5-2021

Quantifying mucosal hemodynamics in a murine model of Ulcerative Colitis with diffuse reflectance spectroscopy

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Quantifying Mucosal Hemodynamics in a Murine Model of Ulcerative Colitis with Diffuse Reflectance Spectroscopy

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Spring 2021

Abstract:

Ulcerative colitis (UC) is a gastrointestinal, autoimmune disease that causes ulceration and inflammation of the colon with an incidence 10 out of every 100,000 people in North America and Western Europe. Though the exact etiology is uncertain, a number of studies have shown that inflammatory cells along with environmental factors, genetics, and lifestyle habits can contribute to the sustained inflammatory response. In order to determine the cellular mechanism behind relapse and remission of UC, researchers have frequently employed immunohistochemistry, western blotting and gene sequencing, but these destructive analysis methods require the removal of a sample, necessarily limiting these methods to non-living tissues. There is an emerging interest in using non-invasive techniques to study the *in vivo*, longitudinal effects of UC on the mucosa in the colon. Here we have developed a mouse model of UC using dextran sulfate sodium and a non-invasive spectroscopy monitoring modality to study the changes in the tissue hemodynamics during active UC.

1. Background

Ulcerative colitis (UC) is an autoimmune disease that causes cyclical inflammation and ulceration of the colon.[1,2] Defects in the mucosal and epithelial barrier within the colon allow the luminal microflora to trigger an immune response[1]. Inflammatory cells such as T helper type 9 lymphocytes (T_H9) cells inhibit mucosal healing while innate lymphoid cells (ILCs) contribute to cytokine production such as interleukin-12 (IL-17) which drives mucosal inflammation.[1] The causes of barrier defects are not fully understood, but genetics, environmental factors, and lifestyle habits are thought to play a role.[1,2] There is emerging interest in using emerging endoscopic optical imaging and spectroscopy methods, along with molecular biology tools, that may be able to examine the mechanisms behind relapse and remission of UC, as well as identify putative therapeutic options.

UC affects about 8 to 14 out of every 100,000 people in North America and Western Europe and is increasing in prevalence.[2,3] UC is a chronic disease, involving many cycles of intestinal inflammation followed by a remission period.[1,2] The gastrointestinal (GI) tract has a unique oxygenation profile. Even at

the baseline, epithelial cells that line the mucosa exist in a low-oxygen environment.[4] Countercurrent oxygen exchange mechanisms show that oxygen from the arterial blood supply diffuses to adjacent venules, along the crypt villus axis, results in graded levels of low oxygen.[4] Steep oxygen gradients have also been documented in the more distal, colonic portions of the GI tract.[4] Based on this, the normal colonic mucosa has a reduced O_2 potential pressure (P_{O2} of approximately 10 mmHg).[4] Interestingly, the mucosa of an animal or human with active inflammation has been shown to be even more hypoxic.[5] The physiology behind the change in oxygen saturation is still debated, but some studies have pointed to a decrease in blood supply from major arteries that supply the rectum as a cause.[5,6] Mucosal hemoglobin concentration has been observed to increase during active colitis, most likely due to mucosal congestion caused by increased levels of proinflammatory chemical mediators such as prostaglandin and leukotrienes.[5,7]

Several murine models of UC have been explored, both chemically induced and through genetic modification. Some common chemically induced models include trinitrobenzene sulfonic acid colitis (TNBS), oxazolone colitis, and dextran sulfate sodium (DSS) colitis, while Interleukin-10 (IL-10) knockout mice can be used as a transgenic model.[8,9] DSS is a sulfated polysaccharide salt that can be dissolved in the animals' drinking water. It is believed to be directly toxic to epithelial cells and results in the breakdown of the intestinal lining.[8,9] This allows bacteria and other luminal organisms to enter the lamina propria, which triggers the body's immune response.[9] Short term exposure (usually five days to a week) in a small animal causes inflammation and ulcer formation.[8,10] The intensity of inflammation can be controlled by the concentration of DSS solution, although individual mouse strains can differ significantly from each other in terms of resistance.[10,11] For example, BALB/C mice are very resistant to DSS-induced inflammation while C3H/HeJBir mice are extremely susceptible[10,11]. Susceptibility may also differ between locations of the GI tract. Mice strains such as C57BL6/J are resistant to DSS in the cecum, but susceptible in the colon.[11] This is a fairly simple and reproducible model to induce ulcerative colitis, however this model is very expensive. In this study, C57BL6/J mice (Jackson Labs) were given

4% DSS (w/v) water solution for 5 days *ad libitum* then 10 days of recovery. The purpose of the recovery period was to simulate the "active" and "remission" cycles of UC in a murine model.

In the clinic, diagnosis and monitoring of UC is commonly done with the help of conventional white light endoscopic inspection, but currently there is no widespread clinical use of high-resolution microendoscopic imaging or spectroscopy techniques.[5] Microendoscopic modalities use optical fibers or fiber bundles in conjunction with microscopy techniques to obtain microscopic images of tissues. This technique can also be used in conjunction with spectroscopy techniques, such as diffuse reflectance spectroscopy, to quantify longitudinal tissue hemodynamics such as oxygen saturation and total hemoglobin. Diffuse reflectance spectroscopy (DRS) is a non-invasive, spectral biopsy technique that is used to estimate optical properties of tissues. The fundamental tissue optical properties are reduced tissue scattering coefficient (μ_s') and the absorption coefficient (μ_a).[12] The reduced scattering coefficient depends on light scattering from cell nuclei, keratin (in skin), lipid membranes of cells and organelles, and collagenous, elastic, and reticular fibers.[13] The absorption coefficient depends on hemoglobin concentration and oxygen saturation.[12] In tissues containing little or no melanin (amelanotic), μ_a in the visible and near infrared spectral ranges functionally depends on the oxygen saturation and total hemoglobin content.[12] Changes in these fundamental optical properties has been shown to occur in epithelial cells with altered morphology.[12]

In the C57 mice, developing DSS ulcers were monitored during "active" and "remission" stages using a commercial colonoscope unit (Karl Storz COLOView) was used in conjunction with a 1 mm diameter multimodal DRS probe.[14] This probe was deployed through a biopsy port of the examination sheath of the COLOView system and touched to the colonic mucosa to obtain spectra.[14] The DRS spectra were then analyzed to obtain measurements for oxygenation, total hemoglobin content, deoxyhemoglobin, and oxyhemoglobin.

2. Materials and Methods

2.1 Animal Studies: DSS model

Eight-week-old female C57BL6/J mice (000664 The Jackson Laboratory, Maine) were randomly separated into two groups: control (n=5) and treatment (n=9). After a week of acclimation to animal facilities, the treatment mice received 4% DSS water *ad libitum* for 5 days. The water was changed on the third day. The control mice received regular tap water *ad libitum.* After the 5-day cycle was completed, the treatment group received regular tap water for 10 days before the cycle was repeated (Figure 1).

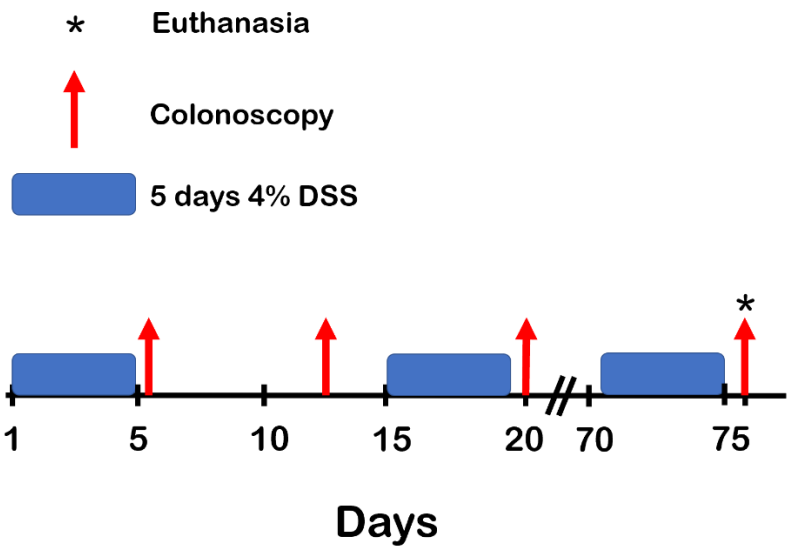

Figure 1. *Sample Timeline of DSS administration with colonoscopies. Control mice are scoped every week without any DSS. DSS treated mice receive 5 days of 4% DSS with a 10-day recovery period between each cycle. This simulates the "active" and "remission" cycling of UC. DSS was administered for 12 weeks and the final colonoscopy and euthanasia occurred on day 76.*

There were six five-day DSS cycles that were completed in about 12 weeks. All mice were weighed every day around the same time in the morning. Mice in the treatment group lost weight at around day 4 or 5 of each treatment cycle. Cages with mice that lost 10% of their pre-DSS weight were supplemented with NutraGel. The longitudinal changes in raw weight of the DSS and control groups are shown in Figure 2.

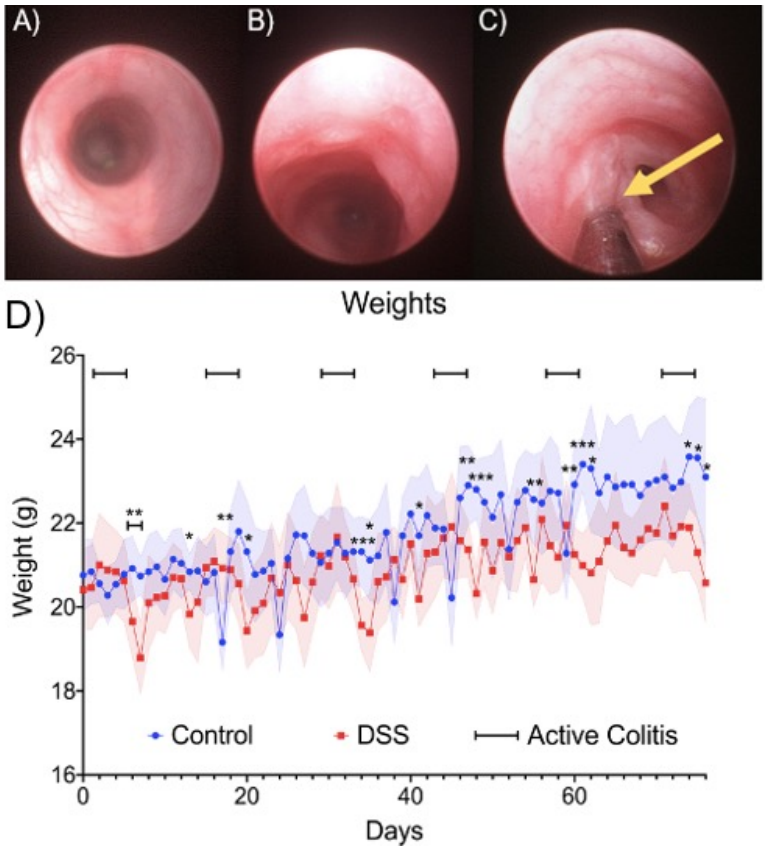

Figure 2. *A) Normal colon mucosa. B) DSS-induced ulcer in the mucosa. C) DRS probe making contact with an ulcer. D) Longitudinal weight changes from C57BL/6J control (n =5) and DSS induced mice (n = 9). Raw weight plots were made in GraphPad Prism©. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$, Mann-Whitney test)*

2.2 Animal Studies: Endoscopic Examination

A commercial colonoscope unit (Karl Storz COLOView) was used in conjunction with a miniaturized, multimodal DRS probe to monitor the development of ulcers in the distal colon.[14] The probe was deployed through the biopsy port of the examination sheath of the COLOView system (Figure 3). The DRS acquisition has been described elsewhere.[14] A shutter was used to block white light from the colonoscopy system while obtaining spectroscopic measurements.

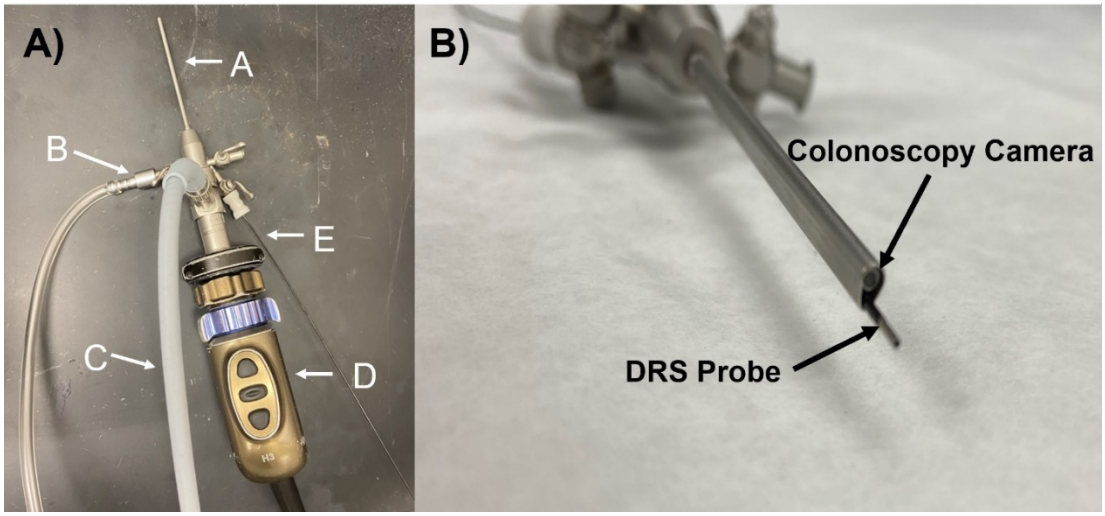

Figure 3. *A) DRS Probe and Endoscope Setup: A – examination sheath B - Air Pump Hose C – Optical fiber D – Camera E – 1mm DRS probe. B) Examination sheath close-up*

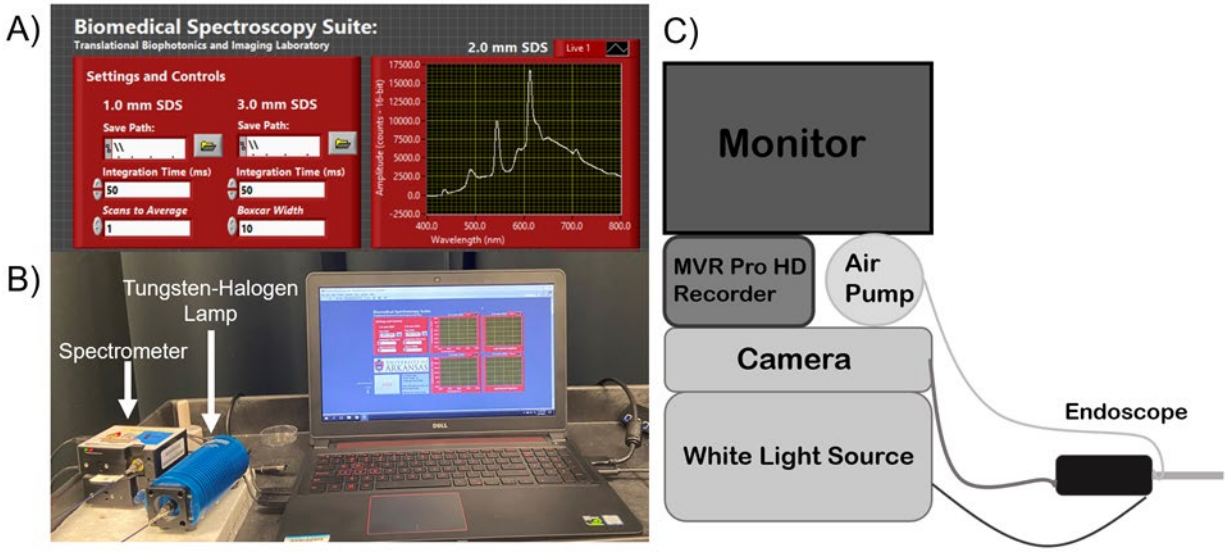

Figure 4. *Spectroscopy and Endoscopy Setup. A) LabView Software and DRS spectra. B) Laptop and probe connections to Ocean Optics spectrometer and Tungsten-Halogen lamp. C) Diagram of Karl Storz ColoView system setup*

Colonoscopies and DRS acquisition took place every week for the first 8 weeks of DSS administration. DSS-treated mice were scoped on the day after the 4% DSS cycle was stopped and then again a week later to monitor any changes in the "active" and "remission" stages. The control mice were also scoped once

every week. The mice were anesthetized at 2.5% isoflurane and underwent colonoscopies to visually monitor developing ulcers and obtain DRS data from the submucosa in the distal colon. The probe was extended to touch the mucosa and the colonoscopy system light was blocked. About 10 DRS spectra were taken at a time. The position of the probe was checked in between each acquisition time by briefly allowing the shutter to open. The process completed until around 100 spectra were collected from the same location. The spectra were collected between 400 and 800 nm at an integration time of 50 ms and a boxcar width (number of pixels to the left and right of individual pixel averaged together) of 10 (Figure 4A).

2.3 DRS Spectral Analysis

Once the spectral data was collected, they were placed into a filtering algorithm (Figure 5) to discard data that had displacement artifacts to avoid biasing of the DRS data. First, if the spectra reflectance was above 0.05 or below 0.01 at 475 nm, it was discarded. Then, the Qratio is calculated.[14] The Q ratio is defined as the ratio between the scattering region at 630 and 575 nm and the second Q band of hemoglobin per each optimal *ex-vivo* spectra defined as

$$Q_{ratio}(i) = \frac{R_{\lambda s(i)}}{R_{\lambda Q(i)}}$$

Where i denotes each recorded reflectance in the 475 to 685 nm range and $R_{\lambda s(i)}$, $R_{\lambda Q(i)}$ are the values of the i'th reflectance at 630 and 575 nm, respectively. The Qratio was calculated for all the *ex-vivo* spectra with different displacement/angular variations that can occur during an endoscopy. If the Qratio was outside the range of previously calculated mean *in vivo* value (< 2.20 and > 3.35), this value would be discarded.[14] Finally, a confidence interval was created for any remaining spectra by calculating the mean and standard deviation (SD) across all wavelengths. Any spectra outside the mean ±1 SD were discarded. Values for oxygen saturation, total hemoglobin, oxyhemoglobin and deoxyhemoglobin were then calculated.[14]

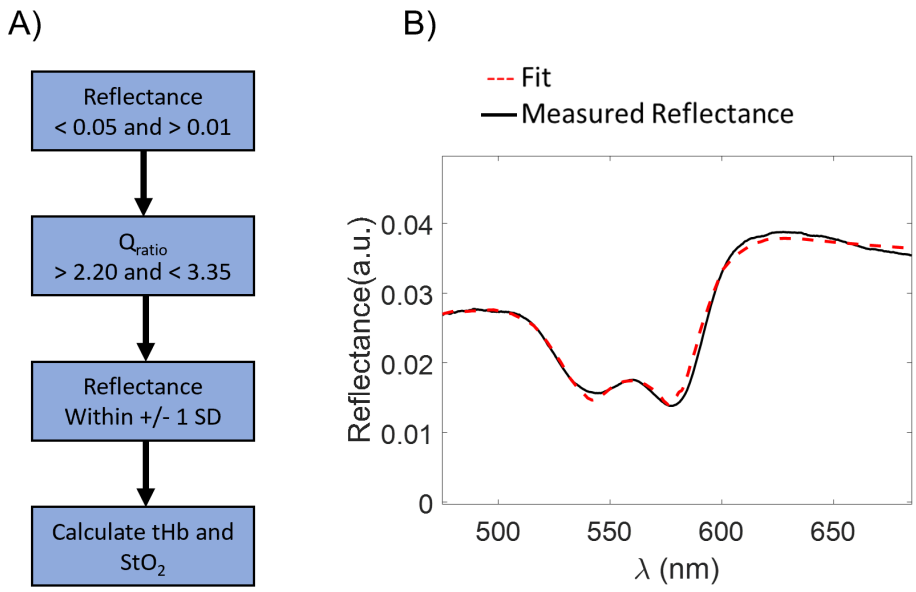

Figure 5. *A) Flowchart used for filtering spectra. B) A spectrum obtained from the distal colon mucosa. The line of best fit (red) generated by MATLAB.*

A Generalized Additive Model (GAM) was used to analyze the DRS-derived perfusion values because the data was not following a linear trend and several observations were missing throughout the course of the study.[15] Repeated measures analysis of variance (rm-ANOVA) is commonly used in biomedical data analysis but assumes complete observations and linearity. Any mouse with incomplete observations would have to be removed from the analysis, which would negatively impact the already small sample size and any important measurements that would contribute to the differences between groups would be lost. GAMs work by using a *basis function* (such as a cubic spline) to approximate the temporal non-linear variations in the data.[16] GAMs do not output a *p-value* that can be used to determine significance, but the confidence intervals can be compared to the smooth to see if treatment has a significant effect. The difference between the smooths can if there are any changes in the parameter at a particular point in time. If two trend lines follow the same pattern, their difference will be zero, thus implying that the treatment has had no significant effect on the parameter. If the difference is not zero at some time point, the treatment is causing a change

in the response. For this study, GAMs were used to compare oxygenation (StO_2), total hemoglobin (tHb), deoxyhemoglobin (dHb), and oxyhemoglobin (HbO_2) values across control and DSS-treated mice. Scatterplots with the raw values of each parameter were overlaid with the trend line calculated by the GAM (Figure 6). There were different smooths observed for oxygenation, deoxyhemoglobin, and oxyhemoglobin, but there was no difference in the trends between total hemoglobin. Intervals at 95% confidence were constructed for each smooth and plotted separately (Figure 7).

3. Results

Oxygenation trends downwards over time for the control mice while the DSS-treated mice experienced an overall increase in trend. Comparison with the confidence interval shows that the biggest difference between the groups occurred at Week 2 and near Week 7, where a pair-wise comparison between the confidence intervals of the smooth was close to zero (Figure 7).

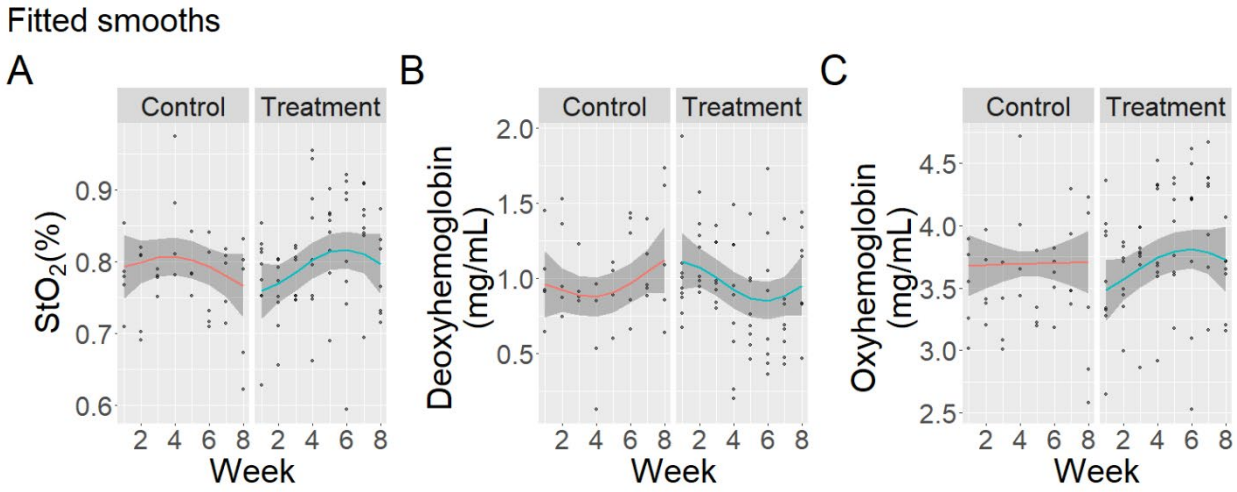

Figure 6: *GAM trend line overlays scatterplot of raw data for A) Oxygenation, B) Deoxyhemoglobin, and C) Oxyhemoglobin. Total hemoglobin did not show any difference between trends, so it is not included here.*

Control mice experienced a slight decrease then increase in deoxyhemoglobin levels while the DSS mice saw a steady decline in dHb until Week 6, when it began to increase. The corresponding confidence interval shows that the greatest difference between the two groups occurred around Weeks 2 and 7, where the upper

and lower limits of the interval are close to zero. Finally, HbO_2 remains constant for the control mice, but DSS mice experienced a steady increase in oxyhemoglobin until Weeks 5 and 6, when it began to taper off. The confidence interval shows that the greatest difference between the groups occurred at around Week 5. All statistical analysis was performed using R (version 1.3.1093)[17], using the packages mgcv[16] and ggplot2 (see Appendix A).[18]

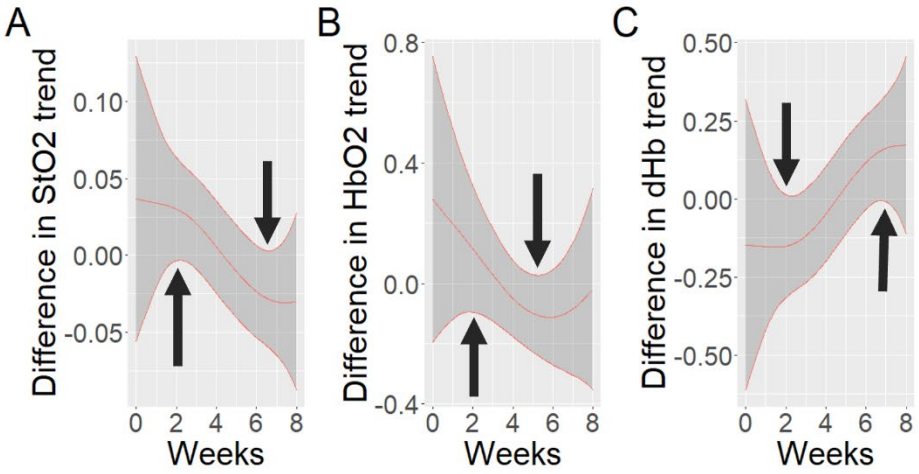

Figure 7: *Confidence Intervals for the smooth curves at the 95% significance level. A) Oxygenation, B) Oxyhemoglobin, and C) Deoxyhemoglobin trend differences across 8 weeks of treatment. Arrows indicate where the confidence level limits almost cross zero meaning that there would be a significant difference between the two groups at that time point.*

4. Discussion

The study found that oxygenation in DSS-treated mice increased over time while the control mice experienced a decrease in oxygenation. The trend in total hemoglobin content did not differ between control and treatment group although there were different trends in deoxyhemoglobin and oxyhemoglobin. As previously mentioned, several studies attest that tissue oxygenation tends to decrease during active colitis

while total hemoglobin content is increased.[5,7] There may have been a combination of several factors that contributed to the result of this study. DSS-induced colitis does not replicate the exact pathophysiology of human UC; it causes inflammation and ulceration by chemical means.[8-11] It may be possible that the difference in symptom mechanism led to a change in the oxygenation or hemoglobin trends. Furthermore, the use of DRS in animal or human UC models is not common in biomedical research, so it can be difficult to compare the results of other studies with different technologies. For future studies, DRS could be employed in alternative colitis models to see if the underlying inflammation mechanism has a significant impact on perfusion values. TNBS colitis is another common murine model, as well as IL-10 knockout mice.[9,10] These methods induce colitis in other ways and may provide better models for human UC.

The positioning of the DRS probe itself while touching the mucosa can also have an impact on the spectra obtained.[14]Although great care was taken to make complete contact with the same section of tissue, some variability will always be present due to user handling and the natural motion artifacts, such as breathing. The filtering algorithm accounts for most variation, but the threshold levels used to filter DRS spectra may need to change depending on mouse species and type of colitis model. As more spectra are collected from a model, it would be prudent to ensure that the threshold values accept most spectra without including anomalies or poor fits. This would be done by extracting the Q-ratio values from each data set and checking the average maximum and minimum before adjusting the second threshold. This step should be examined further in future studies.

Despite that a significant difference between the two groups for all parameters cannot be seen, a difference in trend was observed for three out of four parameters. The small size of the study can obviously be pointed out as a factor. Based off the observed trends, it is likely that further studies with an increased number of subjects would lead to significant differences between control and DSS mice. Once the measurement of physiological parameters with DRS has been sufficiently established for a DSS model of UC, another treatment group may be added to examine the effect of an immunotherapy such as anti-TNF-α on mucosal hemodynamics.[18]

5. Conclusion

This study describes the use of a multimodal DRS probe in conjunction with a veterinary endoscope to measure physiological values in the colonic submucosa of a murine model of Ulcerative Colitis. Colitis was chemically induced with dextran sulfate sodium salt in nine mice. The hemodynamic values were obtained longitudinally for 8 weeks in both DSS treated mice and control mice. The data was analyzed using a Generalized Additive Model and noticeable, though not significant, trends were observed for tissue oxygenation, deoxyhemoglobin, and oxyhemoglobin concentration. The small sample size of the study and the high variability due to the nature of UC symptoms and DRS probe use are factors that may be addressed in future to obtain more statistically significant data. These methodologies may also be used to examine treatments of UC or other types of chronic inflammation.

Acknowledgements

This material is based on work supported by the National Science Foundation (CBET 1751554), the Arkansas Biosciences Institute, and the University of Arkansas Honors College. Special thanks to Dr. Muldoon and the graduate students who often provided guidance and assistance with the colonoscopies.

Competing Interests

The authors declare that there are no conflicts of interests or competing interests related to this article.

Ethics

The study was approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC # 18091, 18093)

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Appendix

(A) R code used for the Generalized Additive Model

```
## This code fits GAMs to different DRS-derived endoscopic perfusion markers.
# Ariel Mundo, Department of Biomedical Engineering, University of Arkansas,
2021.

#loading packages
library(ggplot2)
library(tidyverse)
library(mgcv)
library(ggsci)
library(gratia)
library(patchwork)
library(svglite)

dat<-read.csv("GAM data_weeks.csv") #read the data

#create new column for the Treatment and Control Groups (for plotting
purposes)
dat<-dat %>%

mutate(General_Group=factor(ifelse(grepl("C",Group),"Control","Treatment")))

#create scatter plots for each variable
#StO2
a1<-ggplot(data=dat,aes(x=Week, y=StO2))+
  geom_point(aes(color=General_Group))+
  labs(y=expression(paste(StO[2],'(%)')))+
  scale_color_aaas()+
  theme_classic()+
  facet_wrap(~General_Group)


#tHb
a2<-ggplot(data=dat,aes(x=Week, y=tHb))+
  geom_point(aes(color=General_Group))+
  labs(y=expression(paste(tHb,' (mg/mL)')))+
  scale_color_aaas()+
  theme_classic()+
  facet_wrap(~General_Group)

#HbO2
a3<-ggplot(data=dat,aes(x=Week, y=HbO2))+
  geom_point(aes(color=General_Group))+
  labs(y=expression(paste(HbO[2],' (mg/mL)')))+
  scale_color_aaas()+
  theme_classic()+
  facet_wrap(~General_Group)

#dHb
a4<-ggplot(data=dat,aes(x=Week, y=dHb))+
  geom_point(aes(color=General_Group))+
  labs(y=expression(paste(dHb,' (mg/mL)')))+
  scale_color_aaas()+
```

```
  theme_classic()+
  facet_wrap(~General_Group)

#plot all graphs together
(a1+a2)/(a3+a4)+plot_layout(guides="collect") #plotting all together


#############This section fits the GAM Models#############################
#########################################################################

#Fit model for StO2, by time. Different smooth by group
StO2_GAM<-gam(StO2~s(Week,by=General_Group,k=7),data=dat)
plot(StO2_GAM)
appraise(StO2_GAM)

#Model for tHb by time. Different smooth by group
tHb_GAM<-gam(tHb~s(Week,by=General_Group,k=7),data=dat)
plot(tHb_GAM)
appraise(tHb_GAM)

#Model for HbO2, by time. Different smooth by group
HbO2_GAM<-gam(HbO2~s(Week,by=General_Group,k=7),data=dat)
plot(HbO2_GAM)
appraise(HbO2_GAM)

#Model for dHb,  by time. Different smooth by group
dHb_GAM<-gam(dHb~s(Week,by=General_Group,k=7),data=dat)
plot(dHb_GAM)
appraise(dHb_GAM)

#Create grid of points to use the previously created model and obtain
estimates from the
#previously fitted GAM models

#StO2
StO2_GAM_pred<-
with(dat,expand.grid(StO2=seq(min(StO2),max(StO2),length=400),General_Group=l
evels(General_Group),Week=Week))

StO2_GAM_pred<-cbind(StO2_GAM_pred,predict(StO2_GAM,StO2_GAM_pred,se.fit =
TRUE,type='response'))

#dHb
dHb_GAM_pred<-
with(dat,expand.grid(dHb=seq(min(dHb),max(dHb),length=400),General_Group=leve
ls(General_Group),Week=Week))

dHb_GAM_pred<-cbind(dHb_GAM_pred,predict(dHb_GAM,dHb_GAM_pred,se.fit =
TRUE,type='response'))

#HbO2

HbO2_GAM_pred<-
with(dat,expand.grid(HbO2=seq(min(HbO2),max(HbO2),length=400),General_Group=l
evels(General_Group),Week=Week))
```

```
HbO2_GAM_pred<-cbind(HbO2_GAM_pred,predict(HbO2_GAM,dHb_GAM_pred,se.fit =
TRUE,type='response'))



#Creating the plots
txt<-20 #this controls the text size

pr1<-ggplot(data=dat, aes(x=Week, y=StO2, group=General_Group)) +
  facet_wrap(~General_Group) +
  geom_point(colour='black',size=1,alpha=0.5)+
  geom_ribbon(aes(ymin=(fit - 2*se.fit), ymax=(fit + 2*se.fit), x=Week),
              data=StO2_GAM_pred, alpha=0.3,
              inherit.aes=FALSE) +
  geom_line(aes(y=fit,color=factor(General_Group)),
size=1,data=StO2_GAM_pred,show.legend = FALSE) +
  theme(text=element_text(size=txt))+theme(
    strip.background = element_blank(),
    strip.text.x = element_blank())+

#scale_color_manual(values=c(map_colors(15),map_colors(15),map_colors(15)))+

scale_x_continuous(breaks=seq(0,8,2))+labs(y=expression(paste(StO[2],'(%)')))



pr2<-ggplot(data=dat, aes(x=Week, y=dHb, group=General_Group)) +
  facet_wrap(~General_Group) +
  geom_point(colour='black',size=1,alpha=0.5)+
  geom_ribbon(aes(ymin=(fit - 2*se.fit), ymax=(fit + 2*se.fit), x=Week),
              data=dHb_GAM_pred, alpha=0.3,
              inherit.aes=FALSE) +
  geom_line(aes(y=fit,color=factor(General_Group)),
size=1,data=dHb_GAM_pred,show.legend = FALSE) +
  theme(text=element_text(size=txt))+theme(
    strip.background = element_blank(),
    strip.text.x = element_blank())+

#scale_color_manual(values=c(map_colors(15),map_colors(15),map_colors(15)))+
  scale_x_continuous(breaks=seq(0,8,2))+labs(y='dHb \n (mg/mL)')

#####


pr3<-ggplot(data=dat, aes(x=Week, y=HbO2, group=General_Group)) +
  facet_wrap(~General_Group) +
  geom_point(colour='black',size=1,alpha=0.5)+
  geom_ribbon(aes(ymin=(fit - 2*se.fit), ymax=(fit + 2*se.fit), x=Week),
              data=HbO2_GAM_pred, alpha=0.3,
              inherit.aes=FALSE) +
  geom_line(aes(y=fit,color=factor(General_Group)),
size=1,data=HbO2_GAM_pred,show.legend = TRUE) +
  theme(text=element_text(size=txt))+
  theme(
    strip.background = element_blank(),
    strip.text.x = element_blank(),
    legend.title = element_blank(),
```

```
    legend.position="bottom"
  )+
  guides(colour = guide_legend(override.aes = list(size=10)))+

#scale_color_manual(values=c(map_colors(15),map_colors(15),map_colors(15)))+
  scale_x_continuous(breaks=seq(0,8,2))+labs(y=expression(atop(paste(HbO[2]),
'(mg/mL)')))


###

#plotting them together

fig1<-pr1+pr2+pr3+plot_layout(guides="collect")+
  plot_annotation(
    tag_levels='A') & theme(legend.position = 'bottom')
fig1
ggsave("Smooths for poster.svg",width =28 ,height =8 )


#####################Pair-wise comparisons#####################################
###############################################################################

#creating grid of points of Day to evaluate smooths for timewise comparisons
pdat <- expand.grid(Week = seq(0, 8, length = 400),
                    General_Group = c('Treatment', 'Control'))

smooth_diff <- function(model, newdata, f1, f2, alpha = 0.05,
                        unconditional = FALSE) {
  xp <- predict(model, newdata = newdata, type = 'lpmatrix')
  c1 <- grepl(f1, colnames(xp))
  c2 <- grepl(f2, colnames(xp))
  #r1 <- newdata[[var]] == f1
  #r2 <- newdata[[var]] == f2
  r1 <- with(newdata, General_Group == f1)
  r2 <- with(newdata, General_Group == f2)


  ## difference rows of xp for data from comparison
  X <- xp[r1, ] - xp[r2, ]
  ## zero out cols of X related to splines for other lochs
  X[, ! (c1 | c2)] <- 0
  ## zero out the parametric cols
  X[, !grepl('^s\\(', colnames(xp))] <- 0
  dif <- X %*% coef(model)
  se <- sqrt(rowSums((X %*% vcov(model, unconditional = unconditional)) * X))
  crit <- qt(alpha/2, df.residual(model), lower.tail = FALSE)
  upr <- dif + (crit * se)
  lwr <- dif - (crit * se)
  data.frame(pair = paste(f1, f2, sep = '-'),
             diff = dif,
             se = se,
             upper = upr,
             lower = lwr)
}

#Create objects of pairwise comparisons
```

```
comp1<-smooth_diff(StO2_GAM,pdat,'Control','Treatment')
comp_StO2 <- cbind(Week = seq(0, 8, length = 400),
                   rbind(comp1))


c1<-ggplot(comp_StO2, aes(x = Week, y = diff, group = pair,color=pair)) +
  geom_ribbon(aes(ymin = lower, ymax = upper), alpha = 0.2) + geom_line() +
  facet_wrap(~ pair) + theme(
    strip.background = element_blank(),
    strip.text.x = element_blank()
  )+ theme(text=element_text(size=txt))+
  labs(x ='Weeks', y = expression(paste(StO[2])))


comp2<-smooth_diff(HbO2_GAM,pdat,'Control','Treatment')
comp_HbO2 <- cbind(Week = seq(0, 8, length = 400),
                   rbind(comp2))


c2<-ggplot(comp_HbO2, aes(x = Week, y = diff, group = pair,color=pair)) +
  geom_ribbon(aes(ymin = lower, ymax = upper), alpha = 0.2) + geom_line() +
  facet_wrap(~ pair) +theme(
    strip.background = element_blank(),
    strip.text.x = element_blank()
  )+theme(text=element_text(size=txt))+
  labs(x = 'Weeks', y = expression(paste(HbO[2])))



comp3<-smooth_diff(dHb_GAM,pdat,'Control','Treatment')

comp_dHb <- cbind(Week = seq(0, 8, length = 400),
                  rbind(comp3))


c3<-ggplot(comp_dHb, aes(x = Week, y = diff, group = pair,color=pair)) +
  geom_ribbon(aes(ymin = lower, ymax = upper), alpha = 0.2) + geom_line() +
  facet_wrap(~ pair) +theme(
    strip.background = element_blank(),
    strip.text.x = element_blank()
  )+theme(text=element_text(size=txt))+
  labs(x = 'Weeks', y = 'dHb')

#plot the results
fig2<-c1+c2+c3+plot_layout(guides="collect")+plot_annotation(tag_levels =
'A')


#save plots
ggsave("Confidence Intervals for poster.svg",width =30 ,height =10 )
```