment**

Approval for the study was obtained from the William Beaumont Hospital Institutional Review Board (IRB# 2014-312). The study was approved to collect 12 patients in each arm. Arm 1 consisted of patients who had previously been treated for prostate cancer with definitive radiation treatment either by external beam or a combination of external beam and high-dose brachytherapy (Table 1) who had subsequently developed cystitis and/or proctitis and were being treated by HBO. All patients were diagnosed by cystoscopies and all patients had hematuria. In addition to hematuria some had dysuria (2), frequency (1), nocturia (1) and urethral strictures (2). Arm 2 was originally designed to be prostate cancer patients who had never experienced cystitis after radiation treatment and who were willing to undergo a single “dive” in the hyperbaric oxygen chamber. However, this was not feasible and it was decided to recruit patients who had never been treated by radiation but who were scheduled for HBO treatment for another pathology (Table 1). None of the patients in the control group had urinary symptoms. All patients were studied at the time of their first dive. Blood and urine were collected immediately prior to the dive. Subjects underwent a standard hyperbaric oxygen treatment; the total dive time was 90 min. Vital signs (temperature, blood pressure and pulse) were monitored during and after the HBOT to ensure subject safety. Following the treatment, additional blood and urine samples were collected. Table 1 shows the demographics of the patients who had successful data analysis. There were 10 patients in the radiation cystitis group and 11 in the non-cystitis group. Other patients who were consented were omitted due to issues with blood or urine draws and ineligibility.

**Strategy to select genes of interest**

Selection of genes to be studied was based on Pathway Studio

Table 1

| Non-Radiation Subjects | Radiation-Treated Subjects |
|------------------------|----------------------------|
| ID# | Age | Sex | Diagnosis | RT | Cancer | First Rx |
| 1   | 62  | m   | Chronic osteomyelitis | 51 Gy/10F | Prostate | 1/25/2016 | no |
| 2   | 48  | f   | Failed skin graft | EBRT unknown | Prostate | 10/18/2016 | no |
| 3   | 83  | m   | Diabetic ulcer lower extremity | 79.2 Gy/44F | Rectal | 10/19/2016 | no |
| 4   | 48  | m   | Crush injury | 79.2 Gy/44F | Prostate | 11/2/2016 | no |
| 5   | 68  | m   | Failed skin graft | 79.2 Gy/44F | Prostate | 1/3/2017 | no |
| 6   | 74  | m   | Diabetic ulcer lower extremity | EBRT unknown | Prostate recurrence | 5/9/2018 | no |
| 7   | 90  | f   | Chronic osteomyelitis | 82 Gy/38F | Prostate | 3/13/2018 | no |
| 8   | 65  | m   | Diabetic ulcer lower extremity | EBRT unknown | Prostate | 7/17/2017 | no |
| 9   | 56  | m   | Diabetic ulcer lower extremity | EBRT unknown | Prostate | 4/23/2018 | yes |
| 10  | 50  | m   | Failing skin flap | EBRT and HDR unknown | Prostate | 4/23/2018 | yes |
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 Gene expression

2.5 ml of blood was collected in a PAXgene blood RNA tube (Qiagen, Valencia, CA) before and after the dive and RNA extracted using the manufacturer’s protocol. RNA was quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher, Waltham, MA) and RNA integrity was assessed in a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Complementary DNA (cDNA) was then synthesized by reverse transcription from 40 to 100 ng RNA using the manufacturer’s protocol. The expression levels of all studied genes were detected and identified by real-time PCR using a TaqMan Universal Master Mix (ThermoFisher) with the following genes; VEGF (Hs00900055_m1), SOD2 (Hs00167309_m1), HMOX1 (Hs01110250_m1), NOS2 (Hs01075529_m1), TNFα (Hs00174128_m1), IL-6 (Hs00174131_m1) and TGFβ (Hs00248373_m1). GAPDH control reagents (ThermoFisher) was used as the reference gene. Real-time PCR runs were performed in duplicate for each sample. Expression levels were calculated using QuantiSoft software which sets the best threshold and the cycle at which each amplification curve crossed that threshold was then assigned as the CT for that sample. The ΔCT was calculated by subtracting the CT from the gene from the CT of the housekeeping gene (GAPDH). The data were presented as a ratio of the post-dive ΔCT compared to the pre-dive ΔCT.

Protein expression

For protein expression in plasma and urine, 3 ml of blood was collected in BD Vacutainer tubes and urine collected in a 60 ml collection container pre- and post-dive. The samples were processed and stored at -80 °C until analyzed. Urine samples were spun down for 5 min at 3000 rpm in a tabletop centrifuge to remove any debris. The supernatant was then vortexed before use. Levels of angiogenic growth factors were assessed in undiluted urine. Samples were analyzed using a custom Bio-Plex assay (Bio-Rad, Hercules, CA) using manufacturers protocols. Samples were analyzed in duplicate. Analysis of experimental data was done using five-parametric curve fitting with reference to calibration curves prepared with analyze standards included in the kits.

Creatinine levels

Creatinine determination was performed with a Shimadzu Promini ence high performance liquid chromatography system including a dual pump system, delivering a mobile phase consisting of 25 mM citric acid and 3% acetonitrile (pH 6.0) at a flow rate of 0.5 ml/min. After diluting urine 1:50 in mobile phase, separation was done on a ketinX 2.6 μm C18 100A (100 × 4.60 mm) column (Phenomenex) with UV absorbance detection at 250 nm. Quantification was based on peak height as compared to creatinine standards (Acros Organics).

Statistical analysis

All data was analyzed using JMP 11.2.0 (Cary, NC). Gene expression data was analyzed using a Wilcoxon Rank test with a p-value ≥ 0.05 considered significant with the assumption of equal variances. Paired pre- and post-dive serum and urine samples were analyzed using a paired t-test with a p-value ≥ 0.05 considered significant.

Fig. 1. Pathway analysis to select genes of interest highlighted by the black arrows.
Results

Gene expression changes

The data presented in Fig. 2 shows the relative quantitation for each gene as a ratio of change between the pre-dive compared to the post-dive samples for patients with and without (control) radiation-induced cystitis. Nine samples were successfully analyzed for the cystitis group and 10 for the control patients. The box and whisker plots show that median change in gene expression for the control patients remained unchanged for the post-dive except for NOS2 which showed a 1.52-fold increase after the dive. The majority of genes also showed no change as a result of the dive in the cystitis patients except for NOS2 (0.82-fold) and TNFα (0.78-fold) which were reduced post-dive. This resulted in a significant difference in NOS2 (p = 0.0178) and TNFα (p = 0.037) between the control and cystitis patients (Fig. 2).

Are there intrinsic differences in protein expression between radiation cystitis patients and controls?

Several proteins were either not detectable in plasma or urine at the limit of sensitivity of the ELISA assays. Three proteins, IL-6, VEGF-A and TNFα produced reproducible data in the pre- and post-dive plasma and urine samples and were selected for comparative analysis.

In the plasma samples when comparing patients with and without radiation cystitis, only VEGF-A showed a significant difference in the pre-dive samples (Fig. 3A). The concentration of VEGF-A in the cystitis patients was 40.3 ± 6.6 pg/ml compared to 22.8 ± 6.3 pg/ml in the non-cystitis patients (p = 0.0347). The values for IL-6 were 5.9 ± 1.6 pg/ml and 4.9 ± 1.7 pg/ml in non-cystitis versus cystitis patients and 2.1 ± 1.0 pg/ml and 1.3 ± 1.0 pg/ml for TNFα.

In the urine samples, IL-6 was detectable in only 5 (45.5%) of the control patient samples whilst 8 of 11 (80%) samples were positive in the cystitis patients (Fig. 3B). The mean levels were 6.5 ± 50.7 pg/ml and 157 ± 53.2 pg/ml in controls versus cystitis patients, this resulted in a significant difference (p = 0.027). VEGF levels were detected in all 11 control samples and 9 of 10 cystitis patients and were generally higher in cystitis patients (135.5 ± 27.2 pg/ml) compared to controls (107.2 ± 25.9 pg/ml) but again this did not reach significance (p = 0.25). TNF-α was detectable in 10 of 11 control samples and 9 of 10 cystitis samples but the level of expression was uniformly low with means and S.E.M. of 2.8 ± 1.5 pg/ml and 3.2 ± 1.6 pg/ml in control versus cystitis patients.

Does protein expression change differentially between cystitis and control patients after a HBO dive?

Fig. 4 shows the correlation between the proteins before and after the HBO dive in both plasma and urine samples. In the plasma samples, levels of IL-6, VEGF-A and TNFα were not significantly changed post-dive in both cystitis and non-cystitis patients.

All urine samples were analyzed for creatinine concentration which varied between patients with a mean and S.D. of 1359 ± 482 µg/ml in the control patients and 1859 ± 378 in the cystitis patients in the pre-dive samples compared to 1378 ± 317 and 2255 ± 571 and in the post-dive samples respectively. None of these differences was significant and there was no significant trend in change between pre- and post-dive samples. Analyzing the data as a function of creatinine did not change the significance of results compared to analysis using the protein concentration of each biomarker. When comparing the protein expression data in pre- and post-dive urine samples for VEGF and TNFα, there were no significant differences in the paired samples in either the controls or the cystitis patients. However, IL-6 showed a significant reduction in the cystitis patients post dive. The pre dive levels were 175.3 but were reduced to a mean of 117.7 after the dive (p = 0.0387).

Discussion

In this novel pilot study, we investigated whether the physiological response of patients who have developed radiation-induced bladder cystitis differs to the general population when exposed to mild oxidative stress induced by HBO. It is well accepted that breathing greater than one atmosphere of oxygen will increase production of reactive oxygen species.
species (ROS) [23] and also reactive nitrogen species (RNS). These molecules are involved in signaling transduction cascades for a variety of growth factors, cytokines and hormones. Under normal conditions ROS act in conjunction with several redox systems involving glutathione, thioredoxin and pyridine nucleotides, and play central roles in coordinating cell signaling and also antioxidant protective pathways [24]. In this context oxidative stress is not synonymous with oxygen toxicity.

However, exposure to radiation treatment produces excessive levels of ROS which have been shown to disrupt components of the electron transport chain in mitochondria, induce intracellular redox system imbalances and cause oxidative stress by reacting with biological molecules such as lipids, proteins, and DNA to cause lipid peroxidation, protein misfolding, and DNA strand breaks [25]. Indeed, studies of exposure to radiation treatment have demonstrated a prolonged oxidative stress implying a persistent imbalance between ROS production and antioxidant defense systems [26–28]. The pathophysiology of late radiation cystitis has still to be fully elucidated but endothelial cells appear to play an important role in this process. It has been demonstrated that submucosal vascularity is damaged by fibrosis of the vascular intima leading to vessel obstruction and submucosal/muscular fibrosis [29]. The consequence of these events is ensuing urothelial atrophy, hypoxia with diminished vascularization and ischemia of the bladder which leads to fibrosis and atrophy of the bladder tissue and the emergence of neovascularization in the form of telangiectasia that is susceptible to bleeding [29].

The cellular response to HBO appears to be a combination of systemic events as well as local alterations. Regional angiogenic stimuli influence the efficiency of new blood vessel growth by angiogenesis in local endothelial cells and they stimulate the recruitment and differentiation of circulating stem/progenitor cells (SPCs) to form vessels de novo by vasculogenesis [11]. In radiation-induced cystitis, it is thought that HBO therapy facilitates better oxygen diffusion in tissues and disrupts the continuum between hypoxia and fibrosis through induction of primary neovascularization, secondary growth of healthy granulation tissue, and induces short-term vasoconstriction, which may help control

Fig. 3. Protein expression in plasma (A) and urine (B) samples from cystitis and control patients prior to the HBO dive.

Fig. 4. Protein expression in plasma and urine samples pre- and post-dive specimens for control (gray symbol) and cystitis patients (black symbol). The dashed line in each graph represents the line of unity.
active bleeding [29].

In this study, patients were only exposed to a single dive in the chamber and, therefore, we were not studying the long-term benefits of HBO but assessing the physiological response of patients to this temporary low-level oxidative stress. We showed that gene expression in the control patients remained unchanged for the post-dive except for NO2S which showed a 1.52-fold increase. In contrast, NO2S levels were significantly reduced (0.82-fold) post-dive in the cystitis patients as was TNFα (0.78-fold); the other genes were unchanged. Experimental and clinical wound healing studies have established nitric oxide (NO) as a critical mediator of normal tissue repair [30] and its production is catalyzed by NO2S. Several studies have shown variable effects of HBO on NO2S expression dependent on tissue type and disease context [31–33]. However, the differential response of cystitis to control patients suggests imbalances in this pathway exist which merits further investigation. For instance, it has been shown that NO has dose-dependent effects in the pathogenesis of inflammation where it has an anti-inflammatory effect under normal physiological conditions but is considered as a pro-inflammatory in abnormal situations [34]. Further studies will be required to assess NO and NOS2 levels in bladder tissue to assess the baseline level of these in cystitis and control patients.

Interestingly, VEGF expression levels were not changed in either group of patients whereas synthesis of this most specific growth factor for neovascularization has been shown to be increased in response to hyperbaric oxygen wound healing studies [35]. Again, the lack of effect may be due to the short-term exposure after a single HBO treatment. However, the plasma and urine samples yielded interesting results for this gene. Of note, VEGF protein expression was significantly higher in the plasma of cystitis patients compared to the controls and there was a trend for significant overexpression in urine. This has been observed in previous studies [36,37] of prostate cancer patients who had developed cystitis and parallels data obtained in patients who developed radiation proctitis [38]. Other studies have replicated this finding and suggested that anti-VEGF therapy may be a possible therapy to ameliorate abnormal angiogenesis in this patient population [39,40]. In this context the findings from this present study are thought-provoking as HBO exposure decreased levels of VEGF in the cystitis patients but was without effect in the control population.

The changes in urine and plasma levels after the dive were very modest with only urine levels of IL-6 showing a significant change. Indeed, the levels of IL-6 were highly elevated in the urine of some of the cystitis patients compared to the controls and were much more than that found in plasma indicating the inflammation in the bladder. IL-6 has been reported to be elevated in the urine of interstitial cystitis patients [41] and is associated with bacterial infection. It was interesting that a single HBO dive was able to reduce IL-6 urine levels in cystitis patients but had no effect in control patients.

The study does have limitations. The patient number was relatively small but this reflects that the study was designed to be a pilot study. Second, the original intention to have age-matched controls who had previously had radiation treatment for prostate cancer but who had never experienced cystitis proved impossible to recruit. The compromise was to study patients with other pathologies who were scheduled for HBO treatment as standard of care and therefore they can’t be considered as completely “normal” controls.

In conclusion, this pilot study took the novel approach of studying the response of cystitis and control patients to an in vivo stimulus of HBO exposure to investigate whether there were differences in physiological responses that might shed further light on the pathology of the disease and indicate other treatment options. Further research is ongoing to study these biomarkers in patients prior to radiation treatment and to assess whether the acute exposure to HBO might be a physiological screening tool to identify patients susceptible to chronic radiation toxicity.

Declaration of Competing Interest

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

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