Effects of Different Lasers

On The Fibrotic Tissue

By

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LIST OF ABBREVATIONS

CKD	Chronic Kidney Diseases
ECM	Extracellular Matrix
IL	Interleukin
LLLT	Low Level Laser Therapy
MSC	Mesenchymal Stem Cells
NO	Nitric Oxide
TGF	Transforming Growth Factor
TL	Trilaser
TNF	Tumor Necrosis Factor
UUO	Unilateral Ureteral Obstruction

SUMMARY

Kidney disease, one of the ten major escalating public health problems, affects nearly 20 million people in the United States. This problem is increasing due to an increased prevalence of diabetes and hypertension which leads to chronic kidney disease or end stage renal failure ultimately leading to hemodialysis or reno-transplantation. The progression to chronic ailment is marked by onset and progression of fibrosis with sustained inflammation, overexpression and deposition of collagen in the extracellular matrix making it inhospitable to recovery.

The purpose of this study was to initiate the regeneration of the fibrotic kidney environment with the help of different combinations of low level laser therapy (LLLT) with/without Mesenchymal Stem Cells (MSC) in a mouse model of renal fibrosis induced by unilateral ureter obstruction (UUO). The treatment regimen included a trilaser therapy with a supplemental monolaser treatment as a second dose with/without MSCs. Results were determined by dividing all measurements into kidney cortex and medulla. It was demonstrated that the amount of fibrosis reduced with trilaser + supplemental 635nm + MSC treatment, endothelial quantification increased with trilaser + supplemental 532nm treatment and trilaser + supplemental 635nm+MSC, mitochondrial activation increased with trilaser + supplemental 405nm + MSC treatment. The pro-fibrotic cytokine TGF-β reduced when treated with trilaser + supplemental 405nm + MSC however, a significant increase in the amount of anti-inflammatory cytokine IL-10 was not observed. The results thus indicate a significant effect on the reversal of fibrosis with trilaser therapy supplemented with 635nm wavelength and MSC.

I. INTRODUCTION

A. Kidney disease: An Escalating Public Health Problem

Kidney disease is the 9th leading cause of death in United States. Chronic Kidney Disease (CKD), affects 31 million adults in the US accounting for 10% of the adult population [1]. Progression to chronic kidney disease from subtle physiological changes induced by either chronic hypertension or diabetes, the two main causes, is insidious and often is unrecognized until there is advanced disease. High blood pressure or high serum glucose in the kidney trigger the infiltration of inflammatory macrophages which release cytokines such as tumor necrosis factor (TNF). [2, 3]. Chronic inflammation leads to oxidative stress-related epithelial and endothelial apoptosis [4], myofibroblast proliferation contributing to degeneration of the extracellular matrix and, ultimately, loss of glomerular function. [2, 5] The progression is marked by sustained amount of the pro-sclerotic cytokine, TGF-β, inducing epithelial to mesenchymal transition, with eventual fibrosis. [6, 7] End-stage kidney disease is characterized by a resolution of acute inflammatory activity and replacement of injured tissue with fibrotic tissue [8].

B. Effect of laser on tissue regeneration

Low level laser therapy (LLLT), defined as energy transferred from wavelengths of light between the 350nm-750nm (visible spectrum) has been used as a method to promote lipolysis, superficial wound healing, and acne clearance. [9, 10]. The mechanisms of LLLT are not fully understood. Various studies, on both stem cells and non-stem cells, have demonstrated the effects of LLLT on cells ex vivo [33]. One target of LLLT is the mitochondrial respiratory chain [11]. Following LLLT, increased proton electrochemical potential and ATP

synthesis was observed [12]. Subsequently, LLLT has been shown to modulate gene expression [13], RNA and protein synthesis [14], induce cellular proliferation [15, 16], and promote synthesis or release of growth factors, interleukins, and inflammatory cytokines [17]. Red visible light (635nm) has been FDA approved for clinical applications however extent of green (532nm) and violet (405nm) remains under investigation [9]. Green light photons are used by plants for generating energy, similarly it has been shown that the fibroblasts in chicken embryo grew rapidly to green light stimulus [18].

C. <u>Mesenchymal stem cells and fibrosis</u>

Mesenchymal Stem Cells (MSCs) are multipotent adult stem cells that exhibit regenerative potential due to their capacity to differentiate into a variety of tissue, to reduce inflammation, and promote angiogenesis [19, 20]. MSCs signal several cytokines and growth factors at the site of injury such as vascular endothelial growth factors (VEG-F) to promote vascular remodeling and angiogenesis [21] and transition inflammatory type I macrophages (M1) to pro-regenerative type II macrophages (M2) [22]. Transforming growth factor β (TGF-β), released by macrophages in response to tissue injury, persist in chronic inflammation of kidney disease, and this growth factor has been implicated in the abnormal deposition of matrix leading to the fibrosis observed in end stage renal disease. [23, 24] Interleukin 10 (IL-10) is an anti-inflammatory cytokine released by M2 macrophages and has been involved in the resolution of pro-inflammatory states. [24, 25] While MSCs migrate to the site of acute inflammation via response to CXCR4 [26, 27]. Strategies aimed at modifying the fibrotic

microenvironment to promote MSC recruitment may be effective in initiating regenerative response in the tissue.

In a previous set of studies, the Bartholomew Laboratory tested the photobiomodulation effects of 3 low level light lasers with wavelengths 405nm, 532nm, and 635nm with/without MSCs to initiate regeneration of fibrotic kidney in mouse model by unilateral ureteral obstruction (UUO). Mice underwent UUO, displayed profound fibrotic degeneration of the kidney by 3 weeks, and a single session of laser therapy with or without a single dose of MSC. Twenty-four hours after treatment, fibrotic kidneys were explanted and examined for initial effect. This study indicated that all lasers reduced ongoing apoptosis and enhanced mitochondrial activity with enhanced effect with added MSC. The 535nm wavelength appeared to reduce TGF beta to a greater extent than other laser wavelengths, and the 405nm wavelength led to higher levels of endothelial viability and proliferation, suggesting an improved preservation of endothelium [34].

Given that all three lasers had statistically significantly beneficial effects, we hypothesized that when given together, all three lasers would provide significant benefit over time with evidence of reversal of the fibrotic process. The purpose of these experiments was to test the effect of all three lasers when given together with or without MSC over a four-week period. In addition, we tested whether a specific laser, if given in addition to the trilaser (TL) therapy 4 hours later, would further enhance the laser-specific beneficial effects we observed at 24 hours and how this more individualized treatment would impact fibrosis and the kidney microenvironment after 4 weeks of treatment.

II. MATERIALS AND METHODS

A. Animals

Male C57BL/6 mice, 12 weeks old, were purchased from the Charles River Laboratories (Wilmington, MA), and housed in the University of Illinois at Chicago (UIC) Biologic Resources Laboratory (BRL). All care was given under conditions approved by the Animal Care Committee at UIC. Renal fibrosis was induced through the unilateral ureteral obstruction (UUO) model. Mice were anesthetized with optimum dose of ketamine/xylazine solution, weighed and prepared for the surgery. A 3cm midline incision was made to access the right kidney, and the ureter was isolated from the surrounding tissue. The ureter was ligated by tying one 4-0 silk suture around the ureter 4mm below the kidney. The incision was closed using sterile 4-0 Nylon suture. The mice were then returned to the animal vivarium, sutures were removed at 7 days, and the establishment of fibrosis in a subset of animals was verified 21 days later prior to initiating the four week treatment programs. Animals underwent euthanasia 50 days after UUO for histologic exam of the kidney. Exam also included verification of the intact UUO ligature and occlusion of the ureter. All animals demonstrated intact UUO ligatures with dilated renal pelvis.

B. Mesenchymal stem cell isolation, expansion and administration:

MSCs were prepared as previously described [28]. (Mouse MSC culture media was prepared by filtering 45% alpha minimum essential medium (Life Technologies, Grand Island, NY), 45% F-12 nutrient mixture (Life Technologies), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and 1% antibiotic-antimycotic mixture solution (Life Technologies). MSC were extracted by flushing the bone marrow pellets from the femurs and

tibias of 4 week old mice into the mouse MSC media. The bone marrow cells were filtered through a 70µm filter (BD, Franklin Lakes, NJ), plated as 2×10^7 cells per 9.6 cm^2 in fresh MSC media, and incubated at 37^0C in 5% CO_2. Three days later, the non-adherent cells were removed and resulting cells permitted to grow to 80% confluence at which time, they were passaged using 0.25% Trypsin (Hyclone, Logan UT), purified by the removal of contaminating CD11b+ macrophages with Miltenyi immunomagnetic selection using biotinylated antibodies to CD11b (e-Biosciences, San Diego, CA) and CD45 (e-Biosciences) and MACS anti-biotin beads (Miltenyi Biotec, Auburn, CA). Purity of all negative cells was shown via flow cytometry at <3% contamination with CD45+ cells and were replated at 1×10^6 cells per 175 cm^2 flask. Cells were grown and expanded until used in the experiments at passage 4.

C. <u>Laser therapy</u>

Low-level lasers with dual 7.5mW diodes and variable frequencies (Ercohnia, McKinney, TX) were used to deliver three different wavelengths: 635 nm (red light), 532 nm (green light) and 405 nm (violet light). Mice were randomly assigned to 9 treatment groups lasting 4 weeks each after fibrosis was established: naïve controls with sham surgery, UUO, fluorescently tagged MSC alone, administered once weekly, 2 x 10^6 cells/kg in PBS, trilaser therapy with either a second supplemental 405nm, or 532nm, or 635nm laser treatment 4 hours later with and without MSC (Table 1). Each mouse was lased centrally, near the cauda equina nerve root (L10-L12) using the 635nm wavelength pulsating laser (EML, Erchonia, McKinney, TX) at frequency 9Hz for 60 seconds from a distance of 3cm above the body surface. A rotating laser stand was constructed to administer the three wavelengths to the area of the kidney from ventral and dorsal perspectives, constant wave, 300 seconds,

bilaterally (Zerona, Erchonia, McKinney, TX). After 4 hours, a single laser treatment, specified by treatment group, was administered. Treatments were administered every other day for 4 weeks.

Table I: STUDY DESIGN

Groups	UUO	AM Laser/Trilaser Therapy			PM Laser/Monolaser Therapy			MSC
A minimum of N=3,		405nm	532nm	635nm	405nm	532nm	635nm	
Group 1	-	-	-	-	-	-	-	-
Group 2	+	-	-	-	-	-	-	-
Group 3a	+	+	+	+	-	-	+	
Group 3b	+	+	+	+	-	-	+	+
Group 4a	+	+	+	+	-	+	-	-
Group 4b	+	+	+	+		+	-	+
Group 5a	+	+	+	+	+	-	-	-
Group 5b	+	+	+	+	+	-	-	+
Group 6	+	-	-	-	-	-	-	+

D. <u>Histological measures</u>

Mice were euthanized at the end of 4 weeks of treatment on day 50 via CO2 chamber and cervical dislocation. Right kidneys were collected, weighed and preserved in 4 sections: snap frozen in liquid nitrogen, neutralized 10% formalin (Richard-Allan Scientific, Kalamazoo, MI) to be embedded in paraffin, frozen in O.C.T compound (Tissue-Tek, Torrance, CA) and RNA-later (Sigma-Aldrich, St. Louis, MO).

1. <u>Trichrome stain</u>

The paraffin embedded 3-4mm thick sections were stained with Masson's Trichrome procedure (NovaUltra Special Stain Kit, IHC World, Woodstock, MD, USA) to quantify the

amount of fibrosis occurred. Areas of cortex and medulla were hand drawn for each section and the resultant regions were digitally scanned using VECTRA Automated Quantitative Pathology imaging system with inForm software for digital quantification of fibrotic tissue.

2. Measures of MSC quantification and mitochondrial activity

Prior to euthanasia, each mouse received an intravenous injection of 1mM Mitotracker Red CMXRos solution (Molecular Probes, Eugene, OR). Presence of active mitochondria was detected by counting the immuno-fluorescently labeled cells (10 high power fields per section) using the Vectra Automated Quantitative Pathology Imaging System (PerkinElmer, Waltham, MA). The inForm software system (INFORM International) was used to digitally count the immuno-fluorescent cells based on the pixels and quantification expressed in units of positive areas. CFSE [5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester] (eBioscience, San Diego, CA, USA) was used to fluorescently tag the MSC for their tracking and proliferation studies. Results were determined by similar quantification as for mitochondria.

3. Endothelial quantification

Paraffin embedded 3-4mm thick sections were fluorescently labeled with green fluorescent CD31 Rabbit Polyclonal antibody (Thermo Scientific, Waltham, MA) to analyze and quantify the amount of endothelium present. Quantification was done as per mitochondria quantification method.

E. ELISA

TGF –β1 (Human/ Mouse TGFb1 ELISA Ready-SET-Go! Kit, eBioscience) and IL-10 (Mouse IL-10 ELISA Kit, BDOptEIA, BD Bioscience, CA, USA) were used as per manufacturer's

directions to measure renal levels of cytokine after the kidney was weighed, homogenized, and adjusted for a protein level of 100μg/ml.

F. Statistical Analysis

ANOVA was used to determine significant differences amongst the groups with EXCEL program. A p value less than 0.05 was considered significant.

III. RESULTS

A. Renal fibrosis model

Renal fibrosis following 20 days of UUO was based on the observations of reduced size of glomeruli, expansion of Bowmann's capsule and tubular architecture loss were shown in H&E stain. Renal fibrosis was following 20 days of UUO was selected based on the observations of reduced size glomeruli, expansion of Bowmann's capsule and tubular architecture loss were shown in H&E stain. At 50 days following UUO, sections of explanted kidneys were examined for gross architectural changes and fibrosis using Masson's Trichrome stain to demonstrate the collagen deposition. (Figure 1). As shown in figure 1, UUO kidneys demonstrated a destroyed medulla replaced with fibrotic tissue. Glomeruli were smaller than naïve controls. The overall architecture demonstrated a cavernous space in the area of the medulla. In contrast, animals treated with tri-laser therapy with or without MSC appeared to have improved retention of the medullary region. With increasing wavelength, there appeared to be increased amount of intact medullary epithelium, culminating with the 635nm wavelength treated groups as appearing to have the closest resemblance to naive renal architecture. Of three animals, there appeared to be a non-responder in treatment groups with and without MSC and supplemental 635nm laser. This observation was also noted in other treatment groups, demonstrating some non-responders and some animals which appeared to have some regenerative responses within the medulla.

Statistically significant reduction of collagen deposition was noted in the cortex following trilaser therapy with MSC and supplemental 635nm treatment, with a reduction of approximately, $p=0.01$. While there was a trend for reduction in the medulla with trilaser

therapy supplemented with 532nm and 635nmwith and without MSC, in this pilot study, these differences did not reach significance.

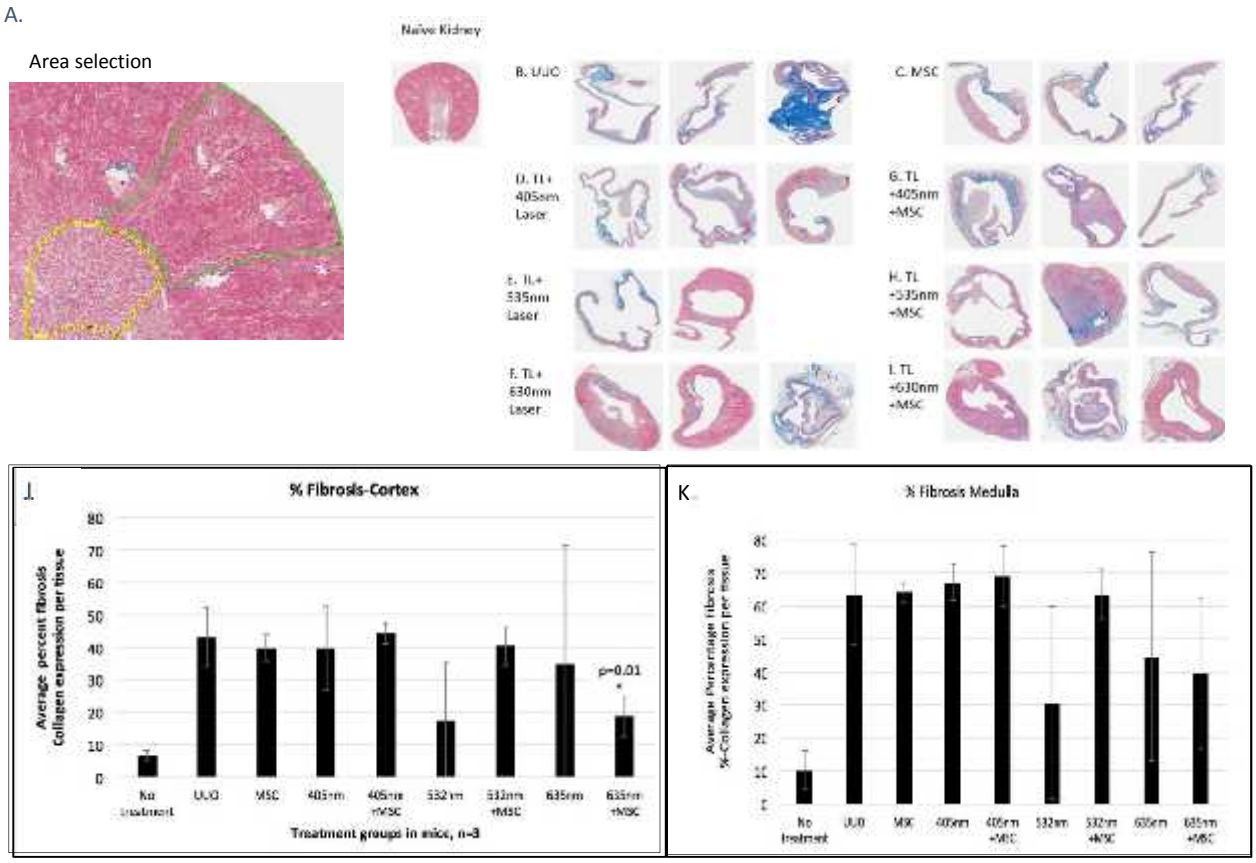

Figure 1: Fibrosis quantification: A) Naïve murine kidney demonstrating delineation between cortex and medullary regions, B) UUO, C) MSC alone, D,E,F) trilaser (TL) therapy with 405nm, 532nm, and 635nm supplemental therapies, respectively, without or G,H,I) with MSC. J) Graph of fibrosis quantification in % positive area for collagen deposit in cortex and K) medulla.

B. <u>Laser effect on mitochondrial activity:</u>

Mitochondrial activity showed improvement over the period of 4 week treatment in the trilaser plus supplemental 405nm with MSC when compared to UUO controls however it was not very

significant as p=0.08. Similarly, increased mitochondrial activity was observed in trilaser plus supplemental 532nm and MSC treatment.

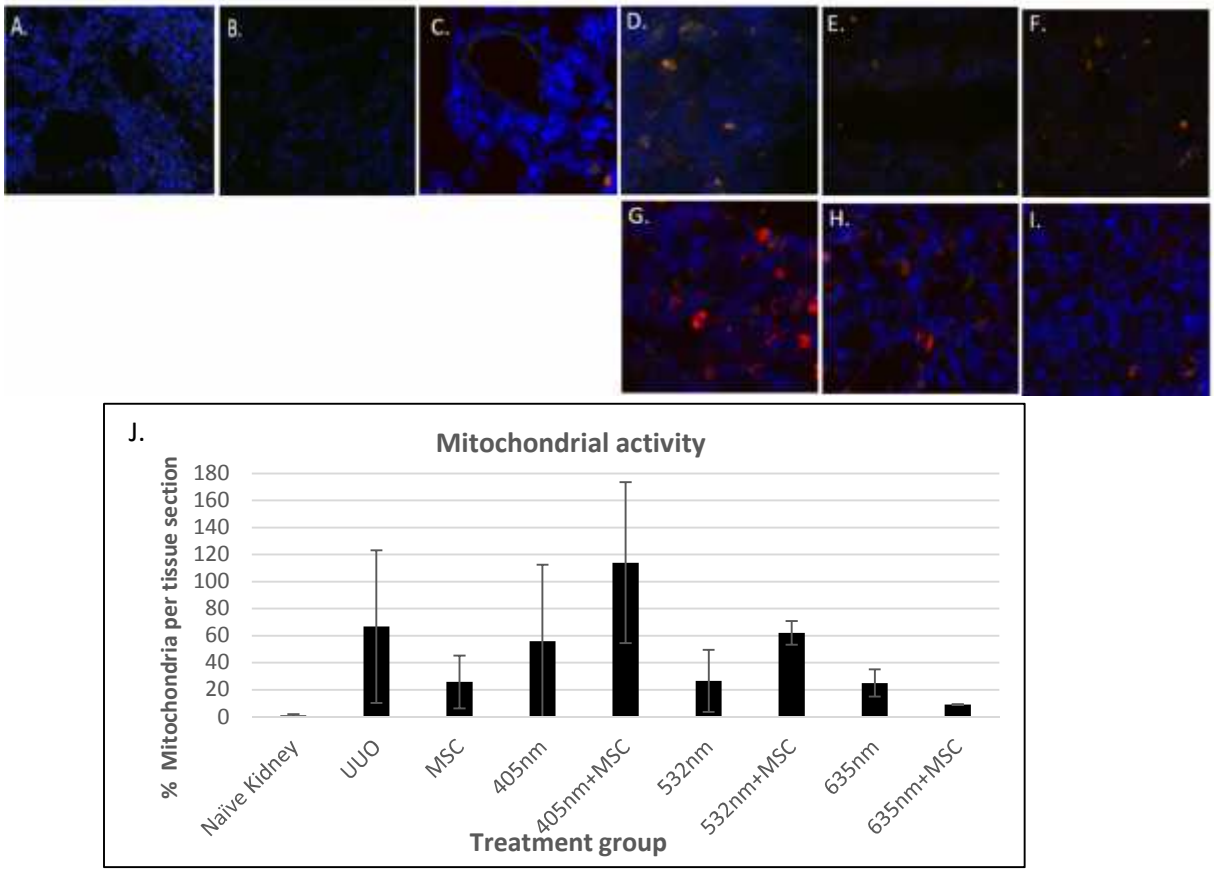

Figure 2: Fluorescent imaging of mitochondrial activation in the kidney for (A) naïve kidney, (B) fibrotic control, (C) MSC, D,E,F) trilaser (TL) therapy with 405nm, 532nm, and 635nm supplemental therapies, respectively, without or G,H,I) with MSC and (J) Results on graph with increase in % positive mitochondrial activity from UUO to TL + 405nm laser treated kidney.

C. Laser effects on Endothelium

Using digital parameters, areas of interest were divided into Cortex with glomeruli and medulla (interstitium). Comparison of naïve kidney to UUO demonstrated a statistically significant depletion of endothelium with P=0.002. There was a trend for increased

endothelial numbers in response to the 405nm laser within the kidney cortex and medulla however this did not reach significance (p =0.09, p=0.08, respectively).

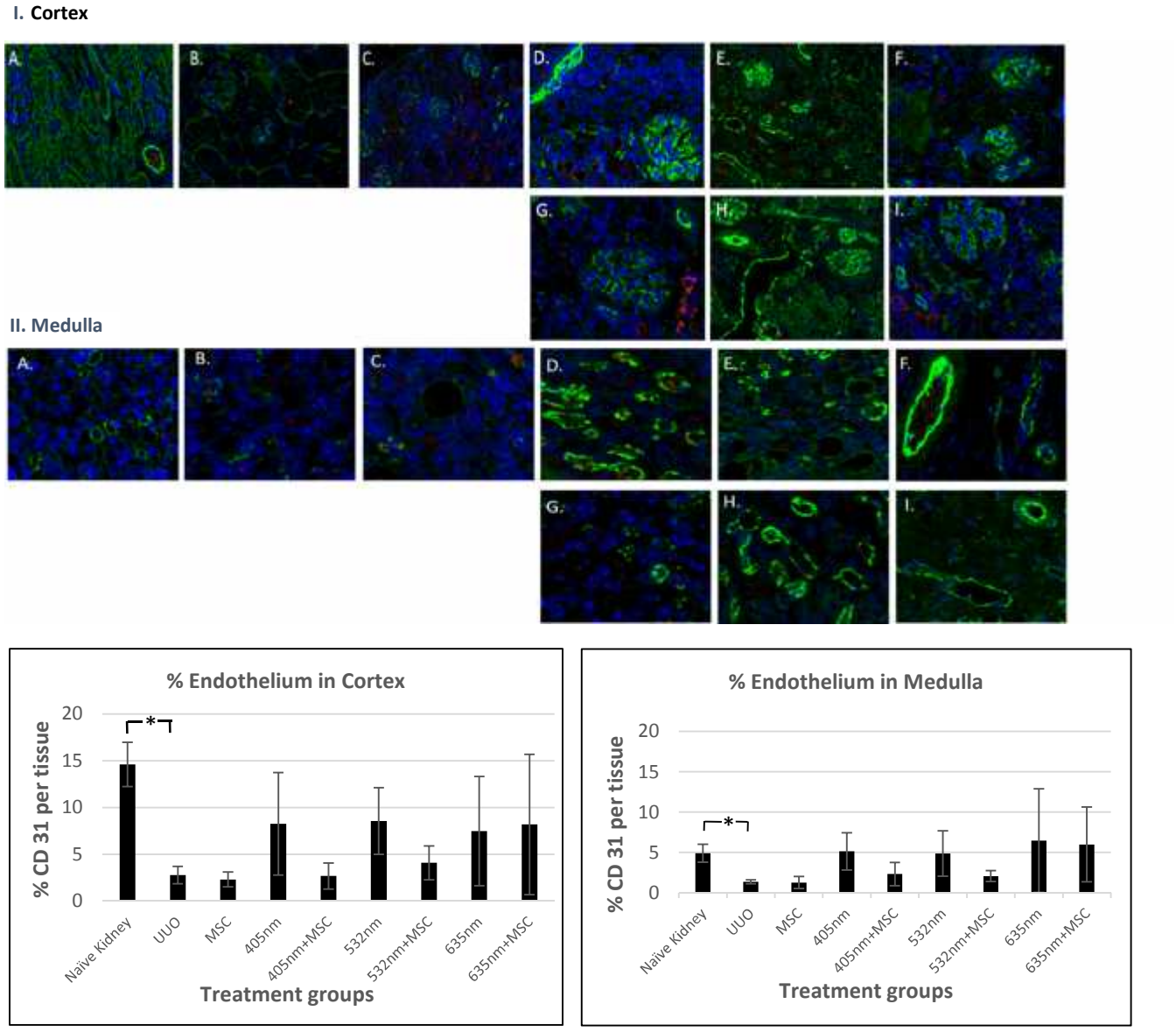

Figure 3: CD 31 staining to show endothelium within the kidney; CD31 labeled green for I. Cortex labelled (A) naïve kidney, (B) UUO, (C) MSC, D,E,F) trilaser (TL) therapy with 405nm, 532nm, and 635nm supplemental therapies, respectively, without or G,H,I) with MSC, and similarly for II. Medulla. Graph J- % positive endothelium in cortex and Graph K- % positive endothelium in medulla of the kidney, both quantified digitally via inForm software. Quantification done for 10 high power fields per tissue section of each mouse.

D. Laser effect on MSC retention

The kidneys were analyzed for the presence of CFSE labeled MSC using digital parameters to know how many MSC infiltrated the fibrotic kidney after injection. The percent area stained as positive for the fluorescent dye is depicted in figure 4 A-D. Graph of the MSC count per sections showed a trend for increased retention of MSCs within the kidney following treatment of trilaser with supplemental 532nm and trilaser with supplemental 635nm and MSC however these counts did not reach statistical significance.

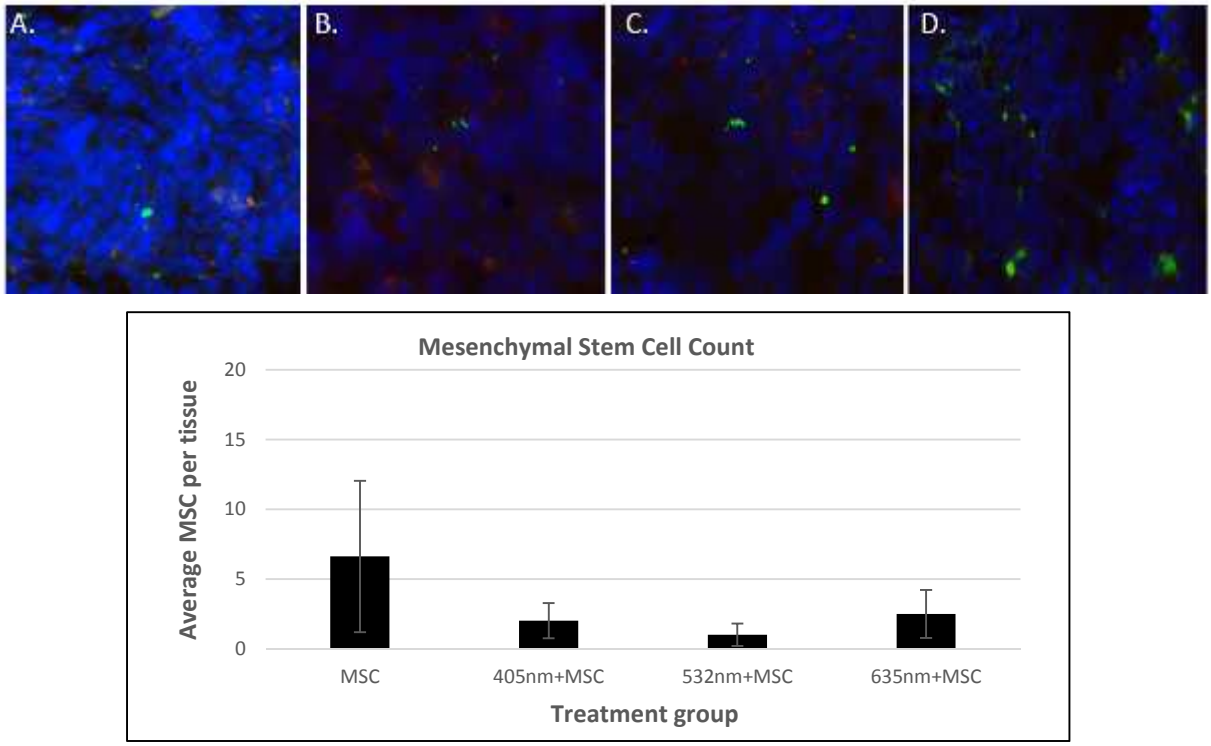

Figure 4: CFSE staining to show MSC present within the kidney; labeled green in order of treatment as (A) MSC, (B)TL + 405nm + MSC, (C) TL + 532nm + MSC, (D) TL + 635nm + MSC and € Graph representing the average %MSC per tissue section.

E. Laser effect on cytokines

As expected, we observed a significant reduction in the amount of IL-10 between UUO and naïve control, suggesting a lack of pro-regenerative type II macrophage activity. Surprisingly, there was a statistically significant decrease in IL-10 between UUO and the 635

laser groups while there was a trend for the 405nm laser to enhance IL-10. There was a significant drop in TGF beta in kidneys treated with trilaser therapy, supplemental 405nm, and MSC. We did not observe a characteristic rise in TGF beta when comparing naïve to UUO treated kidneys.

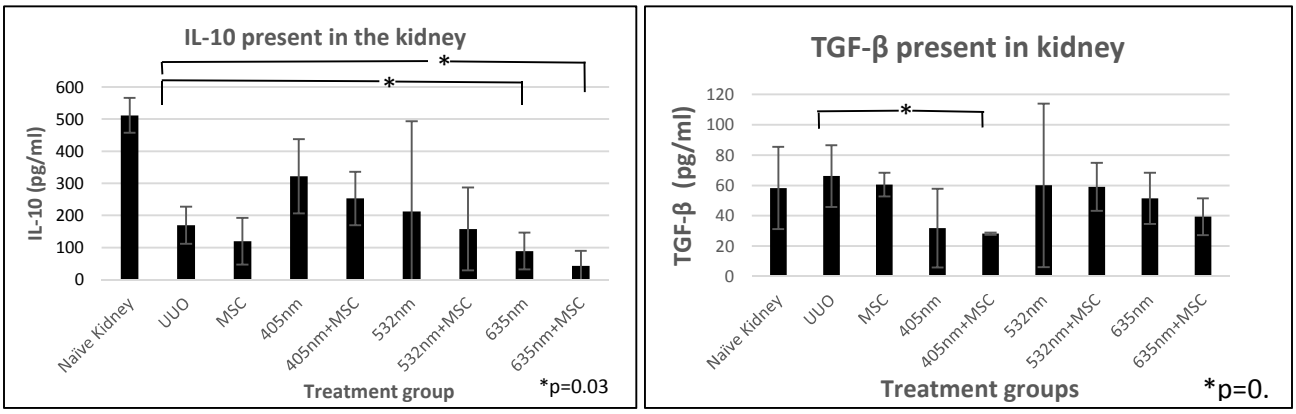

Figure 5: Mean content of IL-10 and TGF-β

IV. DISCUSSION AND CONCLUSIONS

The purpose of this study was to identify the effects of repetitive trilaser treatment in combination with supplemental singular laser treatment and MSC for its effects on the reduction of fibrosis. Secondary outcome measures targeting potential mechanisms included quantification of mitochondrial activation, quantification of endothelium, retention of MSC within the kidney, and renal cytokine levels as surrogates to measure degree of inflammatory and pro-regenerative macrophage activity. One of the initial pathological changes to occur in the beginning of fibrosis is tubular atrophy [29]. Examination of the gross architecture of the kidney 50 days after UUO revealed atretic, small glomeruli, destruction of the medulla with significant tubular atrophy and loss, replaced with dense fibrotic tissue. While all laser treated groups appeared grossly improved from UUO alone, the greatest improvement appeared to be with supplemental 635nm laser treatment with MSC. Notably, there were animals that appeared to have no response, resembling UUO without treatment. This could reflect incomplete ligature of the ureter and subsequent improved regeneration due to less drastic conditions, however the ligatures were examined at time of necropsy, and all were observed to be intact with robustly enlarged kidneys and fluid filled renal pelvises. Alternatively, these findings indicate variability in effect in living organisms which by definition, may not be as uniform as cell culture data. One possible area of variability could be the heterogeneity of MSC which may result in variable potency on macrophage phenotype switching controlled by the pro-inflammatory cytokines [30].

According to previous studies, mitochondria mostly absorb the infrared light photons to produce energy within the cell, hence a target to determine the laser effect on renal fibrosis

[31]. In congruence to this finding, our initial short-term study involving individual lasers and MSC treatment combinations demonstrated significantly increased mitochondrial activation following any of the single laser treatments [34].

With the 4 week treatment period and trilaser therapy the lower wavelength treatment groups, 405nm laser in synergy with MSC and 532nm laser alone, seemed to demonstrate sustained increased mitochondrial activity than 635nm laser (Figure 2, graph). Since these studies are the first of their kind, the significance of this finding in correlation to the histology is not known. The 405nm treated groups had less regenerative activity than the 635 laser groups; these findings may indicate the merits of sequentially utilizing different wavelengths for their specified effects to further refine the process of regenerating the kidney.

Endothelium and its retention or regeneration remains a pivotal factor in regenerative activities. The best way to discern retention due to decreased apoptosis vs new proliferative activity would be to identify proliferating and apoptotic endothelial cells. In this study, while BRDU was used to stain for CD31+ endothelial cells, there was too much non-specific staining and BRDU positive cells could not be reliably identified. Tunnel staining also was difficult to obtain reliable staining results. Instead, we identified the number of CD31+ cells. As expected, UUO led to dramatic loss of endothelium while trilaser treatment supplemented with 405nm and MSC appeared to have the greatest effect on increased endothelium. Additional studies, such as caspase 3 levels in the kidney to define apoptotic activity, will be required to determine if this effect was due to retention and reduction of apoptosis or increase in proliferation.

In regenerative activities, the macrophage has been implicated in playing a pivotal role, providing phagocytosis of tissue debris, laying down orderly matrix, and reducing pro-

inflammatory activities by secretion of IL-10[32]. We hypothesized that the number of MSC recruited to the kidney would correlate with pro-regenerative activities as evidenced by decreased fibrosis, decreased TGF beta and increased IL-10. The number of MSCs retained within the kidney appeared to be highest in the trilaser group treated with MSC and supplemental 532nm and 635 nm laser treatments when compared to the UUO group. This finding along with the histologic findings, may suggest that long-term retention of MSC within the kidney is important in changing the renal microenvironment. The presence of MSC within the kidney is not clear as the IL-10 content did not correlate with this observation in these small sample sizes. Increase in the amount of IL-10 was observed in the group of 405nm laser, 405nm laser + MSC and 532nm laser treatments indicating their role in the reduction of inflammatory effects. TGF-b content was shown to decrease significantly in the kidney when treated with 405nm laser along with MSC. This finding suggests that the 405nm may be particularly effective in reducing TGF-beta mediated collagen deposition and the observation that it did not have a significant impact on overall collagen deposition suggests that the timing of this laser may need to be altered for optimal effects on the fibrotic process.

These studies demonstrate for the first time, the ability of LLLT with MSC to have a marked improvement on the destructive fibrotic processes which occur as a consequence of UUO. These effects appear to involve endothelium, epithelium, mitochondria, and the kinetics of inflammation, suggesting a therapeutic role for this strategy in other conditions of inflammation and fibrosis. Each laser appears to have specific benefits, which can be exploited to further refine this process. Future studies will incorporate these attributes for modification

of the sequence of different wavelength therapy to modify and reverse the kinetics of renal and other types of fibrosis.

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Education

University of Illinois Chicago (UIC): Master of Science 2015

Major: Bioengineering. Specialty: Cell and Tissue Engineering

SRM University: Bachelor of Technology 2011

Major: Biotechnology

Achievements and Awards

- Participant in 98th Indian Science Congress 2010
- Organizer of AARUUSH'10 at SRM University, 2010
- Participant in International Conference on Bioengineering, 2009
- Participant in National conference on Recent Trends in Environmental Biotechnology, 2008

Projects

- Stem Cell characterization, Life Line Institute of Regenerative Medicine, 2011
- Exploration of Techniques of Protein Engineering at Torrent Pharmaceuticals Limited, 2010
- Banana Tissue Culture, Gujarat State Fertilizers & Chemicals Limited, 2009