Title

Effects of Hb-O2 Affinity Modulation and Exogenous Estrogen on Hypoxia and Septic Shock in a Mouse Model

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Effects of Hb-O_2 Affinity Modulation and Exogenous Estrogen on Hypoxia and Septic Shock in a Mouse Model

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

Allyn Matthew Eaker

Committee in charge:

Professor Pedro Cabrales, Chair

Professor Brian Aguado

Professor Lingyan Shi

2021

The thesis of Allyn Matthew Eaker is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

In recognition of all the help given to me,
all of my mentors and peers,
all of the guidance that has helped shape who I am,
I would like to dedicate this thesis to:

my loving family
Paul, Bernice, Carissa, and Joey
for supporting me
through all of my education

all my beautiful friends
both that I made
and that I kept
through college
whose words, love, and care
I could always
and can always
rely on

my amazing senior design group
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for all the hard work, studying, stress,
kindness, support, and jokes
that we all shared

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Christian, Andrew, Quoc
Peter, and Noelle
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for sharing, teaching, and laughing

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no matter how long it's been
and brighten my day
whenever I see them

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for welcoming me
and introducing me
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and warmly, patiently
teaching me

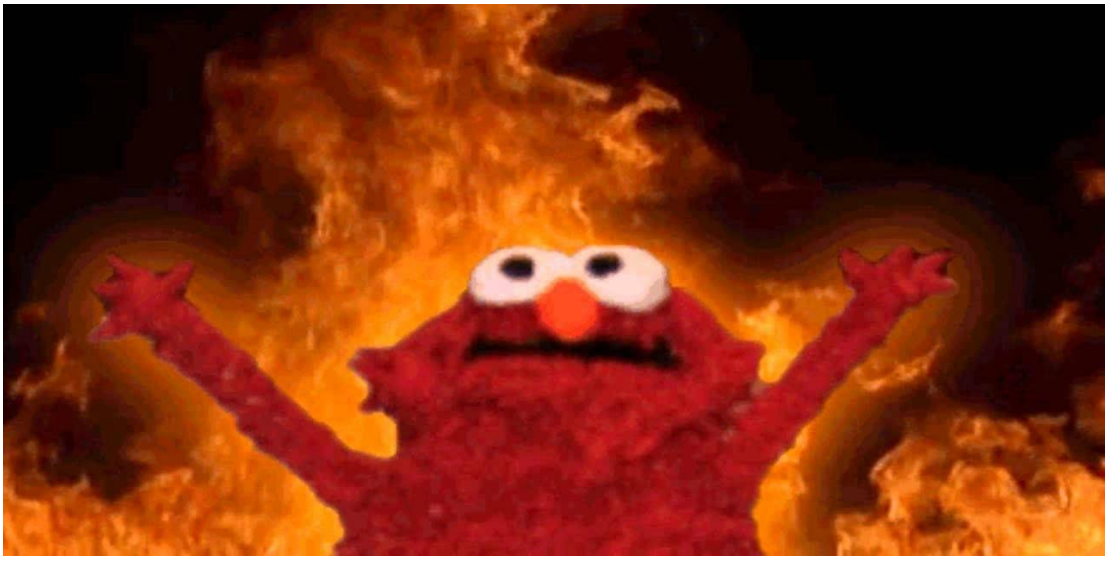
my mentors,
training partners,
and peers
in a most warm, and loving
jiujitsu, wrestling, and grappling
community

and lastly,

for always making me smile

Fire Elmo

(Elmo Rise)



Thank you all

for everything

for all of the kindness, love, support

and for helping me smile

as much as I can

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LIST OF ABBREVIATIONS

Hb.....hemoglobin

O_2 .....oxygen

SCD.....sickle cell disease

RBC.....red blood cell

GBT.....Global Blood Therapeutics, a biopharmaceutical company

pO_2 .....partial pressure of oxygen; oxygen tension

P50 .....partial pressure of oxygen at 50% Hb-O_2 saturation

CO.....cardiac output

HbS .....sickle hemoglobin

HbF .....fetal hemoglobin

oxyHb.....oxygenated hemoglobin

deoxyHbS.....deoxygenated sickle hemoglobin

WT .....wild type

Hct.....hematocrit

MAP.....mean arterial pressure

HR.....heart rate

FiO_2.....fraction of inspired oxygen

BPM.....beats per minute

PO .....per os; by mouth

PaO_2 .....arterial partial pressure of oxygen

PvO_2.....venous partial pressure of oxygen

sO_2.....oxygen saturation of hemoglobin

SaO_2.....arterial oxygen saturation of hemoglobin

SvO_2.....venous oxygen saturation of hemoglobin

DO_2.....oxygen delivery

VO_2.....oxygen consumption

IQR.....interquartile range

pCO_2.....partial pressure of carbon dioxide

K^+.....potassium ion

Ca^{2+}.....calcium ion

Cl^-.....chloride ion

Na^+.....sodium ion

lac.....lactate

cBase.....base excess in blood

MRI.....magnetic resonance imaging

SV.....stroke volume

CBC.....complete blood count

WBC.....white blood cell

PLT.....platelet

MCV.....mean corpuscular volume

MCH.....mean corpuscular hemoglobin

MCHC.....mean corpuscular hemoglobin concentration

RDW.....red blood cell distribution width

NE.....neutrophil

MO .....monocyte

EO .....eosinophil

LY .....lymphocyte

BA .....basophil

NO.....nitric oxide

HIF-1α.....hypoxia-inducible factor 1-alpha

LPS.....lipopolysaccharide

LPL .....lipoprotein lipase

NF-κB .....nuclear factor kappa-light-chain-enhancer of activated B cells

TNF .....tumor necrosis factor; also called TNF-α

C5a .....complement component 5a

IL-6 .....interleukin 6

IL-8 .....interleukin 8

IL-1ra .....interleukin 1 receptor antagonist

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Section 1: GBT1118 in Hypoxia and SCD Mice, is a reprint of the material as it appears in the American Journal of Physiology: Heart and Circulatory Physiology, 2021; volume 321, issue 2: page H400-H411. Dufu, Kobina; Williams, Alexander T.; Muller, Cynthia R.; Walser, Cynthia M.; Lucas, Alfredo; Eaker, Allyn M.; Alt, Carsten; Cathers, Brian E.; Oksenberg, Donna; Cabrales, Pedro. Increased hemoglobin affinity for oxygen with GBT1118 improves hypoxia tolerance in sickle cell mice. The thesis author was a coauthor of this paper. Associated methods sections are listed either as reprints or adapted in section 6 of this thesis.

ABSTRACT OF THE THESIS

Effects of Hb-O_2 Affinity Modulation and Exogenous Estrogen on Hypoxia and Septic Shock in a Mouse Model

by

Allyn Matthew Eaker

Master of Science in Bioengineering

University of California San Diego, 2021

Professor Pedro Cabrales, Chair

Chapter 1. Hb-O_2 Binding Affinity Modulating Treatments

Pharmaceutical modulation of Hb-O_2 binding affinity may improve hypoxia tolerance and decrease sickling in Townes transgenic sickle cell disease (SCD) mice. Two voxelotor (GBT440) analogs, GBT1118 and GBT21601, were tested and found to increase Hb-O_2 affinity

without affecting oxygen-sensitive cortical tissue. When treated with either drug, SCD mice displayed greatly improved tolerance to hypoxia both with chronic and acute treatments. Additionally, increased Hb-O_2 affinity improved cardiac function of SCD mice during hypoxia while also reducing red blood cell (RBC) sickling.

Chapter 2. Exogenous Estradiol Treatments

Sexual biases have long been observed in many cardiovascular diseases. Estrogen has been found to impart cardioprotective benefit and estradiol is known to play regulatory roles in hypoxia and inflammatory pathway. Chronic treatments of estradiol were administered to female mice to determine if there are significant changes when compared to either male or untreated female mice during both hypoxia and sepsis. Exogenous estradiol treatments may improve cardiac function during hypoxia as well as induce changes in lipid and fatty acid production that may provide a protection during sepsis and inflammatory response.

Chapter 1: Hb-O_2 Binding Affinity Modulating Treatments

Section 1: GBT1118 in Hypoxia and SCD Mice

1.1 Abstract

Therapeutic agents that increase the Hb affinity for oxygen (O_2) could, in theory, lead to decreased O_2 release from Hb and impose a hypoxic risk to tissues. In this study, GBT1118, an allosteric modifier of Hb affinity for O_2, was used to assess the impact of increasing Hb affinity for O_2 on brain tissue oxygenation, blood pressure, heart rate, O_2 delivery, and tolerance to hypoxia in Townes transgenic sickle cell disease (SCD) mice. Brain oxygenation and O_2 delivery were studied during normoxia and severe hypoxic challenges. Chronic treatment with GBT1118 increased Hb affinity for O_2, reducing the PO_2 for 50% HbO_2 saturation (P50) in SCD mice from 31 mmHg to 18 mmHg. This treatment significantly reduced anemia, increasing hematocrit by 33%, improved cardiac output (CO), and O_2 delivery and extraction. Chronically increasing Hb affinity for O_2 with GBT1118 preserved cortical O_2 tension during normoxia, improved cortical O_2 tension during hypoxia, and increased tolerance to severe hypoxia in SCD mice. Independent of hematological changes induced by chronic treatment, a single dose of GBT1118 significantly improved tolerance to hypoxia, highlighting the benefits of increasing Hb affinity for O_2 and consequently reducing sickling of RBCs in blood during hypoxia in SCD.

1.2 Introduction

Sickle cell disease (SCD) is caused by a single point mutation in the DNA encoding the sixth amino acid of the hemoglobin (Hb) β-chain resulting in the production of sickle hemoglobin (HbS) (1). Upon deoxygenation, HbS aggregates due to the formation of multistranded helical polymers, which results in rigid and deformed red blood cells (RBCs) that are prone to lysis. Sickled RBCs can rapidly and permanently obstruct the microvasculature thus decreasing tissue oxygen (O_2) delivery, leading to ischemic injury and painful vaso-occlusive crises. The resulting organ damage is a major cause of pain, morbidity, and mortality associated with SCD (1–6). Central nervous system vascular complications are one of the most devastating problems in SCD, where overt stroke or repeated silent cerebral infarcts lead to significant physical and neurocognitive consequences (7–9). In addition, anemia (low Hb concentration) and increased cerebral blood flow in SCD are associated with increased stroke risk, suggesting that cerebral hypoxia is an important factor contributing to SCD morbidity (10).

As polymerization of deoxy-HbS in RBCs drives the pathogenesis of SCD, inhibiting HbS polymerization has proven to reduce the burden of the disease. Therapeutic strategies to reduce HbS polymerization in SCD have been based on increasing the RBCs' concentration of fetal hemoglobin (HbF), reducing the cellular concentration of HbS, or by chemically modifying HbS (1, 3). Because oxygenated Hb (oxyHb) is a potent inhibitor of deoxygenated HbS (deoxyHbS) polymerization, allosteric modification of Hb to increase the proportion of oxyHb in RBCs is another promising strategy to inhibit HbS polymerization (11). This approach led to the development of voxelotor (GBT440; Oxbryta), which was approved in 2019 by the Food and Drug Administration for the treatment of SCD in patients ages 12 years and older. Voxelotor reversibly binds to Hb and allosterically increases Hb affinity for O_2 (12, 13). Consequently,

voxelotor increases the concentration of oxyHb and thereby inhibits deoxyHbS polymerization. Oral administration of voxelotor reduces RBC sickling, extends RBC half-life, and reduces anemia and hemolysis in vivo (12–15). In the Phase III HOPE trial, voxelotor significantly increased Hb levels and reduced markers of hemolysis in patients with SCD compared with placebo (16).

An excessive increase in Hb affinity for O_2 could, in theory, lead to decreased O_2 release from Hb and impose a hypoxic risk to tissues. This would be especially deleterious in the brain, as it is the most energy-demanding and metabolically active organ of the body. Compared with healthy individuals, this theoretical hypoxic risk may be increased in patients with SCD who already suffer from impaired arteriolar vasoregulation and hence cannot compensate by increasing cerebral blood flow (17–19). The Townes transgenic SCD mouse represents a well-established model, whereby murine α and β globin genes have been replaced by human α and β^s globin genes (20). The Townes mice develop hemolytic anemia and severe organ injury consistent with human SCD (20). The purpose of this study was to assess the impact of a pharmacologically mediated increase in Hb affinity for O_2 on O_2 delivery and extraction, cerebral tissue oxygenation during normoxia and hypoxia, and tolerance to severe hypoxia in Townes transgenic SCD mice. To increase Hb affinity for O_2, mice were dosed with 2-hydroxy-6-[(2S)-1-(pyridine-3-carbonyl)piperidin-2yl]methoxy (GBT1118), an analog of voxelotor with the same mechanism of action but with improved pharmacokinetic properties that allow it to achieve in SCD mice the same degree of Hb modification voxelotor targets clinically.

1.3 Results

1.3.1 Tolerance to Hypoxia in SCD Mice and Healthy Controls

As shown in Fig. 1A, Townes transgenic SCD mice have a lower P50 in blood than WT mice (31 mmHg in SCD mice versus 43mmHg in C57BL/6 mice) (13, 21). In addition, SCD mice are anemic with a hematocrit (Hct) of 35 ± 3.3% (25) and thus have reduced O_2-carrying capacity relative to WT mice, which have an Hct of ~45% (26). To study the impact of the SCD pathophysiology on tolerance to hypoxia, we subjected Townes SCD mice and their WT counterparts to progressive hypoxia (Fig. 1, B–D). SCD mice were significantly less tolerant to severe (5% O_2) hypoxia compared with WT mice (Fig. 1B). During the hypoxic challenge protocol, although all WT mice survived the full duration of the 10% O_2 hypoxic challenge stage, a significant number of SCD mice succumbed at 10% O_2 hypoxia. Measurements of mean arterial blood pressure (MAP) during the hypoxic challenge indicated that SCD mice possessed a significantly lower MAP at normoxia (21% O_2) and at 15% O_2 hypoxia compared with their WT counterparts (Fig. 1C). In addition, both WT and SCD mice experienced a significant decrease in MAP at 10% and 5% O_2 hypoxia. Similarly, the heart rate (HR) in SCD mice was significantly lower than that in WT mice at all hypoxia levels (Fig. 1D). HR decreased significantly in WT mice at 5% O_2 hypoxia. In contrast, there were not enough SCD mice tolerant to 5% O_2 hypoxia to detect a significant difference in HR (Fig. 1D). These results confirm that the SCD pathophysiology reduces tolerance to hypoxia in SCD mice.

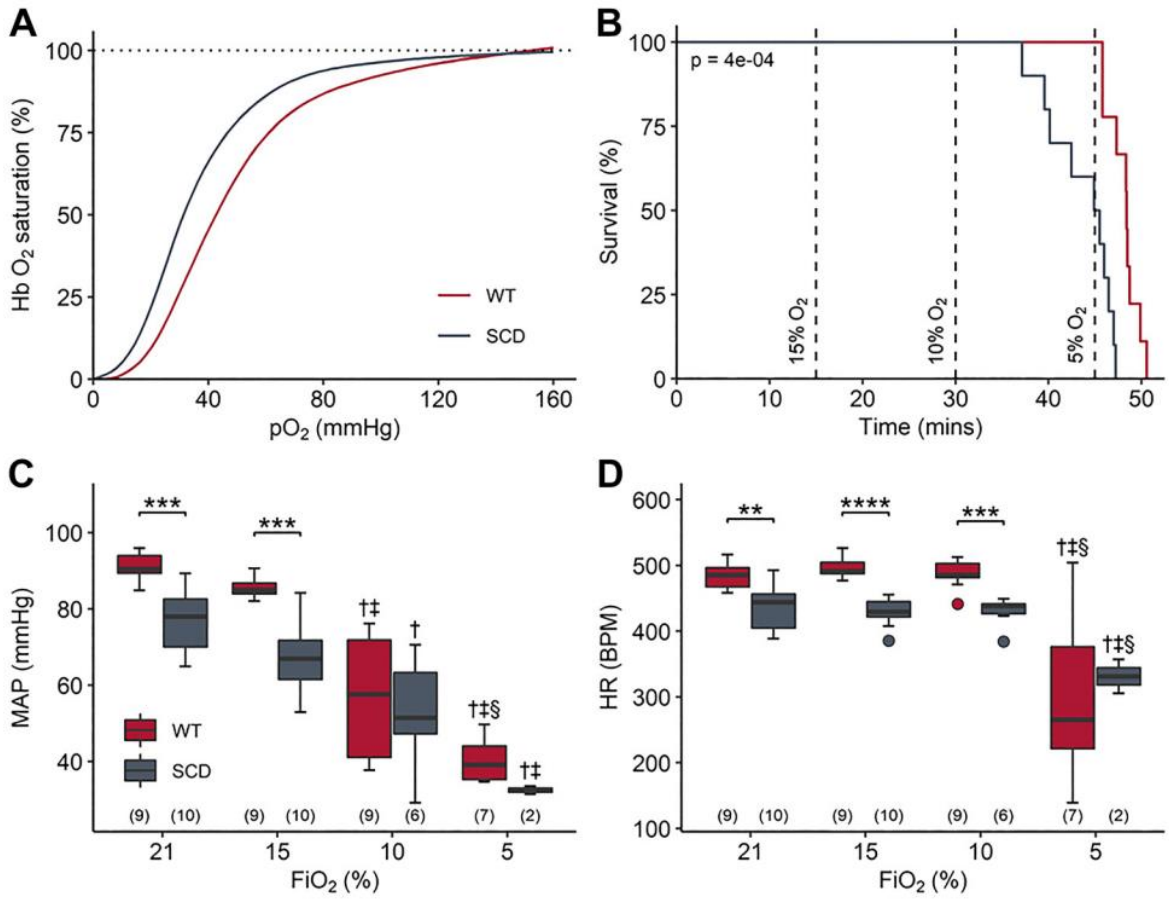

Figure 1: Transgenic sickle cell disease (SCD) mice are significantly less tolerant to hypoxia than wild-type (WT) mice. A: Transgenic SCD mice express only human Hb and have a lower P50 than their WT controls. B-D: Tolerance to progressive hypoxia (B), changes in mean arterial pressure (MAP; C), and heart rate (HR; D) during tolerance to progressive hypoxia. The number of animals surviving per group, per time point, are displayed underneath the respective boxplots in parentheses at the bottom of the graphs. Survival P value was calculated via the log-rank test. **P<0.01, ***P<0.001, ****P< 0.0001, †P<0.05 vs. 21% FiO_2, ‡P<0.05 vs. 15% FiO_2, and §P<0.05 vs. 10% FiO_2 for two-way ANOVA with Tukey's multiple comparisons test. BPM, beats per minute; HbO_2, oxyhemoglobin; P50, PO_2 for 50% HbO_2 saturation.

1.3.2 Selection of Compound to Increase Hb Affinity for O_2 in SCD Mice

The clinical application of voxelotor is targeted to an Hb occupancy of ~30% to ensure protection from RBC sickling based on clinical studies in patients with SCD (15, 16). Voxelotor and its analog GBT1118 increase human Hb affinity for O_2 with similar potency (Fig. 2A). However, unlike in patients with SCD, the pharmacokinetic characteristics of voxelotor in SCD mice indicated that voxelotor could not consistently achieve a targeted Hb occupancy of 30%

(Fig. 2B). During chronic oral treatment (100mg/kg twice a day for 14 days), voxelotor concentration in the blood was only 25% of the concentration of GBT1118 at equal dose (Fig. 2C). This observation is consistent with the relatively short T1/2 of voxelotor (9h) driven by its rapid clearance in this murine model. As GBT1118 is able to consistently achieve >30% Hb occupancy following oral chronic treatment in SCD mice, it was selected to pharmacologically increase the Hb affinity for O_2 in Townes SCD mice for our studies.

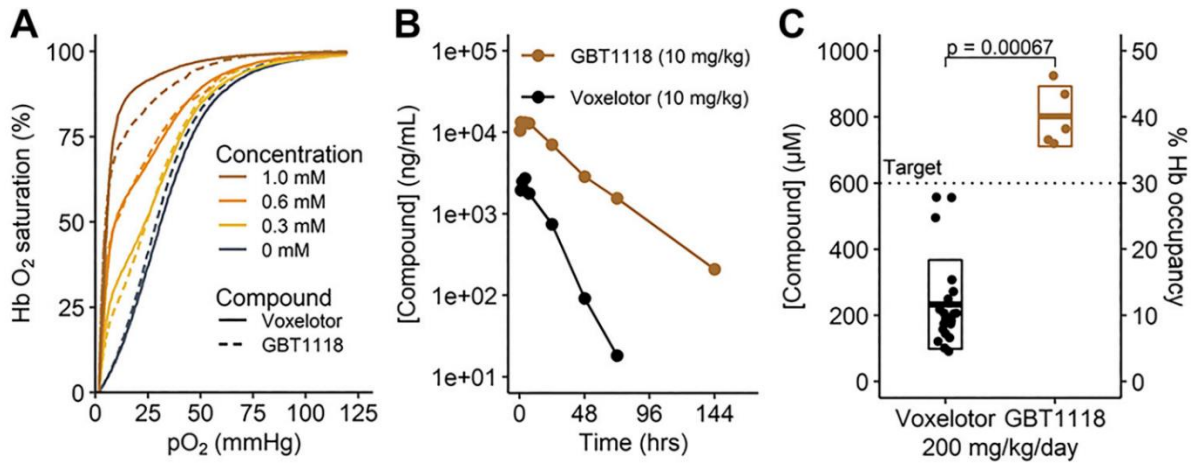

Figure 2: Comparison of in vitro Hb affinity for O_2-modifying properties and in vivo pharmacokinetic properties of voxelotor and GBT1118. A: in vitro modification of human SCD Hb with voxelotor or GBT1118 results in similar changes in Hb affinity for O_2 at equivalent concentrations. B: time-concentration profiles following single oral doses (10 mg/kg) in SCD mice indicate that GBT1118 has higher exposure and longer half-life compared with voxelotor in SCD mice. C: GBT1118 achieved higher blood concentrations than voxelotor following repeated oral dosing in SCD mice; the dashed line represents the target occupancy. Mean ± SD for n = 20 and n = 5 SCD mice treated with 200 mg/kg PO for 14 days with voxelotor and GBT1118, respectively, and P value was calculated via unpaired Welch's t test. GBT1118, 2-hydroxy-6-[(2S)-1-(pyridine-3-carbonyl)piperidin-2yl]methoxy; SCD, sickle cell disease.

1.3.3 Chronic Treatment of SCD Mice with GBT1118

1.3.3A Effects on SCD Pathophysiology in SCD Mice

The hematological impact of chronic GBT1118 treatment in SCD mice is presented in Table 1. Chronic oral treatment with GBT1118 for 24 days achieved blood concentrations

equivalent to an Hb occupancy of 43 ± 6.5% (median ± SD) and reduced the P50 of blood from 31 to 18 mmHg, indicating increased Hb affinity for O_2 (Table 1). As a result, GBT1118 reduced ex vivo sickling under hypoxic conditions (20mmHg), suggesting that by increasing the concentration of oxyHb in RBCs, GBT1118 reduces HbS polymerization. In accordance with its anti-polymerization and anti-sickling activities, GBT1118 reduced hemolysis as demonstrated by the concurrent increase in total Hb, RBC counts, and Hct, as well as reduced reticulocyte counts (Table 1). Consistent with an improvement in RBC health, GBT1118 increased RBC half-life from a median of 1.9 to 3.9 days (Table 1). Together, these results demonstrate that chronic modification of HbS with GBT1118 leads to inhibition of HbS polymerization, while reducing anemia in SCD mice, all consistent with the outcomes observed in patients with SCD chronically treated with voxelotor (15, 16).

Table 1: Hematological parameters showing beneficial effects of chronic GBT1118 treatment in SCD mice. *P < 0.05 vs vehicles-treated mice for Welch's t test. SD, standard deviation.

Parameter	Vehicle		GBT1118, 200 mg/kg	
	Median (SD)	**n**	**Median (SD)**	**N**
Blood concentration, µM	N/A		750.5 (100)	4
Hb occupancy, %	N/A		42.9 (6.5)	4
Blood P50, mmHg	30.6		17.6	
Ex-vivo sickling, %	36.3		18.0	
RBC half-life, days	1.9 (0.2)	4	3.9 (0.5)*	3
Hematocrit, %	29.3 (2.8)	3	36.6 (4.7)*	4
Hemoglobin, g/dL	10.6 (0.7)	3	12.9 (1.2)*	4
RBC count, millions/µL	6.6 (1)	3	8.8 (1.2)*	4
Reticulocytes, %	53.3 (6.5)	4	37.8 (0.7)*	4

1.3.3B Effects on Hypoxia Tolerance, Oxygenation, and Acid-Base Balance

To determine the effects of chronic dosing of GBT1118 on tolerance to hypoxia, oxygenation, and acid-base balance, SCD mice were dosed with GBT1118 for 14 days followed by an acute hypoxic challenge protocol (see section 6: Materials and Methods, Hypoxia

Protocols). SCD mice achieved GBT1118 blood concentrations of 802 ± 81 mM corresponding to an Hb occupancy of 44 ± 5% (Fig. 2C), which reduced the P50 of blood from ~31 mmHg in vehicle-treated SCD mice to 18 mmHg in GBT1118-treated SCD mice (Fig. 3A). Notably, the same Hb occupancy (~44%) was achieved when SCD mice were dosed with GBT1118 for 14 or 24 days, indicating that stable Hb modification with GBT1118 was attained by day 14 of chronic dosing.

1.3.3C Tolerance to Hypoxia

The impact of chronic GBT1118 treatment on tolerance to hypoxia, mean arterial pressure (MAP), and heart rate (HR) in SCD mice are shown in Fig. 3, B–D. Chronic GBT1118 treatment significantly improved tolerance to hypoxia in SCD mice. Although only two vehicle-treated SCD mice survived 2 min. of exposure to 5% O_2 hypoxia, all GBT1118-treated SCD mice survived for at least 2 min. during the 5% O_2 hypoxic challenge (Fig. 3B). Additionally, GBT1118-treated SCD mice were more tolerant to the hypoxic challenge compared with their WT counterparts (shown as the red dashed data set on the survival curve; Fig. 3B). The MAP of vehicle-treated and GBT1118-treated SCD mice decreased significantly during 10% and 5% O_2 hypoxia compared with normoxia; however, GBT1118-treated SCD mice maintained significantly higher MAP than vehicle-treated controls during 10% and 5% O_2 hypoxia (Fig. 3C). Additionally, although HR was preserved in GBT1118-treated SCD mice during the entire hypoxia protocol, HR significantly decreased for control animals at 5% O_2 hypoxia relative to normoxia (Fig. 3D). These results indicate that increasing the Hb affinity for O_2 with GBT1118 in SCD mice improves tolerance to severe hypoxia and is cardioprotective during severe hypoxia.

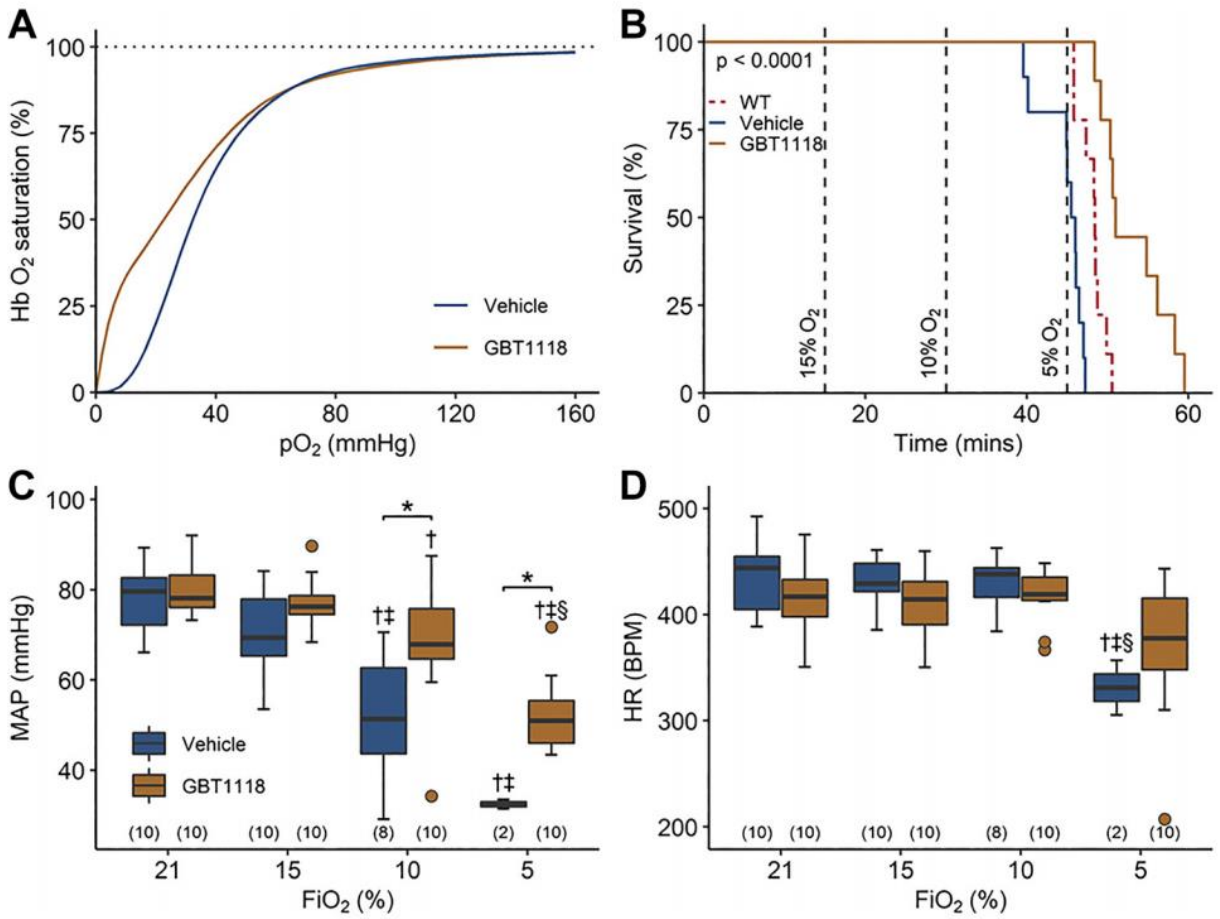

Figure 3: Chronic treatment with GBT1118 significantly increases the Hb affinity for O_2 and improves tolerance to progressive hypoxia in SCD mice. A: chronic treatment with GBT1118 increases the affinity of Hb for O_2. B: chronic treatment with GBT1118 significantly improves survival to extreme hypoxia in SCD mice, beyond the survival level of even WT mice. For reference, the WT group is shown on the survival curve as the red dashed data set. C: mean arterial pressure (MAP) is preserved at lower FiO_2 levels for SCD mice chronically dosed with GBT1118. D: heart rate (HR) is significantly decreased for vehicle-treated SCD mice but not for GBT1118-treated SCD mice. The number of animals surviving per group, per time point, are displayed underneath the respective boxplots in parentheses at the bottom of the graph. Survival P value was calculated via the log-rank test. *P<0.05, †P<0.05 vs. 21% FiO_2, ‡P<0.05 vs. 15% FiO_2, and §P<0.05 vs. 10% FiO_2 for two-way ANOVA with Tukey's multiple comparisons test. BPM, beats per minute; GBT1118, 2-hydroxy-6-[(2S)-1-(pyridine-3-carbonyl) piperidin-2yl]methoxy; HbO_2, oxyhemoglobin; SCD, sickle cell disease; WT, wild type.

1.3.3D Oxygenation

The impact of chronic GBT1118 treatment on systemic and cerebral oxygenation under normoxia (21% O_2) and 10% O_2 hypoxia is shown in Figs. 4 and 5. Chronic treatment with GBT1118 significantly increased total Hb levels in SCD mice (Fig. 4A). Differences in total Hb

between normoxia and hypoxia are likely due to blood sampling (Fig. 4A). GBT1118 treatment increased cardiac output (CO) at normoxia and 10% O_2 hypoxia (Fig. 4B). Arterial and venous PO_2 (PaO_2 and PvO_2, respectively) values were similar for GBT1118- and vehicle-treated SCD mice at normoxia, but GBT1118-treated SCD mice maintained higher PaO_2 and PvO_2 at 10% O_2 hypoxia. Additionally, PaO_2 and PvO_2 significantly decreased during exposure to 10% O_2 hypoxia for both GBT1118- and vehicle-treated SCD mice (Fig. 4, C and E). GBT1118-treated SCD mice had significantly higher arterial and venous Hb-O_2 saturation (SaO_2 and SvO_2, respectively) compared with vehicle controls at normoxia and 10% O_2 hypoxia. Although SaO_2 decreased significantly for both groups during 10% O_2 hypoxia, GBT1118-treated animals maintained SaO_2 above 90% even at 10% O_2 hypoxia (Fig. 4D). Additionally, SvO_2 only decreased from 78% to 69% for GBT1118-treated SCD mice during hypoxia, but the SvO_2 in vehicle-treated mice dropped from 46% to 11% during hypoxia (Fig. 4F).

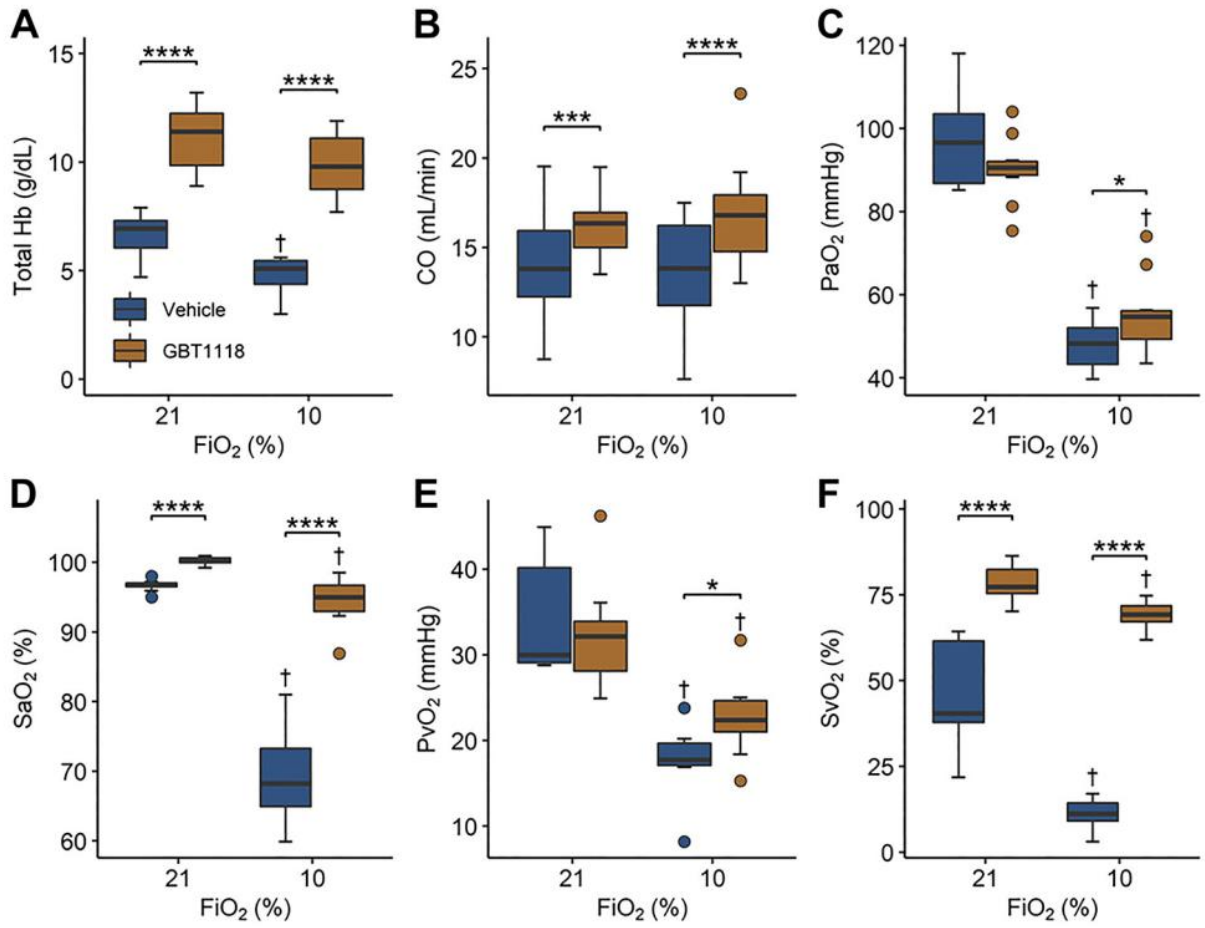

Figure 4: Chronic treatment with GBT1118 improves blood oxygenation during hypoxia in SCD mice. A: chronic treatment with GBT1118 significantly increases total Hb levels by increasing RBC half-life and reduce sickling. B: GBT1118 significantly increases cardiac output (CO), but CO remains the same during hypoxia. Arterial PO_2 (PaO_2; C) and venous PO_2 (PvO_2; E) are similar for both vehicle-treated and GBT1118-treated SCD mice during normoxia, but higher for GBT1118-treated SCD during hypoxia. Additionally, GBT1118 increases arterial SO_2 (SaO_2; D) and venous SO_2 (SvO_2; F) during normoxia and hypoxia. n= 10 animals/group; *P<0.05, ****P<0.001, ****P<0.0001, and †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Tukey's multiple comparisons test. GBT1118, 2-hydroxy-6-[(2S)-1-(pyridine-3-carbonyl)piperidin-2yl]methoxy; RBC, red blood cell; SCD, sickle cell disease.

Resulting from the increased total Hb, CO, and SaO_2, GBT1118-treated SCD mice possessed a significantly higher O_2 delivery (DO_2) at normoxia and at 10% O_2 hypoxia compared with vehicle-treated animals (Fig. 5A). GBT1118-treated SCD mice maintained O_2 extraction (VO_2) at normoxia and 10% O_2 hypoxia (Fig. 5B). The increase in DO_2 for GBT1118-treated SCD mice allowed for a significantly lower (i.e., more efficient) arterial to venous extraction ratio for GBT1118-treated SCD mice compared with vehicle-treated SCD mice (Fig. 5C). The preserved O_2 delivery:extraction ratio during hypoxia could explain the improved tolerance to

hypoxia of GBT1118-treated over vehicle-treated SCD mice, as a higher percentage of HbS remains oxygenated, thus preventing sickling during exposure to hypoxia. Notably, the increased Hb affinity for O_2 induced by GBT1118 did not impair vital tissue PO_2 levels at normoxia and hypoxia, as indicated by cortical PO_2 measurements (Fig. 5, D and E). Although hypoxia significantly decreased cortical PO_2 levels for both GBT1118- and vehicle-treated SCD mice, the GBT1118 group presented a significantly higher cortical PO_2 during hypoxia compared with the control group (Fig. 5, D and E). Additionally, GBT1118 treatment decreased the percentage of hypoxic areas within the brain compared with the control group, as observed by the reduced pimonidazole staining in the treated group compared with the control group (Fig. 5F).

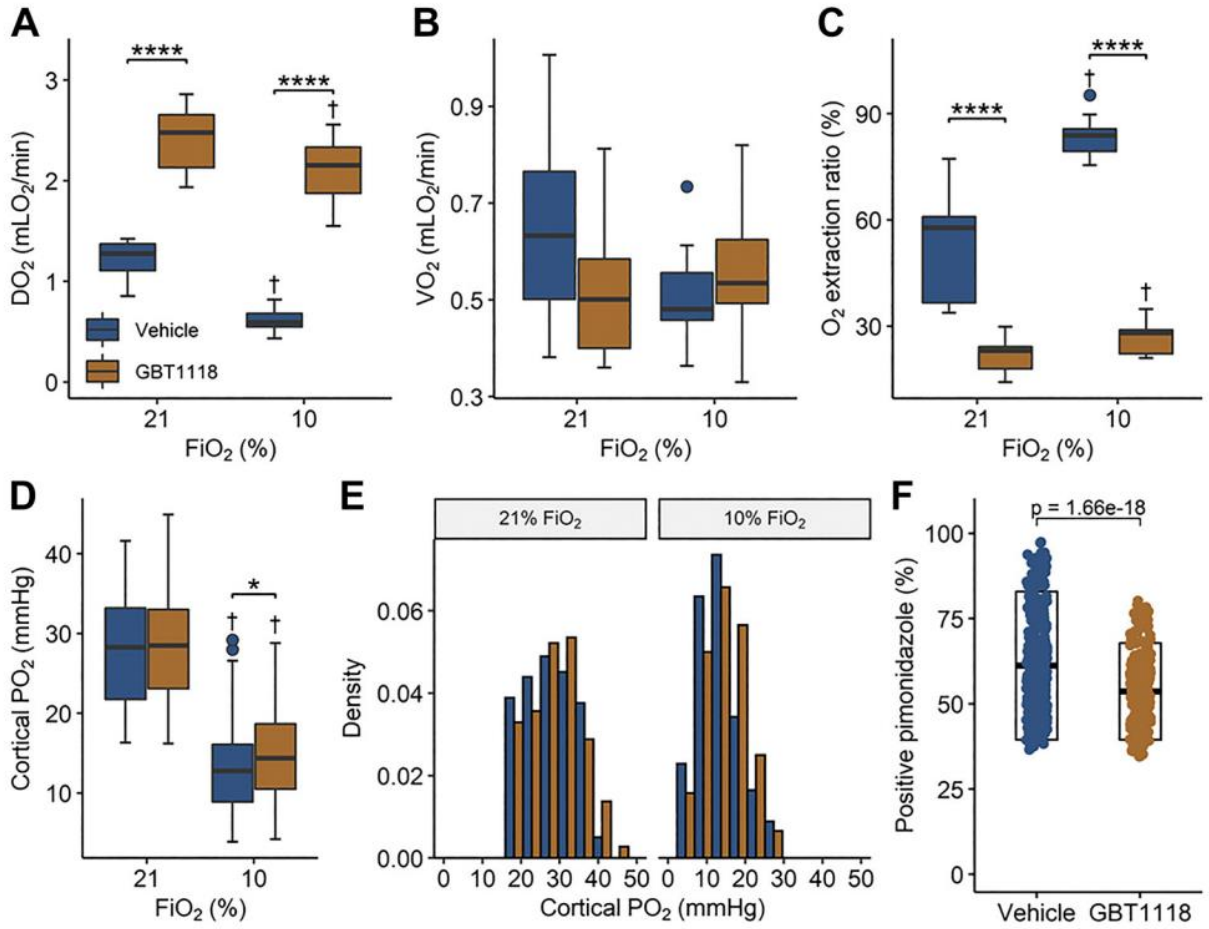

Figure 5: Chronic treatment with GBT1118 improves oxygen delivery and does not impact tissue oxygen extraction or reduce tissue PO_2 in SCD mice. A: oxygen delivery (DO_2) during normoxia and hypoxia is significantly increased for SCD mice chronically treated with GBT1118 compared with control. B: oxygen extraction (VO_2) is not reduced by increasing the Hb affinity for O_2 of over 40% of the Hb. C: the O_2 extraction ratio indicates that most O_2 delivered is extracted in vehicle-treated SCD mice during hypoxia (10% FiO_2), whereas only ~30% of the O_2 delivered was extracted for the GBT1118 chronically treated SCD mice. D and E: cortical PO_1 levels are significantly decreased during hypoxia but increased Hb affinity for O_2 as chronic GBT1118 treatment does not impair cortical PO_2 during normoxia and helps preserve cortical PO_2 during hypoxia. F: vehicle-treated SCD mice show significantly higher tissue hypoxia in the brain than GBT1118-treated SCD mice following extended hypoxia, as indicated by pimonidazole staining. Data are shown as the percentage of cells positively labeled for pimonidazole in a microscopic field; individual values, as well as the median and IQR are displayed; P value was calculated via unpaired Welch's t test. n = 10 animals/group (A–C); n = 8 animals/group (D and E); n = 8 animals/group, 40 microscopic fields/animal (F). *P<0.05, ****P<0.0001, and †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Tukey's multiple comparisons test. GBT1118, 2-hydroxy-6-[(2S)-1-(pyridine-3-carbonyl)piperidin-2yl]methoxy; IQR, interquartile range; SCD, sickle cell disease.

1.3.3E Acid-Base and Electrolyte Balance

The effects of chronic GBT1118 treatment on blood acid-base balance during normoxia and hypoxia are shown in Fig. 6. The reduced arterial pCO_2 in the GBT1118 group narrowed the range of systemic respiratory acidosis in the SCD mice via normalizing arterial pH at normoxia and 10% O_2 hypoxia (Fig. 6, A and B). Lactate was not different between GBT1118- and vehicle-treated SCD mice at normoxia; however, vehicle-treated SCD mice experienced a significant increase in lactate during hypoxia not found in GBT1118-treated SCD mice (Fig. 6C). This finding is consistent with the improved DO_2 afforded by GBT1118 treatment leading to reduction in anaerobic metabolism during the hypoxic challenge. Interestingly, GBT1118 prevented changes in acid-base balance and potassium compared with vehicle treatment. Healthy and SCD RBCs exhibit increased electrogenic cation permeability, particularly following deoxygenation, which results in increased plasma potassium (27). Consistent with the increase in Hb affinity for O_2 and the reduction of anaerobic metabolism (Fig. 6C), GBT1118 treatment reduced RBC deoxygenation and sickling, preserved DO_2, and reduced associated acid-base and electrolytic disturbances (Fig. 6, D–F).

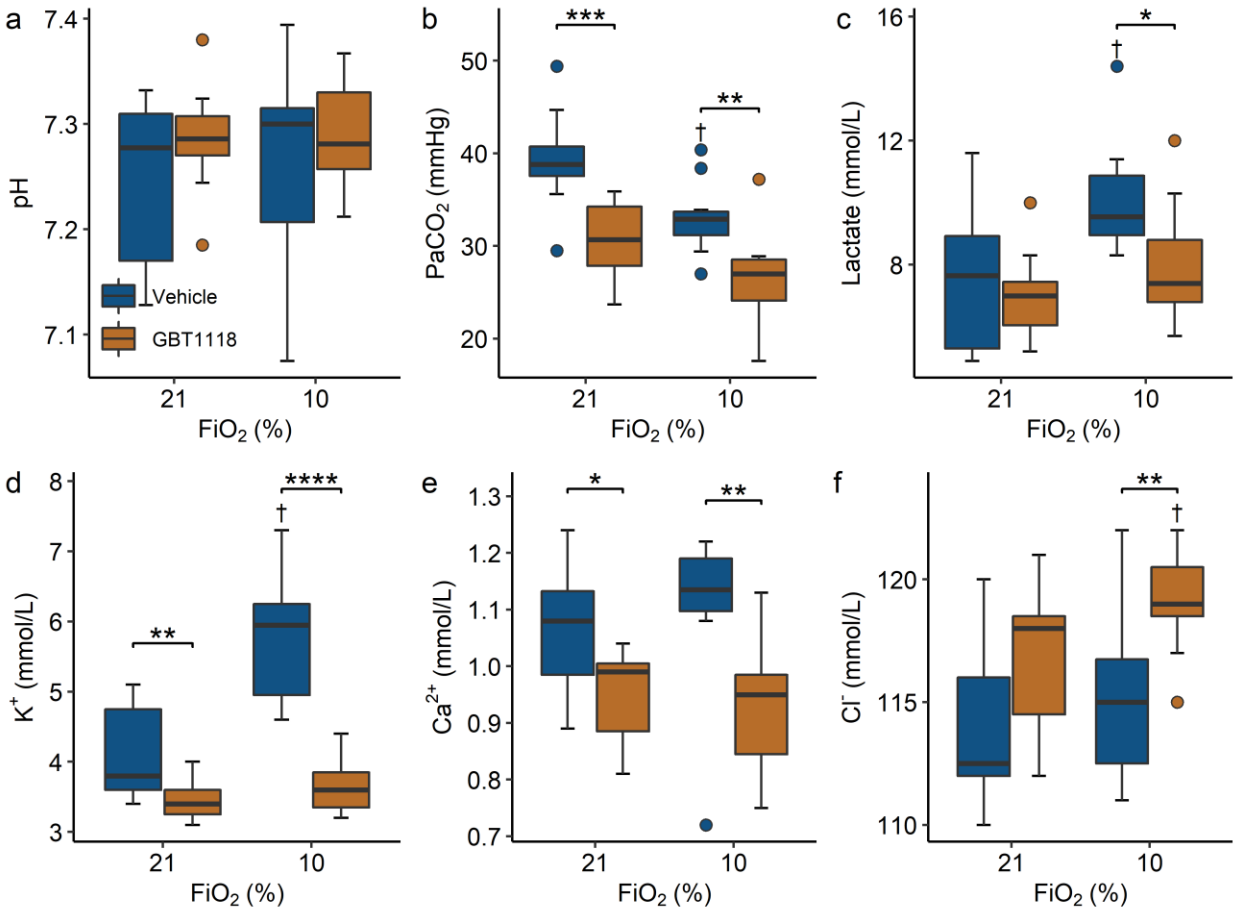

Figure 6: Acid/base balance of SCD mice following chronic treatment with GBT1118. (a) Arterial pH is largely unaffected by GBT1118 treatment (b) Arterial pCO_2 ($PaCO_2$) is lower for SCD mice chronically treated with GBT1118. (c) Lactate levels for both vehicle and GBT1118-treated animals are higher than WT controls (not shown), but GBT1118-treated SCD mice do not experience an increase in lactate, and have lower lactate than vehicle-treated controls, during hypoxia. (d, e, f) Vehicle-treated SCD mice show significantly higher plasma cations, and lower anions than GBT1118 chronically treated SCD mice. n = 10/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and †P<0.05 vs 21% FiO_2 for two-way ANOVA with Tukey's multiple comparisons test.

1.3.4 Single-Dose Treatment of SCD Mice with GBT1118

We examined the impact of an increase in Hb affinity for O_2 without increased O_2-carrying capacity by treating SCD mice with a single oral dose of GBT1118 (100mg/kg) followed by hypoxic challenge. The single dose of GBT1118 had no impact on Hb or Hct but significantly increased Hb affinity for O_2 for at least 4 h (Fig. 7A) and improved survival

compared with vehicle controls within this period (Fig. 7B). Although the single dose of GBT1118 slightly increased arterial O_2 saturation (SaO_2), it significantly improved venous O_2 saturation (SvO_2) (Fig. 8, A and B) without a significant impact on hemodynamics during progressive hypoxia (Fig. 7, C and D). A single oral dose of GBT1118 did not increase arterial O_2 tension, but it increased venular O_2 tension during normoxia and 10% O_2 hypoxia (Fig. 8, C and D). These results suggest that the protective effects of GBT1118 are more immediately related to minimizing hypoxemia and preserving SvO_2, and during chronic treatment, these effects are complemented by the hematological downstream pharmacodynamic effects induced by preventing RBC sickling.

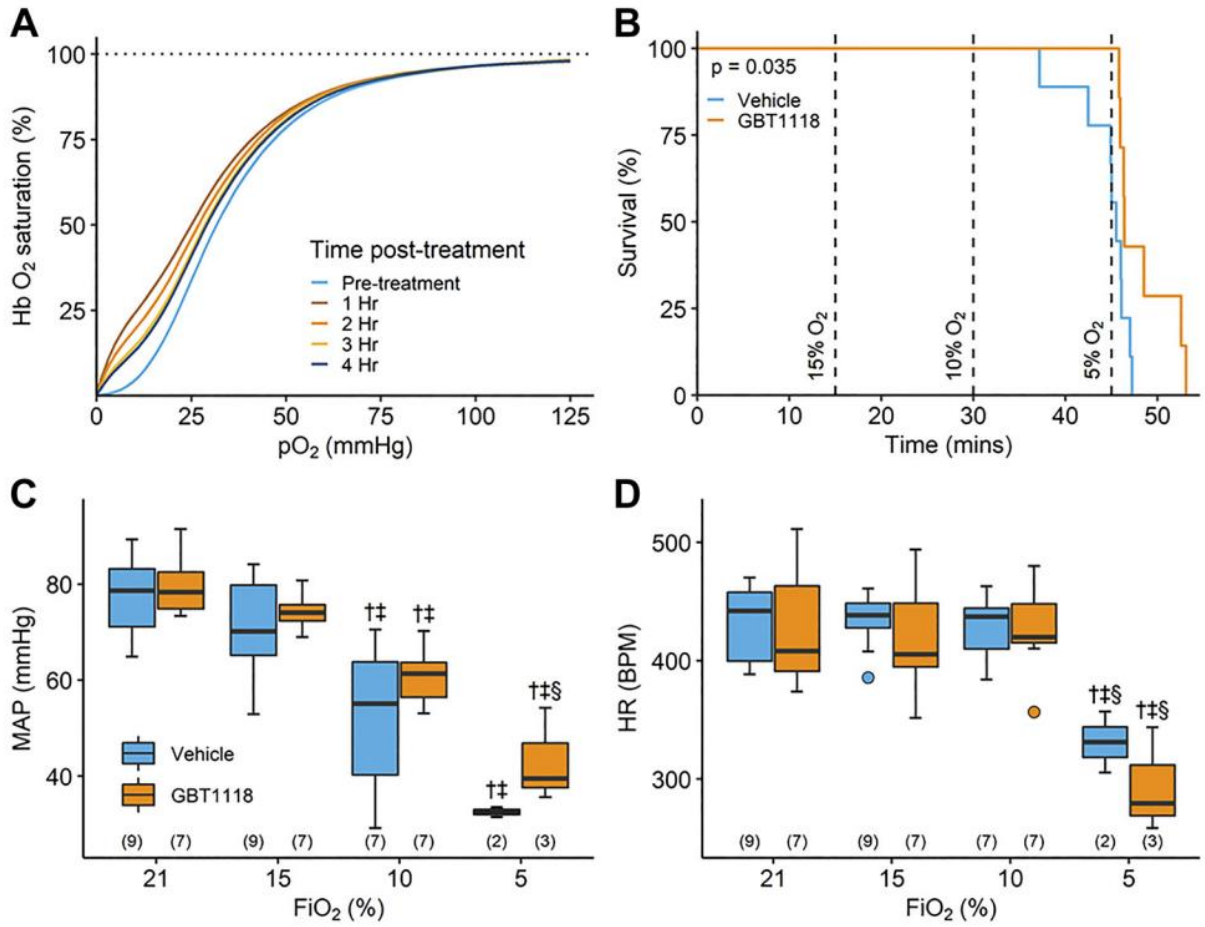

Figure 7: Acute (single-dose) treatment with GBT1118 improves survival in SCD mice but has minimal effect on hemodynamics. A: a single dose of GBT1118 significantly increases the Hb affinity for O_2 for over 4h. The P50 decreases from 31.4 ± 0.4 mmHg pretreatment to 21.3 ± 1.1 mmHg 1-h post-treatment, and then slowly increases over 4 h (P50 = 24.1 ± 0.8, 26.9 ± 0.9, and 27.7 ± 0.7 mmHg for 2, 3, and 4 h posttreatment, respectively; data represented as mean ± SD). All comparisons of P50, other than 3 vs. 4h, were statistically significant following an ANOVA with Tukey's post hoc test (n = 3 animals/curve). B: the acute dosage of GBT1118 mildly improves survival to extreme hypoxia. C and D: the acute dosage of GBT1118 has minimal impact on hemodynamics. The numbers of animals surviving per group, per time point, are displayed underneath the respective boxplots in parentheses at the bottom of the graphs. Survival P value was calculated via the log-rank test. †P<0.05 vs. 21% FiO_2, ‡P<0.05 vs. 15% FiO_2, and §P<0.05 vs. 10% FiO_2 for two-way ANOVA with Tukey's multiple comparisons test. GBT1118, 2-hydroxy-6-[(2S)-1-(pyridine-3-carbonyl)piperidin-2yl]methoxy; HbO_2, oxyhemoglobin; HR, heart rate; MAP, mean arterial pressure; P50, PO_2 for 50% HbO_2 saturation; SCD, sickle cell disease.

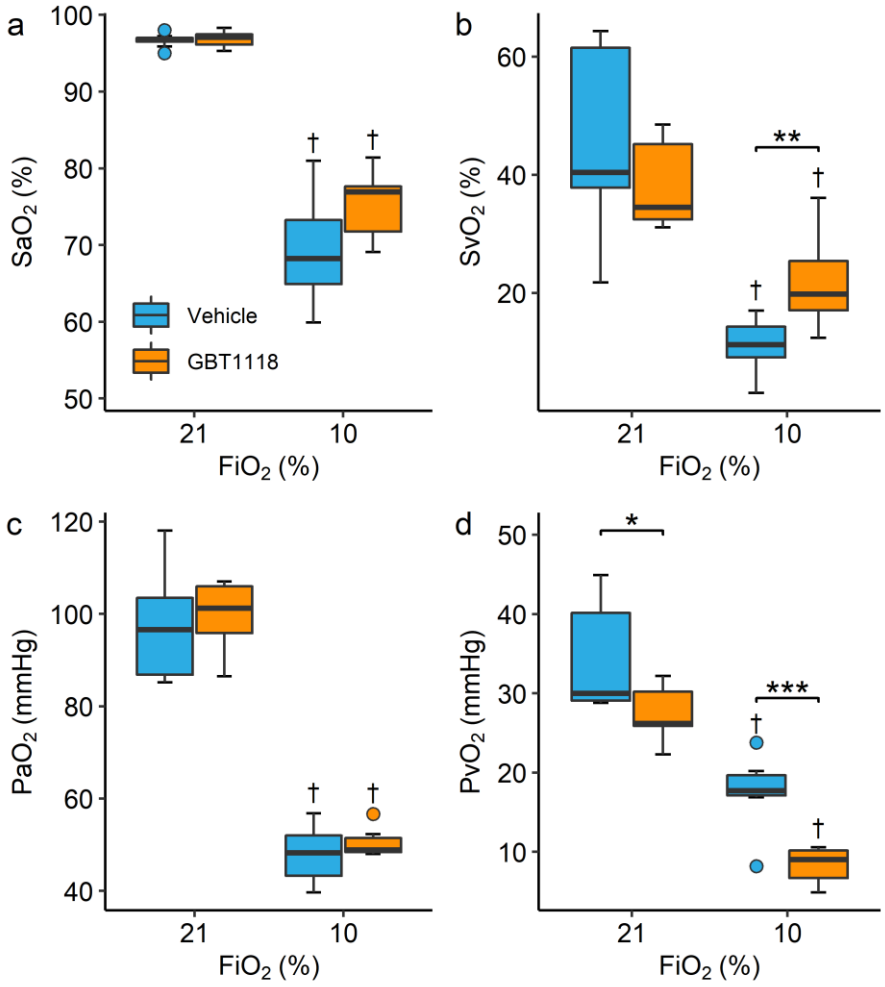

Figure 8: Acute (single dose) treatment with GBT1118 affects blood oxygenation parameters. (a) Arterial saturation (SaO_2) is slightly improved during hypoxia (10% FiO_2). (b) Single treatment of GBT1118 increases venous saturation (SvO_2) compared to vehicle-treated controls during hypoxia. (c) Arterial PO_2 (PaO_2) decreases similarly for both groups. (d) Venous PO_2 (PvO_2) remains significantly higher for vehicle-treated animals than GBT1118-treated SCD mice. n = 10 & 6 (Vehicle (single dose) & GBT1118 (single dose), respectively. *P<0.05, **P<0.01, ***P<0.001, and †P<0.05 vs 21% FiO_2 for two-way ANOVA with Tukey's multiple comparisons test.

1.4 Discussion

It has been previously reported that pharmacologically increasing Hb affinity for O_2 improves O_2 delivery, reduces tissue hypoxia, decreases lactate and acidemia, and improves survival during hypoxia in healthy animals (21, 23, 24). The impact of increasing Hb affinity for O_2-on-O_2 transport, hemodynamics, and tissue oxygenation is poorly understood in SCD due to the pathophysiological changes induced by the disease, where both O_2-carrying capacity and O_2 delivery are compromised. Questions have been raised about whether drugs that increase Hb affinity for O_2 impair O_2 off-loading in tissues in SCD.

As expected, chronic treatment with GBT1118 increased RBC count, total Hb, and RBC half-life, while simultaneously decreasing reticulocytes and preventing the sickling of RBCs ex vivo. Increasing Hb affinity for O_2 in a fraction of the Hb decreases polymerization of HbS and relieves SCD mice from hematological consequences resulting from lingering sickling. Importantly, results from this study suggest that the GBT1118-mediated increase in Hb affinity for O_2 at target Hb modification of ~44% does not affect the release of O_2 or the tissue O_2 tension required to offload physiologically needed O_2. Although a single dose of GBT1118 did not produce measurable hematological changes, it increased Hb affinity for O_2 and significantly improved tolerance to extreme hypoxia. Lastly, our results demonstrate that pharmacologically increasing the Hb affinity for O_2 does not decrease tissue PO_2 in areas with high physiological O_2 demands, such as the brain, during normoxia and hypoxia.

These various pharmacodynamic effects resulted in improved hemodynamics and increased survival during extreme hypoxia. Taken together, this data indicates that the theoretical concern that a drug like voxelotor would compromise tissue O_2 delivery is not supported by the evidence obtained in a SCD mouse model treated with GBT1118. This mechanism may instead

confer cardioprotective effects both during normoxia and hypoxia. Pharmacologically increasing the Hb affinity for O_2 by treatment with GBT1118 improves the tolerance of SCD mice to hypoxic conditions to a greater extent than untreated WT animals. In addition, pharmacologically increasing the O_2 affinity of 44% of the Hb in SCD mice did not impair cortical tissue PO_2 under normoxia but rather improved cortical tissue PO_2 during hypoxia relative to vehicle-treated SCD mice.

GBT1118 treatment reduced metabolic acid-base disruption and prevented electrolytic disturbances, which are observed during deoxygenation of sickle cells by increasing cation permeabilities (K^+ and Ca^{2+}). Thus, GBT1118 prevented red cell dehydration and supported the x, blood flow, inflammation, and organ function. For example, the significant increase in Hb (>2 g/dL) observed in this study was a large contributor to the observed increase in O_2 delivery. In addition to the Hb increase, the concurrent reduction in reticulocyte counts and increase in RBC half-life associated with GBT1118 treatment indicates decreased hemolysis and therefore reduced tissue exposure to heme, a known prooxidant. Together, these effects improved SaO_2, blood flow, and O_2 delivery to tissues; however, the increase in Hb and hematocrit cannot entirely explain the improvements in tissue oxygenation and tolerance to hypoxia.

In acute single-dose studies with GBT1118, the increase in the fraction of oxyHb during hypoxia and thus, the consequent inhibition of polymerization is the primary mechanism that improved hypoxia tolerance in SCD mice as there were no measurable changes in the Hb and hematocrit between GBT1118-treated and vehicle-treated SCD mice in the acute single-dose studies. GBT1118 treatment may have also prevented the abnormal activation of the potassium and chloride cotransport system (Gardos channel) in RBCs, which has been proposed to be involved in RBC permeability changes during RBC sickling, as these permeability changes

increase RBC dehydration in SCD and aggravate clinical complications (28). This change in RBC permeability may have acted as a secondary mechanism that improved hypoxia tolerance in SCD mice.

Chronic increase of Hb affinity for oxygen in SCD mice has additional benefits related to the prevention of RBC sickling and damage to RBC structure and function. SCD therapies that increase Hb affinity for O_2 reduce RBC sickling and hemolysis and alleviate the comorbidities associated with plasma Hb, heme, and iron, such as vascular inflammation, coagulopathies, and subsequent pulmonary hypertension and infection (29, 30). In this study, we see evidence of decreased pulmonary hypertension, as animals chronically treated with GBT1118 showed significantly increased CO compared with controls during normoxia and hypoxia (31).

Novel treatments reducing Hb, heme, and iron toxicity based on hepcidin, hemopexin, and haptoglobin have been experimentally validated to reduce some side effects of SCDs but fail to address the underlying problem that drives hemolysis in SCD (32). Increasing Hb affinity for O_2 in SCD prevents HbS polymerization, thus inhibiting RBC sickling and lysis, which addresses both the underlying origin of SCD comorbidities and prevents the downstream sequalae associated with chronic hemolysis, anemia, and vasoocclusion.

These experimental results have several potential limitations. Results in SCD mice cannot be directly extrapolated to humans, and GBT1118 pharmacokinetics in the SCD mice is different compared with that of voxelotor. In addition, relatively young and healthy SCD mice may be able to adapt and tolerate modification of over 40% of their Hb by GBT1118, but older and sicker animals may have a different response. Cardiovascular and respiratory response to hypoxic hypoxia in rodents allows for increases in cardiac output and respiratory rates and leads

to alterations in arterial blood gases. Therefore, direct extrapolation of the degree of protection to hypoxia provided by GBT1118 still needs to be evaluated in larger animals and humans.

Arterial blood pCO_2 and HCO_3^- were higher in GBT1118-treated groups than in the control group during hypoxia. Analysis of arterial blood gases indicates that the GBT1118 prevented metabolic acidosis and accumulation of lactate. It is important to recognize the measurement of O_2 affinity from blood samples presents the overall affinity for O_2, without distinguishing between the fraction of Hb modified by GBT1118 (or Hb with high affinity) and the unmodified Hb (with normal Hb affinity for O_2). In addition, the measurement of O_2 affinity requires dilution in a strongly buffered solution (TCS Hemox solution, pH 7.3) and deoxygenation is performed in the absence of CO_2, so these measurements do not reflect the Bohr and Haldane effects.

Another limitation of the study is that WT mice have a significantly higher P50 (~42–43 mmHg) compared with SCD mice (P50 of 32mmHg), challenging the comparison between SCD and WT mice. Although the HbAA-Townes (mice expressing human Hb) mice were created similarly to the HbSS-Townes mice, by replacing murine globin genes with the human globin genes, HbAA-Townes mice do not represent healthy normal mice. To different degrees, the HbAA-Townes and HbSS-Townes mice have differences in their hematology, kidney and liver function, inflammatory markers, haptoglobin and hemopexin levels, red cell half-lives, and organ histopathology compared with healthy mice (33). For example, the HbAA-Townes mice have lower Hb levels, higher reticulocyte counts, and their livers and spleens are significantly enlarged compared with healthy WT mice. In these studies, we used WT mice as control to establish the implications of changes in O_2 affinity in HbSS to physiological responses to hypoxia. It may be valuable to include studies in the HbAA-Townes mouse models, but their

results will need to be interpreted within the limitations of the model, further complicating the understanding of results. Lastly, the results presented here are specific to GBT1118 in SCD mice.

1.5 Conclusion

The main finding of this study is that in a murine model of SCD, chronic pharmacological increases of Hb affinity for O_2 increases O_2-carrying capacity by raising Hb levels and minimizes hypoxemia at low O_2 concentrations. In addition, it also improves tolerance to hypoxia by preventing RBC sickling, well before inducing any hematological changes. Although SCD mice have substantial differences from patients with SCD, SCD mice provide opportunities to further explore the mechanisms of this disease, and previous clinical studies with voxelotor confirm the pharmacodynamic effects of increasing Hb affinity for O_2 in patients similar to those observed in this SCD mouse study (15, 16). In summary, chronic modification of total Hb resulting in ~44% high-affinity form not only led to increased O_2-carrying capacity (i.e., increased total Hb) and O_2 delivery but also preserved O_2 extraction by tissues and ultimately improved hypoxia tolerance in SCD mice. The results from these studies support the accumulated evidence to date that in SCD, agents such as voxelotor that increase Hb affinity for O_2 can improve the pathophysiology of SCD by inhibiting HbS polymerization and RBC sickling without compromising tissue oxygenation. Lastly, the physiological balance between O_2 supply and extraction in cerebral, gastrointestinal, coronary, and skeletal muscle tissues is importantly determined by blood flow, and it appears that modifying less than 50% of the Hb with GBT1118 in the SCD mice seems to enable hematological and hemodynamics improvements that preserve oxygenation. We cannot speculate that these conditions can be reproduced in humans and carefully advise not to draw this conclusion for the presented results.

1.6 Acknowledgement of Published Material

Section 1: GBT1118 in Hypoxia and SCD Mice, is a reprint of the material as it appears in the American Journal of Physiology: Heart and Circulatory Physiology, 2021; volume 321, issue 2: page H400-H411. Dufu, Kobina; Williams, Alexander T.; Muller, Cynthia R.; Walser, Cynthia M.; Lucas, Alfredo; Eaker, Allyn M.; Alt, Carsten; Cathers, Brian E.; Oksenberg, Donna; Cabrales, Pedro. The thesis author is a coauthor of this paper. Associated methods sections are listed either as reprints or adapted in section 6 of this thesis. The full citation is listed under the references as (34).

Section 2: GBT21601 in Hypoxia and SCD Mice

2.1 Introduction

Having established the beneficial effects of Hb-O_2 affinity modulating pharmaceuticals in a murine SCD model, we set out to determine the effect of dose size on the model. For this continuation study, we treated the mice with GBT21601, a newer formulation analog of voxelotor with a higher efficacy. GBT21601 uses the same mechanism of action to increase Hb-O_2 binding affinity within a fraction of Hb. In this study, we treated Townes SCD mice with either 75 mg/kg or 150 mg/kg of GBT21601. Similar to before, we observed the effects of both chronic and acute treatment.

2.2 Results

2.2.1 Chronic Treatment of SCD Mice with GBT21601

2.2.1A Tolerance to Hypoxia

As shown in Fig. 9A, chronic dosing of GBT21601 caused the SCD mice to have a lowered P50 compared to vehicle-treated mice, regardless of dose size. With GBT21601, the P50 of blood from the SCD mice was ~20 mmHg and ~15 mmHg (for 75 mg/kg and 150 mg/kg respectively) compared to vehicle-treated, in which the P50 of blood was ~32. GBT21601-treated groups had similar Hb saturation curves at both dose sizes, with both being elevated compared to vehicle-treated Hb saturation curves. Dose size did not affect the hypoxia tolerance of the GBT21601-treated mice, with both groups showing similar survival curves (Fig. 9B). As observed previously, the SCD that received a treatment with Hb-O_2 affinity modulating drugs were better able to tolerate hypoxic conditions, compared to their vehicle-treated counterparts. From the results of the previous study, we expected the GBT21601-treated SCD mice to

maintain higher MAP values during hypoxia, and we found that the lower dosed (75 mg/kg) mice had a significantly higher MAP at 5% hypoxia compared to the vehicle-treated group (Fig. 9C). MAP corresponding to the 150 mg/kg dose was significantly different from both the vehicle-treated and the 75 mg/kg dose at normoxia (21% FiO_2 – fraction of inspired O_2) and at all severities of hypoxia (15, 10, and 5% FiO_2) (Fig. 9C). HR was found to be most different in the 75 mg/kg dose group which was significantly lower than both other groups at normoxia, as well as significantly lower than the vehicle-treated mice at low and moderate hypoxia (15, 10% FiO_2) (Fig 9D). At the most severely hypoxic conditions tested, all three groups showed no statistical difference in HR (Fig. 9D).

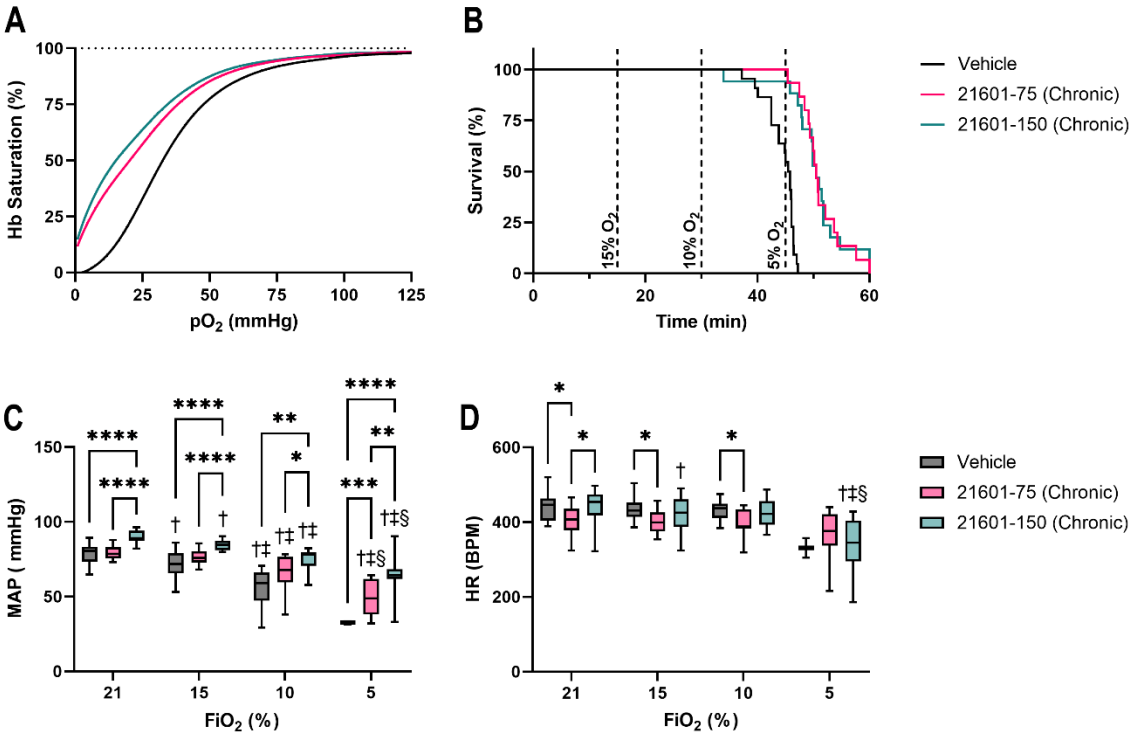

Figure 9: Impact of chronic treatment of GBT21601 on hypoxia tolerance, comparing two dose sizes, 75 and 150 mg/kg. A: GBT21601 treatment increases Hb affinity for O_2. B: Both dose sizes of GBT21601 improve survival of SCD mice under severe hypoxia. C: GBT21601 aids in preservation of MAP in low oxygen conditions. D: GBT21601 slightly decreased HR with the 75 mg/kg dose treatment. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2, ‡P<0.05 vs. 15% FiO_2, and §P<0.05 vs. 10% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; MAP, mean arterial pressure; HR, heart rate; BPM, beats per minute.

2.2.1B Oxygenation

The effects of the different doses of GBT21601 on oxygenation in normoxia and 10% O_2 hypoxia are shown in Figs. 10 and 11. All groups showed significantly different total Hb levels when compared to either of the other groups (Fig. 10A). All groups of SCD mice experienced depressed total Hb during 10% O_2 hypoxia, but both GBT21601-treated groups maintained higher total Hb levels. The effects of GBT21601 on total Hb was greatest in the mice that received higher doses of the drug. Shown in Figs. 10B and C, all SCD mice had decreased arterial pO_2 and sO_2 at 10% FiO_2. Neither GBT21601-treated group had significantly different arterial pO_2 compared to the vehicle-treated control, but the 150 mg/kg dosed mice had significantly lower arterial pO_2 than the 75 mg/kg dosed mice at both normoxia and 10% FiO_2. Both groups of GBT21601-treated mice had significantly higher arterial sO_2 compared to the vehicle-treated mice at 10% FiO_2, but showed no significant difference from each other. Venous pO_2 only differed at normoxia, with both GBT21601-treated groups being significantly lower than the vehicle-treated mice (Fig. 10 D). Both GBT21601-treated groups showed significantly higher sO_2 than the vehicle-treated mice, as expected, but showed no statistical difference between the two different doses. Cortical pO_2 levels are shown in Fig. 11. Similar to the previous study with GBT1118, the pharmacologically increased Hb-O_2 affinity does not seem to greatly impact cortical pO_2, with both GBT21601-treated groups showing similar density curves and median values as compared to the vehicle-treated mice.

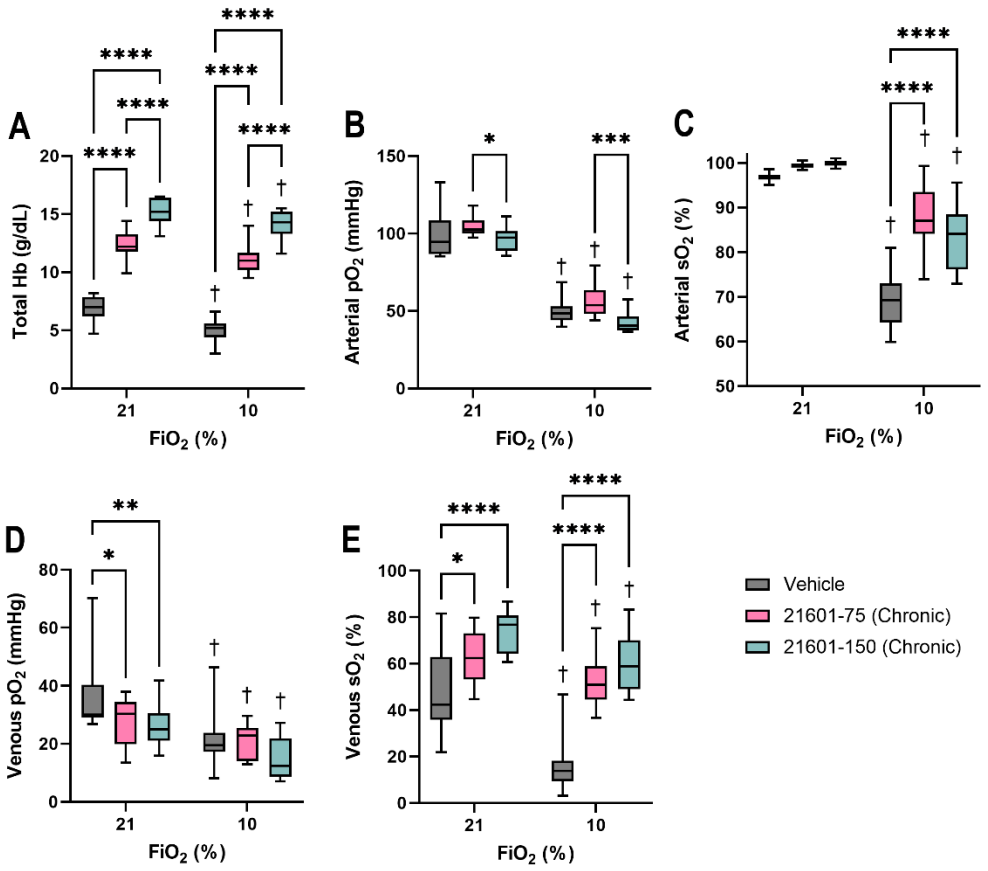

Figure 10: Effects of chronic treatment of GBT21601 on oxygenation, comparing two dose sizes, 75 and 150 mg/kg. A: GBT21601 greatly increases total Hb and exhibits dose dependent behavior. B: The lower 75 mg/kg dose maintains higher arterial pO_2 than the 150 mg/kg dose. C: GBT21601 treatment improves arterial oxygen saturation, with no difference between doses. C: Vehicle-treated mice have a higher venous pO_2 while under normoxia. D: Both doses of GBT21601 significantly increase venous oxygen saturation. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; pO_2, partial pressure of oxygen, sO_2, oxygen saturation of Hb.

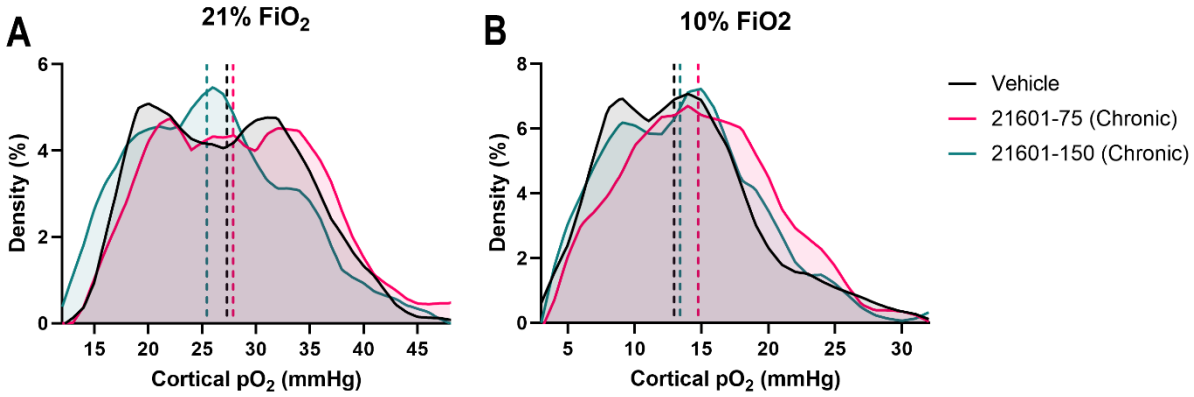

Figure 11: Density curve of cortical pO_2 levels for SCD at normoxia (A) and 10% hypoxia (B) following chronic oral treatment of GBT21601. Cortical tissue oxygenation does not seem to be impacted by the GBT21601 modulated Hb-O_2 affinity.

Oxygen delivery and consumption is shown in Fig 12. Chronic treatment of GBT21601 greatly improves both oxygen delivery to tissues and oxygen consumption (Figs. 12A and B). The increased total Hb following GBT21601 treatment is likely the cause of the three-fold increase in oxygen delivery, allowing for more O_2 to be consumed by tissues and reducing the risk of tissue damage from hypoxia as well as reducing the risk of RBC sickling. GBT21601-treated SCD mice demonstrate reduced O_2 extraction ratios as a consequence of the increase in oxygen delivery (DO_2) being comparatively larger than the increase in oxygen consumption (VO_2) (Fig. 12C). This also confirms the results of the GBT1118 studies, showing that increased Hb-O_2 binding affinity can improve tissue oxygenation and reduce risk of hypoxia-induced tissue damage.

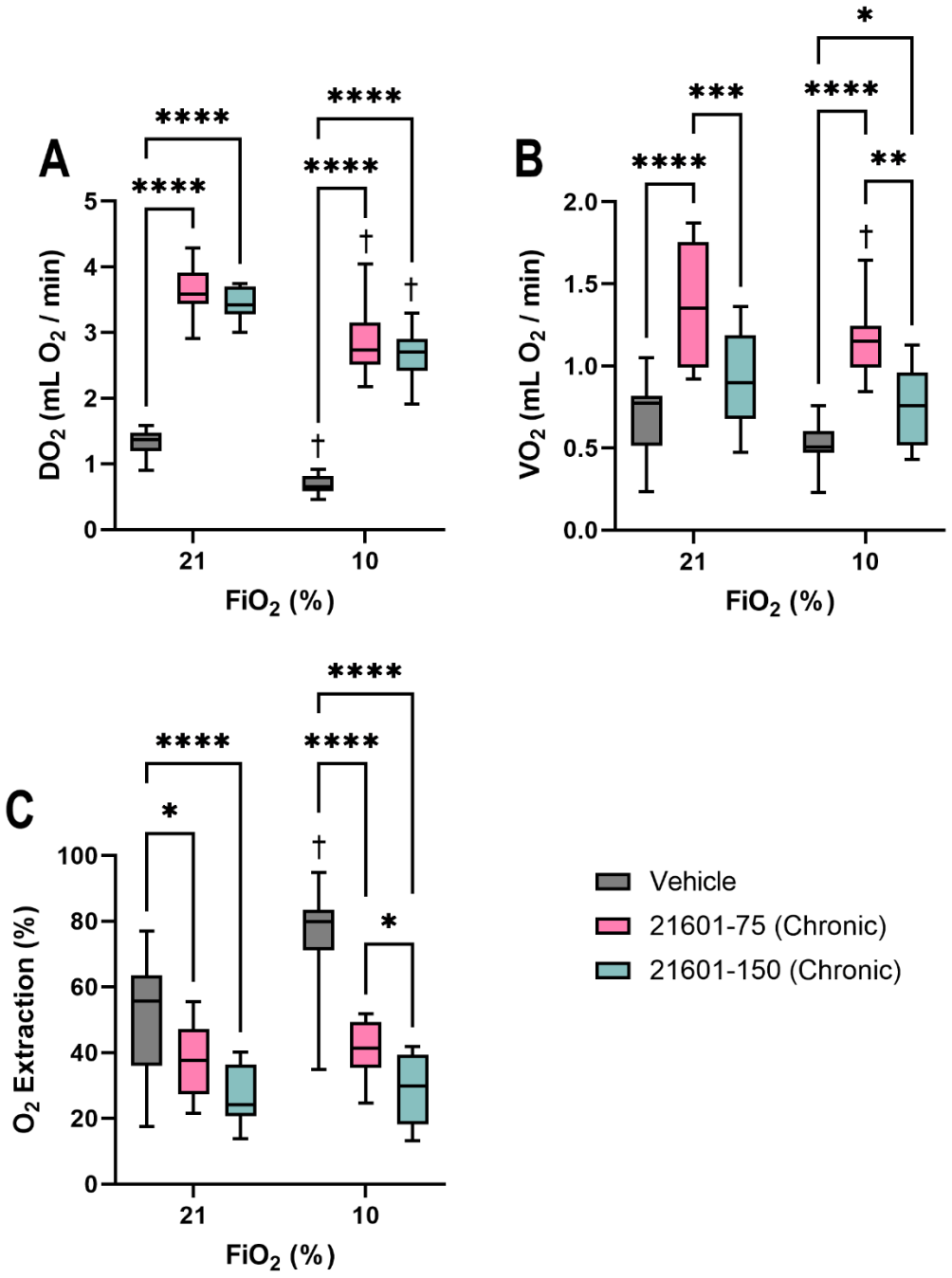

Figure 12: Effects of chronic treatment of GBT21601 on oxygen delivery and consumption. A: Global O_2 delivery is greatly improved following chronic treatment, both at normoxia and 10% hypoxia. B: The 75 mg/kg dose of GBT21601 increases O_2 consumption at normoxia, and both dose sizes improve tissue O_2 consumption during hypoxia, reducing chance of hypoxic tissue damage. C: The comparatively larger increase in DO_2 compared to VO_2 leads to lower O_2 extraction ratios in GBT21601-treated SCD mice. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; DO_2, oxygen delivery; VO_2, oxygen consumption.

2.2.1C Blood Acid-Base and Electrolyte Balance

The impact of chronic GBT21601treatment on the acid-base balance within the blood of SCD mice is shown in Fig. 13. The 150 mg/kg dosed SCD mice were found to have significantly different arterial and venous pH at normoxia compared to both the vehicle-treated and the 75 mg/kg dosed mice (Fig. 13A and D). When compared to the vehicle-treated control during normoxia, both the 75 mg/kg dosed group and the 150 mg/kg dosed group showed significantly lower arterial pCO_2, but the 150 mg/kg group also showed lowered venous pCO_2 (Fig. 13B and E). All groups showed no statistical difference in pCO_2 to each other during 10% hypoxia; however, the 75 mg/kg group maintained no change in both arterial and venous pCO_2 at 10% FiO_2 compared to normoxia while the 150mg/kg-treated group showed a significant rise in arterial pCO_2 at 10% FiO_2. For reference, the vehicle-treated control had significantly decreased arterial and venous pCO_2 at 10% FiO_2 compared to normoxia. Lactate levels showed no difference between the 75 mg/kg dosed group and vehicle-treated control at either FiO_2 level, but there was a significant difference found between the 75 mg/kg and 150 mg/kg dosed groups at both FiO_2 levels (Fig. 13C). Additionally, the 150mg/kg dosed group had significantly lower lactate levels than the vehicle-treated group at 10% hypoxia. All three groups experienced significantly decreased base excess measurements at 10% hypoxia, but no significant difference between the groups was found at either FiO_2 level (Fig. 13F).

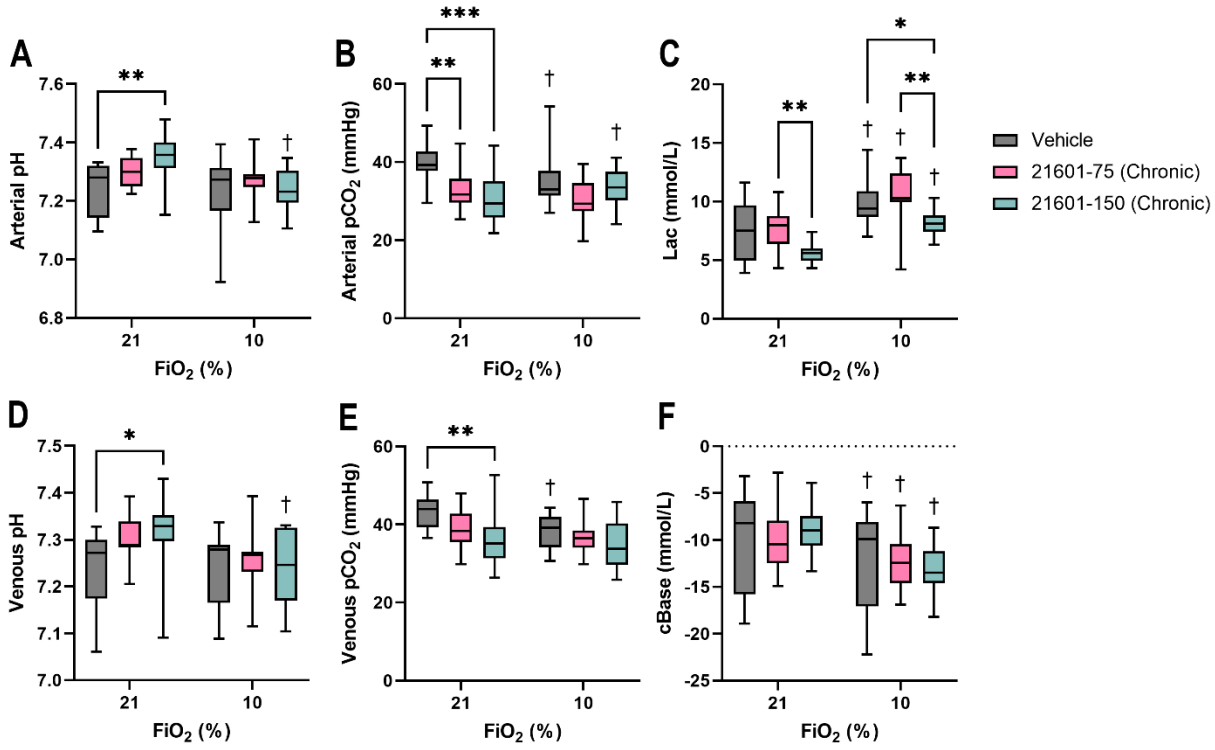

Figure 13: Blood acid-base balance as impacted by chronic treatment of GBT21601. A: The 150 mg/kg treatment increases arterial pH at normoxia; however, no difference is found between groups at 10% hypoxia. B: GBT21601 treatment lowers arterial partial pressure of CO_2 during normoxia. C: The 150 mg/kg treatment lowers lactate during hypoxia. D: The 150 mg/kg treatment increases venous pH at normoxia. E: Lower venous pCO_2 is observed in the 150 mg/kg treatment group at normoxia. Both GBT21601-treated groups have no change in venous pCO_2 during 10% hypoxia. F: GBT21601 does not impact base excess. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; pCO_2, partial pressure of CO_2; lac, lactate; cBase, base excess.

The effects of chronic treatment of GBT21601 on major electrolytes are shown in Fig. 14. While blood potassium measurements increased significantly for all groups during 10% hypoxia, both GBT21601-treated groups showed no difference from each other and both showed significant reductions in increase as compared to the vehicle-treated mice (Fig. 14A). When observing the sodium levels, no significant difference was found between the 75 mg/kg dosed mice and the vehicle-treated control mice (Fig. 14B). The 150 mg/kg dosed mice showed a significant difference compared to only the vehicle-treated mice during 10% hypoxia. Calcium

and chloride ions were consistent between all groups at all timepoints and showed no change due to treatment or hypoxia (Fig. 14C and D).

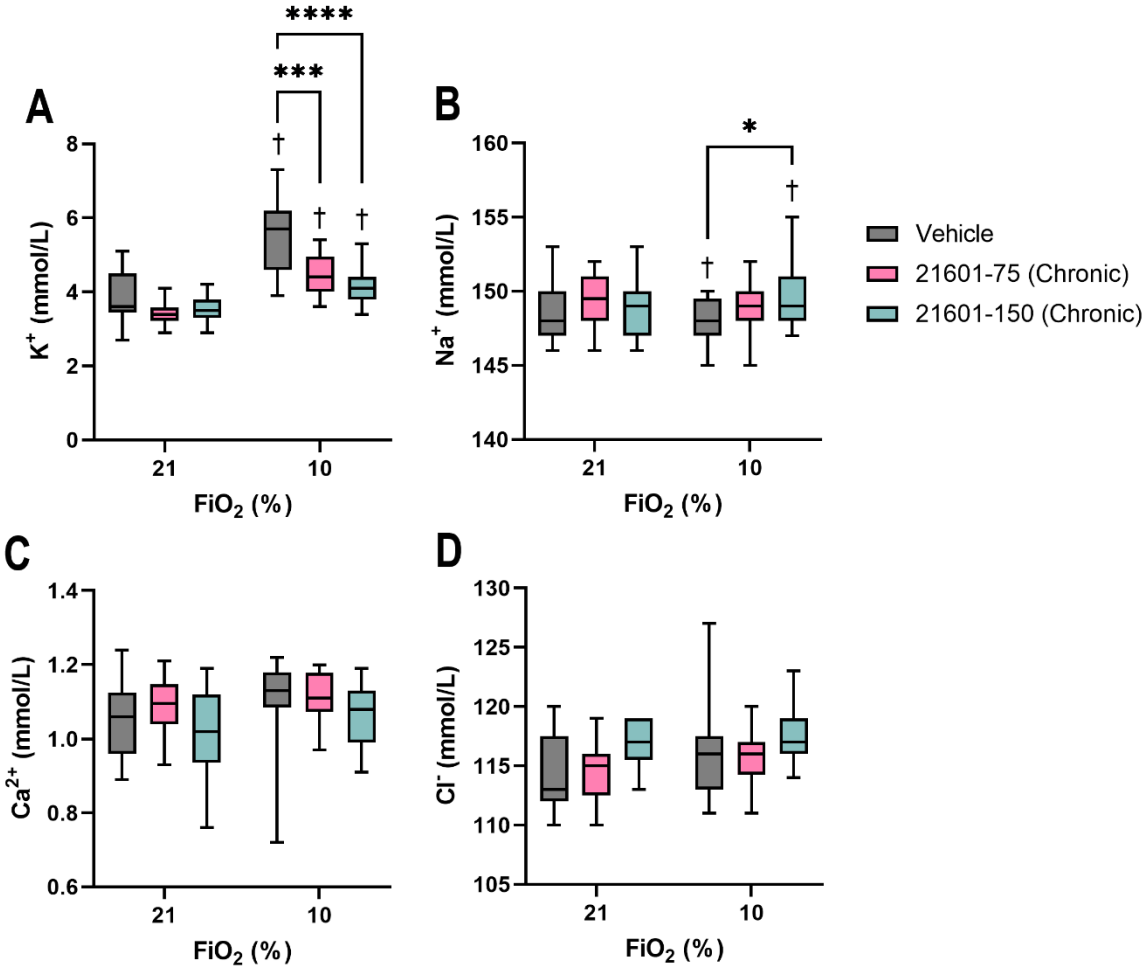

Figure 14: Major electrolytes as affected by chronic GBT21601 treatment at 75 and 150 mg/kg. A: The increase in potassium cations during hypoxia is attenuated by GBT21601. B: The 150 mg/kg treatment increases sodium cations present during hypoxia. C: Calcium ions are unaffected by GBT21601 treatment. D: Chloride ions also show no change with GBT21601 treatment. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2.

2.2.1D Effects on Cardiac MRI

Shown in Fig. 15, GBT21601 greatly affects cardiac function during hypoxia in SCD mice. All groups had significant decreases in HR during 10% hypoxia; however, the lower 75 mg/kg dosed GBT21601 mice maintained the highest HR during hypoxia (Fig.15A). Interestingly, the higher dose of GBT21601 caused a significantly depressed HR at normoxia compared to both the vehicle-treated control as well as the 75 mg/kg-treated group. Although the 150 mg/kg GBT21601-treated group did not experience the same magnitude of decrease observed in the vehicle-treated mice, since the 150 mg/kg group already had decreased HR at baseline, both groups showed similar HR at 10% hypoxia. GBT21601 seems to increase the stroke volume (SV) of SCD mice. The higher dose of 150 mg/kg was observed to have less of an impact when compared to their lower dosed counterparts (Fig. 14B). Consequently, the cardiac output (CO) is highest in the GBT21601-treated mice, again with the lower dosed 75 mg/kg mice experiencing significantly higher CO than the higher dosed 150 mg/kg mice (Fig. 14C). Both GBT21601-treated groups maintained similar CO levels at 10% hypoxia as compared to normoxia, while the vehicle-treated mice experienced a drop in CO during hypoxia. GBT21601 generally seems to increase cardiac function in SCD mice. The higher dose of 150 mg/kg does not increase this effect, however, instead attenuating the increases in cardiac function displayed by mice receiving the lower dose of 75 mg/kg.

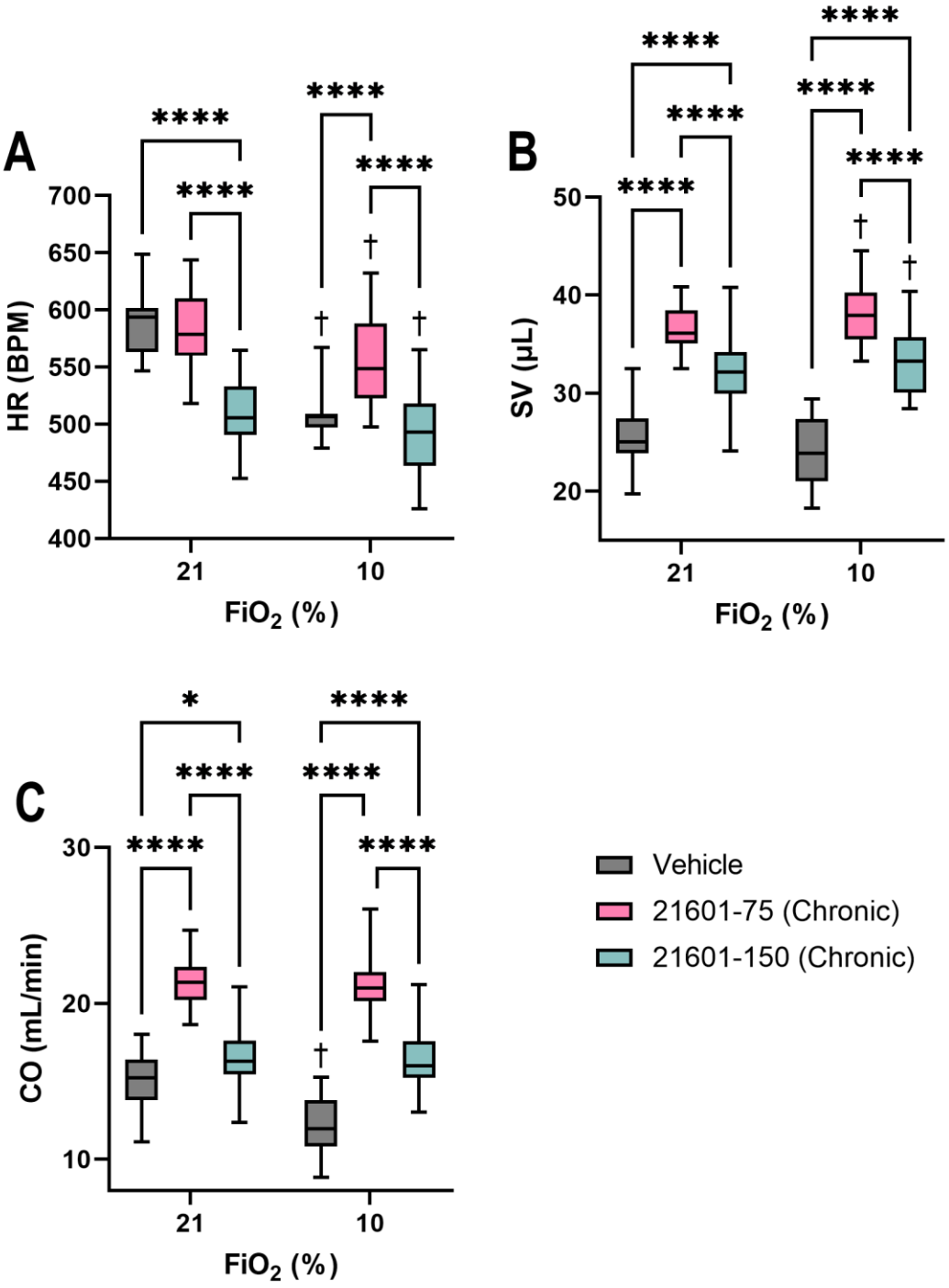

Figure 15: Chronic treatment of GBT21601 may improve cardiac function during hypoxia. A: The larger dose of 150 mg/kg lowered baseline HR, while the lower 75 mg/kg dose better attenuated the decrease in HR during hypoxia. B. Both doses of GBT21601 increased stroke volume with chronic treatment; however, the 75 mg/kg dose had a significantly higher impact. C: Cardiac output is increased by both dose sizes, but the SCD mice given the 75 mg/kg dose had much higher output. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO2, fraction of inspired O2; HR, heart rate; BPM, beats per minute; SV, stroke volume; CO, cardiac output

2.2.1E Effects on CBC

Fig. 16 shows the results of the complete blood count (CBC) done with samples obtained after chronic treatment of SCD with GBT21601 or a vehicle control. Chronic treatment with GBT21601 increased RBC count, and consequently Hb and Hct (Figs. 16A, B, and C). This increased Hb reflects what was previously found during the hypoxia tolerance tests. GBT21601 seems to have a dose-dependency on the magnitude of increase in RBC count, with the 150 mg/kg dosed SCD mice having significantly more RBCs than their lower dosed counterparts.

When examining the effects of GBT21601 on WBC and platelet count, the 75 mg/kg GBT21601-treated group had the lowest measurements of all three groups (Figs. 16D and E). The 150 mg/kg dose of GBT21601 had no effect on WBC count compared to the vehicle-treated mice, but it significantly increased the number of platelets present. This could imply that although the higher dose of GBT21601 has a greater effect on RBC count and Hb concentration, the lower dose may cause less inflammatory and immune response.

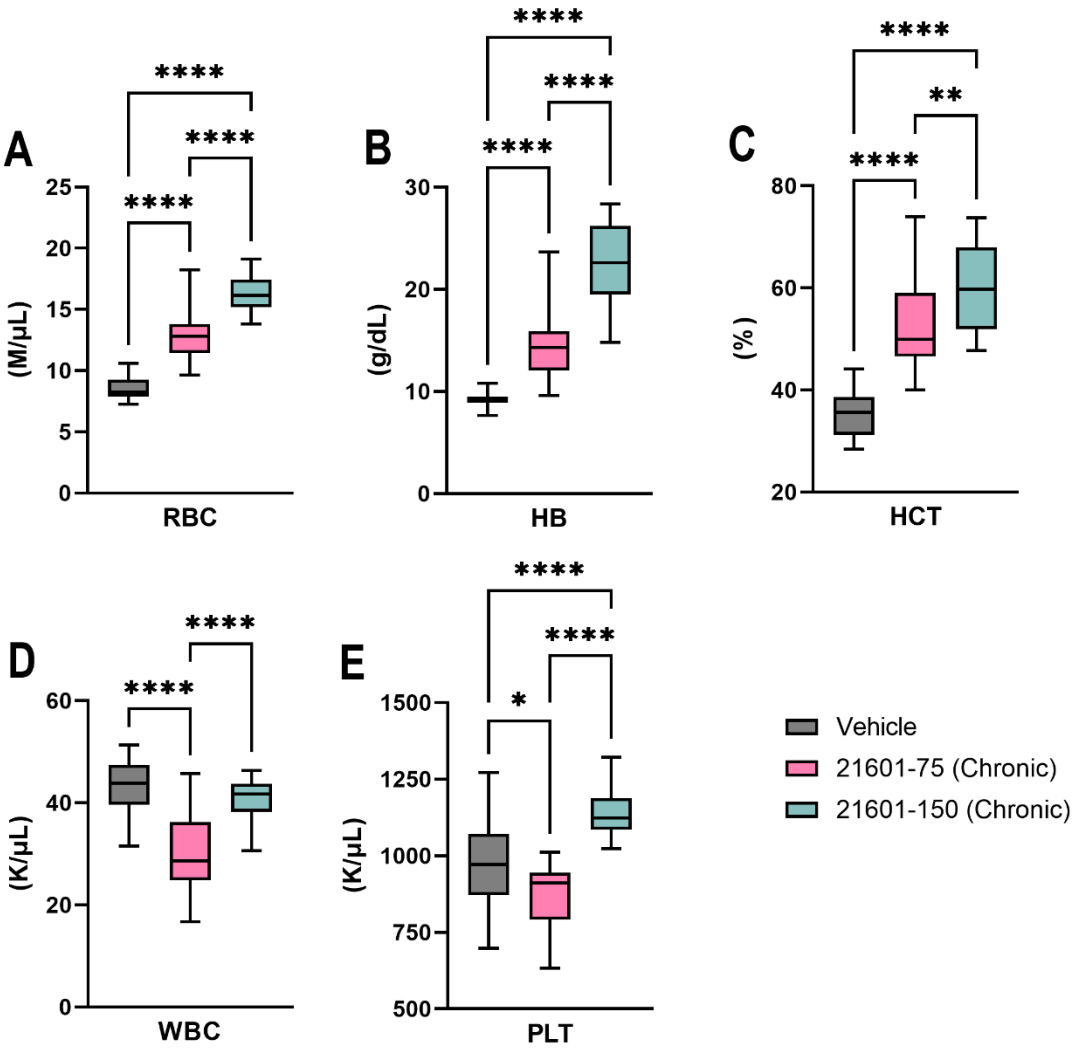

Figure 16: Effects of chronic GBT21601 treatment on complete blood count (CBC). A: Chronic treatment of GBT21061 increase RBC count and shows dose dependent behavior. B: Hb levels are much higher in SCD mice who received treatment with GBT21601. C: Fraction of hematocrit is higher with GBT21601 treatment, as a result of the increased RBC presence. D: WBC count is unaffected by the 150 mg/kg dose, but lowered by the 75 mg/kg dose. E: The 75 mg/kg-treated mice had the lowest number of platelets, while the 150 mg/kg-treated mice had a significantly higher number, possibly indicating adverse immune response. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for one-way ANOVA with Tukey's multiple comparisons test between test groups. WBC, white blood cell; PLT, platelet.

Mean corpuscular volume (MCV) is decreased with chronic treatment of GBT21601 (Fig. 17A). A low MCV could signify iron or nutritional deficiency. Although the 150 mg/kg GBT21601-treated mice had the highest RBC counts, a significantly lower MCV is observed in the 75 mg/kg group. Fig. 17B shows the mean corpuscular hemoglobin (MCH) observed in the

three groups of SCD mice. Chronic treatment of GBT21601 increased both RBC count and Hb, but the average mass of Hb in each RBC is lower in GBT21601-treated mice compared to that of the vehicle-treated. GBT21601 seems to affect MCH identically with both dose sizes. The mean corpuscular hemoglobin concentration (MCHC) is only changed in the 75 mg/kg GBT21601-treated mice, with a significantly lower concentration than both the vehicle-treated and 150 mg/kg -treated mice (Fig. 17C). Although both sized doses of GBT21601 reduce the average mass of Hb present in RBCs, the concentration of Hb present within an RBC is unchanged in the higher 150 mg/kg dose treatment. This is likely due to the significantly lower volume within RBCs shown in Fig. 17A. The range of RBC volume variation is shown in Fig. 17D, the plot of RBC distribution width (RDW). At the 75 mg/kg dose, chronic treatment of GBT21601 results in a significantly lower RDW than both the vehicle-treated and 150 mg/kg -treated mice.

Treatment with GBT21601 increased the amount of RBC, Hb, and Hct in SCD mice. GBT21601 also caused RBCs to be smaller and contain less Hb on average. The 150 mg/kg-treated mice had the same concentration of Hb for their volume, compared to the vehicle-treated control mice, but the 75 mg/kg-treated mice displayed lower Hb concentration. The 75 mg/kg dose of GBT21601 also increased the uniformity of size between RBCs. This effect was not evident in the 150 mg/kg group, which demonstrated decreased RBC volume but similar uniformity compared to the vehicle-treated control.

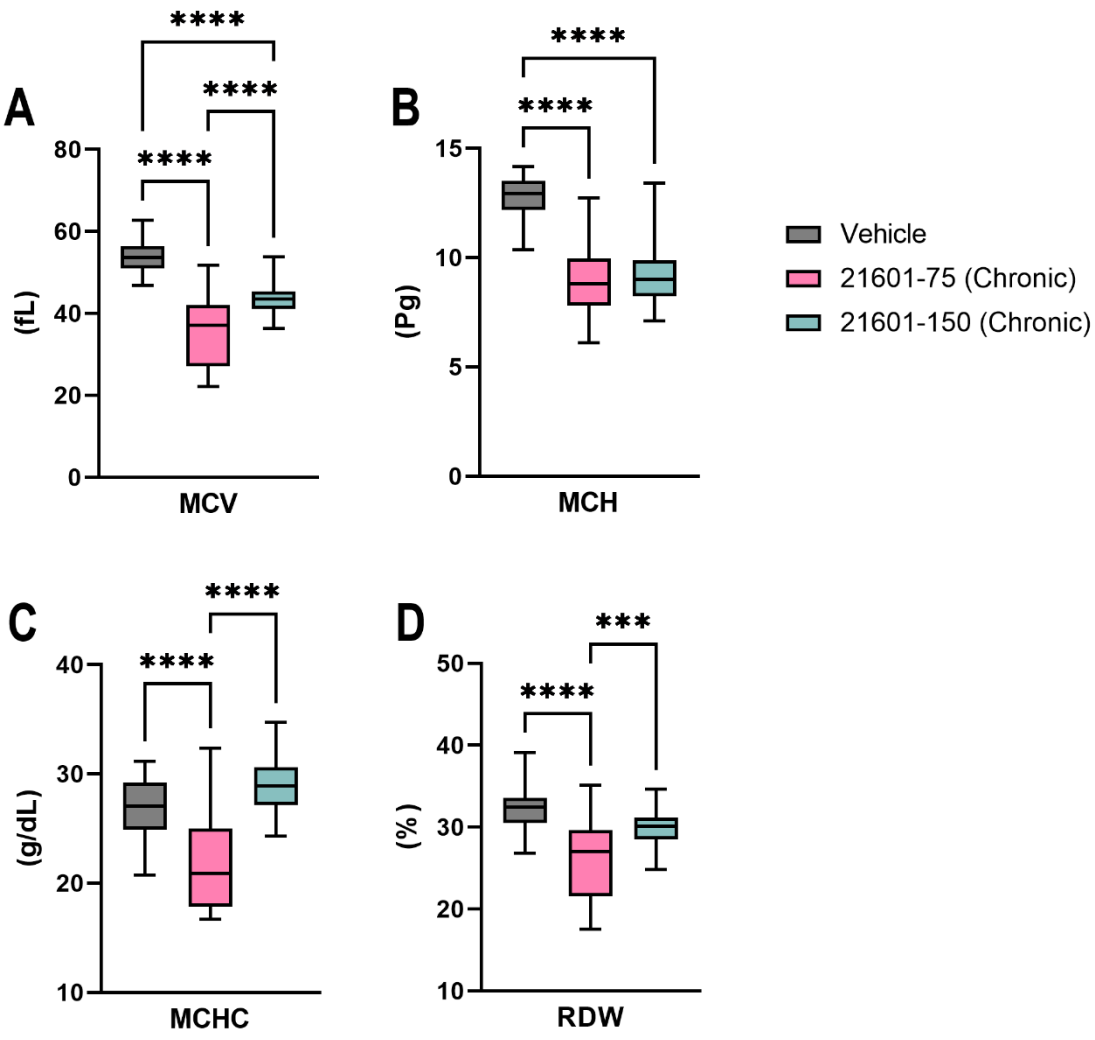

Figure 17: Additional CBC test results showing effects of chronic GBT21601 treatment on RBCs. A: GBT21601 treatment decreases RBC size; however, the higher dose of 150 mg/kg seems to have less of an effect on RBC size than the 75 mg/kg dose. B: Average RBC hemoglobin is equally decreased in both GBT21601 treatments compared to the vehicle-treated SCD mice. C: Concentration of RBC hemoglobin is only affected by the 75 mg/kg-treatment and is significantly lower than both other groups. D. Distribution of RBC size is significantly lower in the 75 mg/kg GBT21601-treated mice, which display more uniformly sized RBCs. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for one-way ANOVA with Tukey's multiple comparisons test between test groups. MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, RBC distribution width.

The effects of chronic treatment of GBT21601 in SCD mice is shown in Fig. 18. Neutrophil (NE) count increases with both dose sizes, compared to vehicle-treated mice, with the 150 mg/kg dose having a significantly greater increase over the 75 mg/kg dose (Fig. 18A).

Similar changes are seen in monocyte (MO) and eosinophil (EO) counts as well. GBT21601 treatment significantly increases the number of cells present and the 150 mg/kg dose size has a significantly larger effect than the 75 mg/kg dose (Figs. 18C and D). Lymphocyte (LY) count is decreased in both GBT21601-treated groups, with the 75 mg/kg dose group having the greatest decrease in lymphocyte presence (Fig. 18B). Basophil (BA) count is increased with GBT21601 treatment, and no difference is found with dose size (Fig. 18E). High NE, MO, EO, and BA presence could indicate infection or inflammation. These results may indicate that the 150 mg/kg dose places more stress upon the immune system.

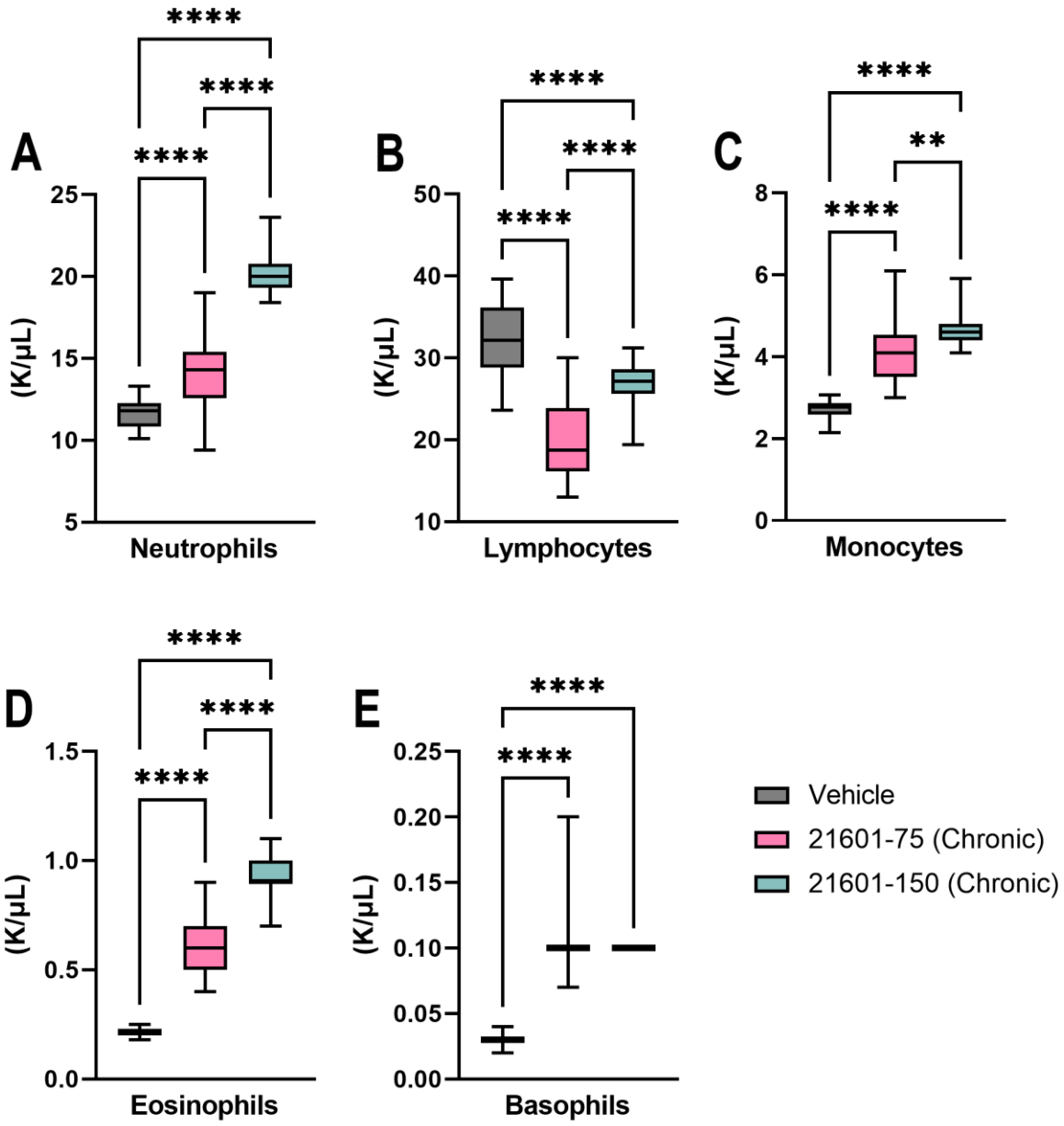

Figure 18: Impact of chronic GBT21601 treatment on presence of various WBCs. A: Both GBT21601 doses increase NE presence; however, the 150 mg/kg dose causes a large increase in NE presence. B: LY counts are decreased with GBT21601 treatment, with the lower dose having a much great drop in LY presence. C: MO is increased with both doses, with a higher MO presence observed with the 150 mg/kg treatment. D: GBT21601 causes increased presence in EO, again the 150 mg/kg dose has a larger effect than the 75 mg/kg dose. E: BA is increased in both GBT21601-treated groups. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for one-way ANOVA with Tukey's multiple comparisons test between test groups. NE, neutrophil; LY, lymphocyte; MO, monocyte; EO, eosinophil; BA, basophil.

2.2.2 Single Dose (Acute) Treatment of SCD Mice with GBT21601

After examining the effects of chronic oral treatment of varying dose sizes of GBT21601, we then repeated the hypoxia tolerance model acute (single-dose) treatments of GBT21601 at different dose sizes. As with the previous tests, we treated Townes SCD mice with either 75 or 150 mg/kg of GBT21601 to observe the effects of different dose sizes.

2.2.2A Tolerance to Hypoxia

The modulation in Hb-O_2 saturation by a single dose of GBT21601 is shown in Fig. 19. In both the 75 mg/kg and 150 mg/kg doses, GBT21601 increases the binding affinity of Hb for O_2 for at least six hours following oral treatment. Fig. 20 shows the partial pressure of O_2 at 20% and 50% Hb saturation over time following a single dose of GBT21601, demonstrating the behavior of the Hb-O_2 affinity over time. Hb-O_2 binding affinity greatly increases during the first two hours, where it stays stable for about an hour before slowly decreasing towards the baseline affinity shown by vehicle-treated mice. The difference between the effect of the two dose sizes are shown in Fig. 21. The higher dose of GBT21601 causes a greater increase in Hb-O_2 binding affinity, most visible during the first few hours of treatment. This change in P50, however, is not significant between the dose sizes.

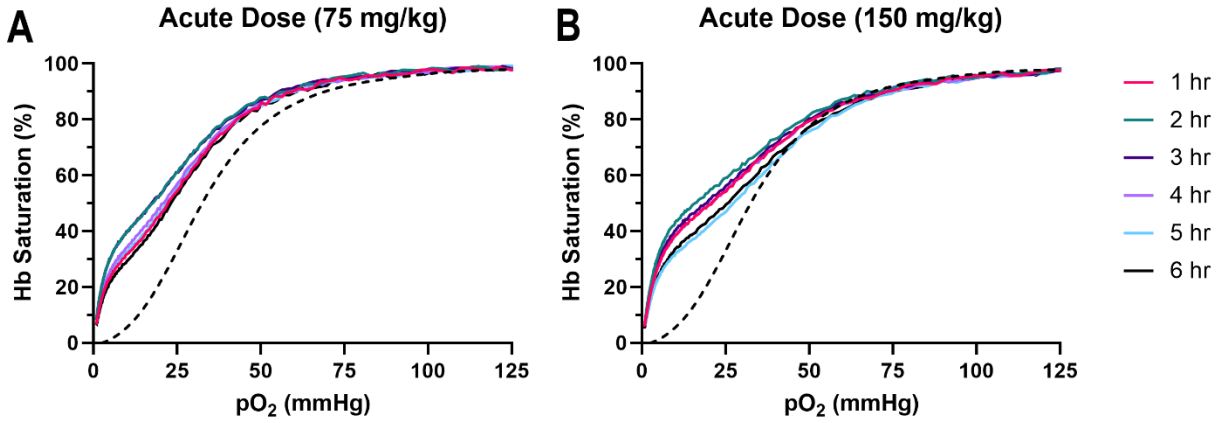

Figure 19: Acute (single dose) treatment of GBT21601 increases Hb-O_2 affinity for at least 6 hours following treatment. Vehicle-treated control is shown with a dashed line. A: At a dose size of 75 mg/kg, acute treatment of GBT21601 shows the largest increases of Hb-O_2 affinity at 2-3 hours following treatment. B: The 150 mg/kg dose shows the greatest increase in Hb-O_2 affinity at 1-3 hours following treatment.

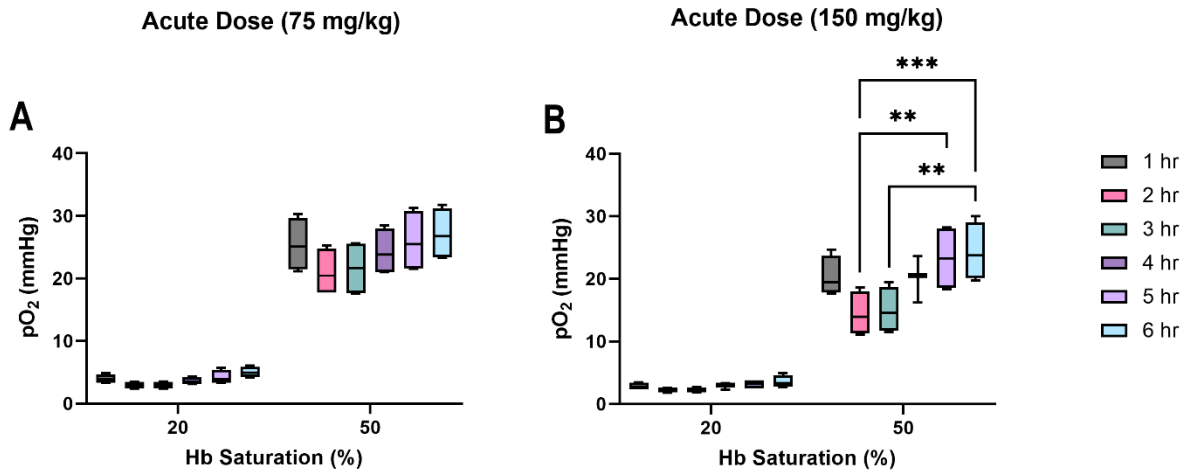

Figure 20: Changes in Hb-O_2 affinity over time following an acute (single dose) treatment of GBT21601. A: A single dose of GBT21601 at 75 mg/kg shows no significant changes in P50 through the course of 6 hours. B. A single dose of GBT21601 at 150 mg/kg shows decreased Hb-O_2 affinity at 6 hours post-treatment compared to the 2- and 3-hours post-treatment. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between timepoints. pO_2, partial pressure of O_2; P50, partial pressure of O_2 at 50% Hb saturation.

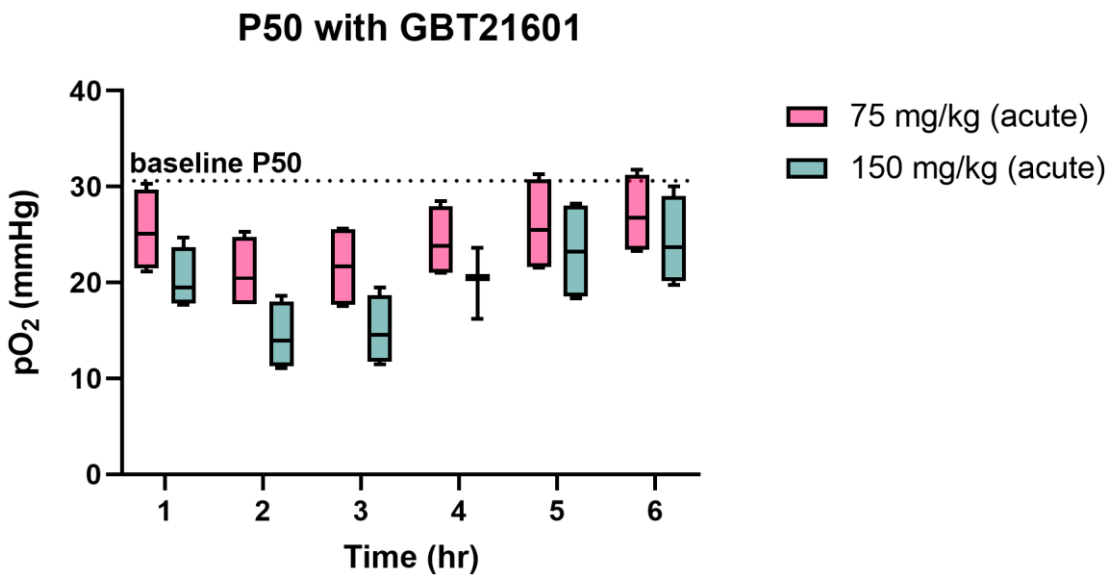

Figure 21: Blood P50 over time following a single dose of GBT21601. Both doses increase Hb-O_2 binding affinity, peaking around 2-3 hours. The lower dose has a higher mean P50 at every timepoint following treatment. No significant difference between dose sizes is found for two-way ANOVA with Šidák multiple comparisons test. pO_2, partial pressure of O_2; P50, partial pressure of O_2 at 50% Hb saturation.

Further results of our hypoxia tolerance tests with acutely dosed SCD mice are shown in Fig. 22. As expected from the previous study, acute treatments of GBT21601 improve hypoxia tolerance of SCD mice in severely low O_2 environments. Again, no difference is seen in the survival curve between the two dose sizes (Fig. 22A). When observing MAP, GBT21601 attenuates pressure decrease incurred during hypoxia, with no significant difference found between acute treatments of either dose size (Fig. 22B). At normoxia, the lower dose of GBT21601 seems to slightly increase MAP. Shown in Fig. 22C, acute treatment of GBT21601 only seems to affect HR in the SCD mice treated with the lower 75 mg/kg dose size. These mice were found to maintain significantly higher HR during normoxia as well as 15% and 10% hypoxia. No difference in HR is found between any group at 5% hypoxia.

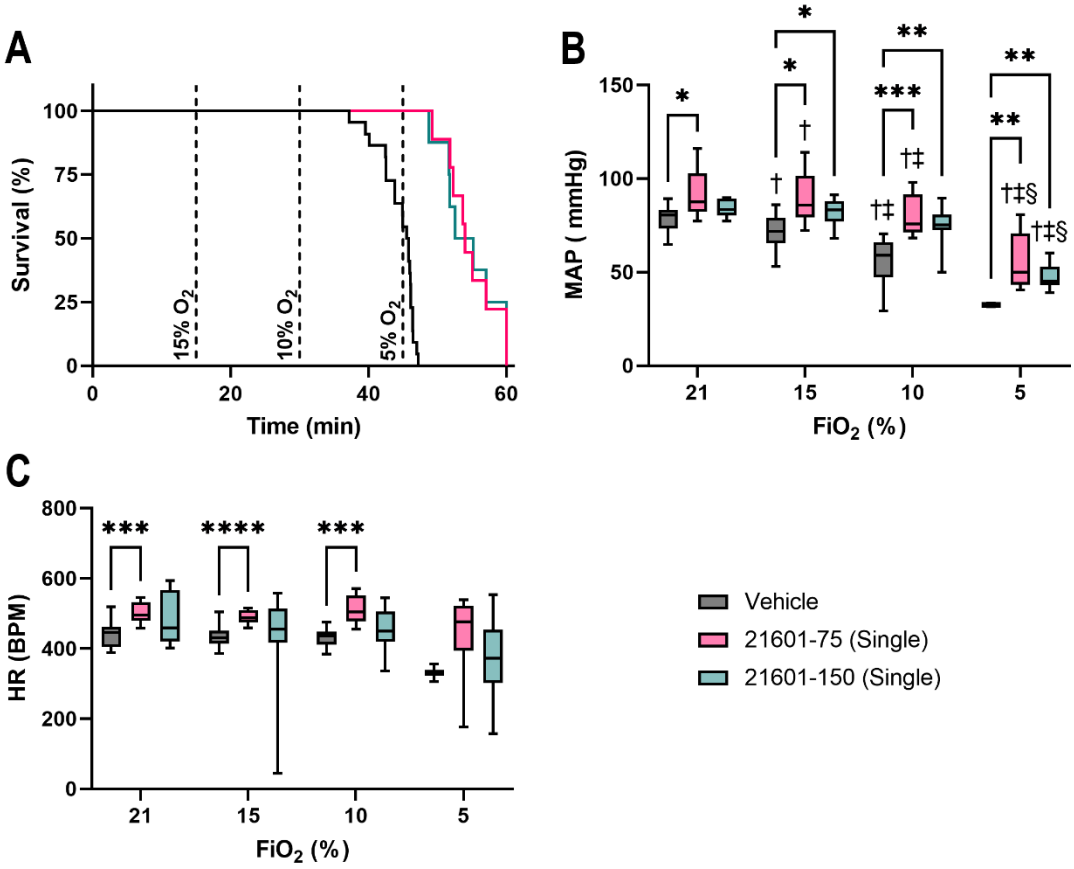

Figure 22: Impact of acute treatment of GBT21601 on hypoxia tolerance, comparing two dose sizes, 75 and 150 mg/kg. A: Both dose sizes of GBT21601 improve survival of SCD mice under severe hypoxia. B: GBT21601 aids in preservation of MAP in low oxygen conditions. C: GBT21601 slightly decreased HR with the 75 mg/kg dose treatment. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2, ‡P<0.05 vs. 15% FiO_2, and §P<0.05 vs. 10% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; MAP, mean arterial pressure; HR, heart rate; BPM, beats per minute.

2.2.2B Oxygenation

When comparing the blood P50 between the chronic and acute treatments, the different dose sizes demonstrate similar effects of Hb-O_2 affinity modulation (Fig. 23). Neither the 75 mg/kg or 150 mg/kg dose sizes are statistically different from each other in their effect on P50 during normoxia with either chronic or acute treatment. Chronic treatment with the 150 mg/kg

dose lowers P50 during 10% hypoxia significantly more than chronic treatment with the 75 mg/kg dose. This was not observed in the single-dose studies, in which no distinguishable difference is observed between the two dose sizes during 10% hypoxia.

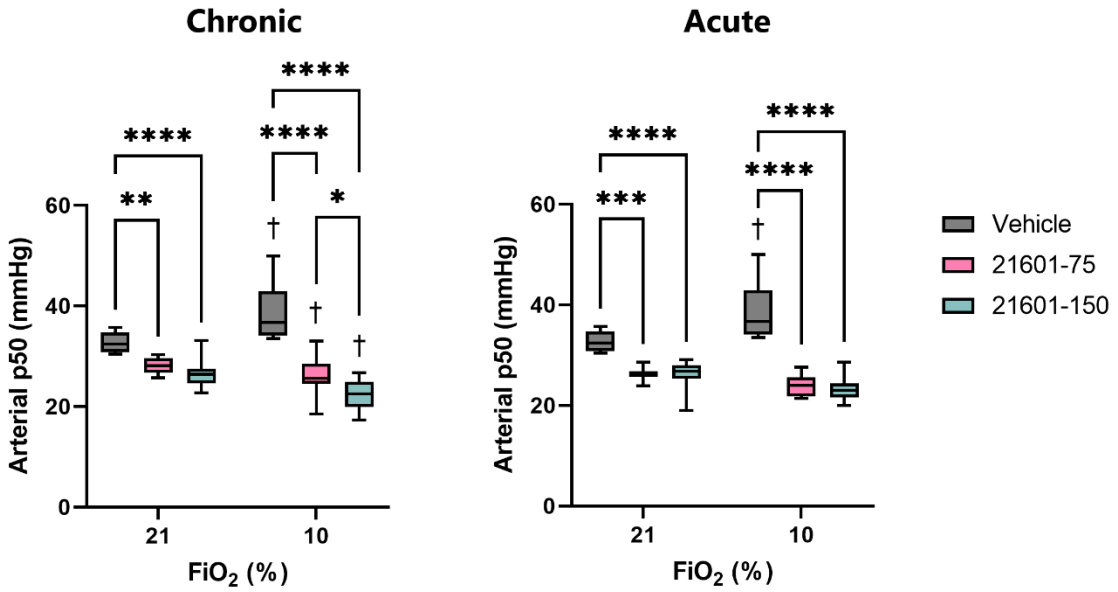

Figure 23: Blood P50 compared between chronic and acute treatments of GBT21601. GBT21601 shows similar effects on blood P50 regardless of dose size or length of treatment. The 150 mg/kg dose only shows significantly greater Hb-O_2 affinity than the 75 mg/kg dose during hypoxia with chronic treatment of GBT21601. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; P50, partial pressure of O_2 required for 50% saturation of Hb.

With acute treatment of GBT21601, total Hb in SCD mice is improved during both normoxia and 10% hypoxia (Fig. 24A). Although all groups experienced decreased total Hb during hypoxia, the GBT21601-treated groups seem to maintain Hb levels closer to normoxia. GBT21601 does not have any significant effect on either arterial or venous pO_2 during 10% hypoxia (Figs. 24B and D). Arterial and venous sO_2 is greatly improved by GBT21601, with both the 75 mg/kg and 150 mg/kg treatments attenuating the drop in sO_2 experienced by the vehicle-treated control (Figs. 24C and E). While the range of values recorded is wider in the 75

mg/kg dosed groups for many of these parameters, no significant difference is found between either dose size. GBT21601 improves oxygenation in SCD mice during hypoxia by first improving both arterial and venous sO_2. This improvement in sO_2 with an acute dose is similar to the improvement observed in GBT1118. It seems, however, that GBT21601 does not affect vessel O_2 tension in the same manner. Density curves for cortical pO_2 measurements are shown in Fig. 25. The curves for all groups are greatly overlapped at both 21% and 10% FiO_2, similar to the previous tests.

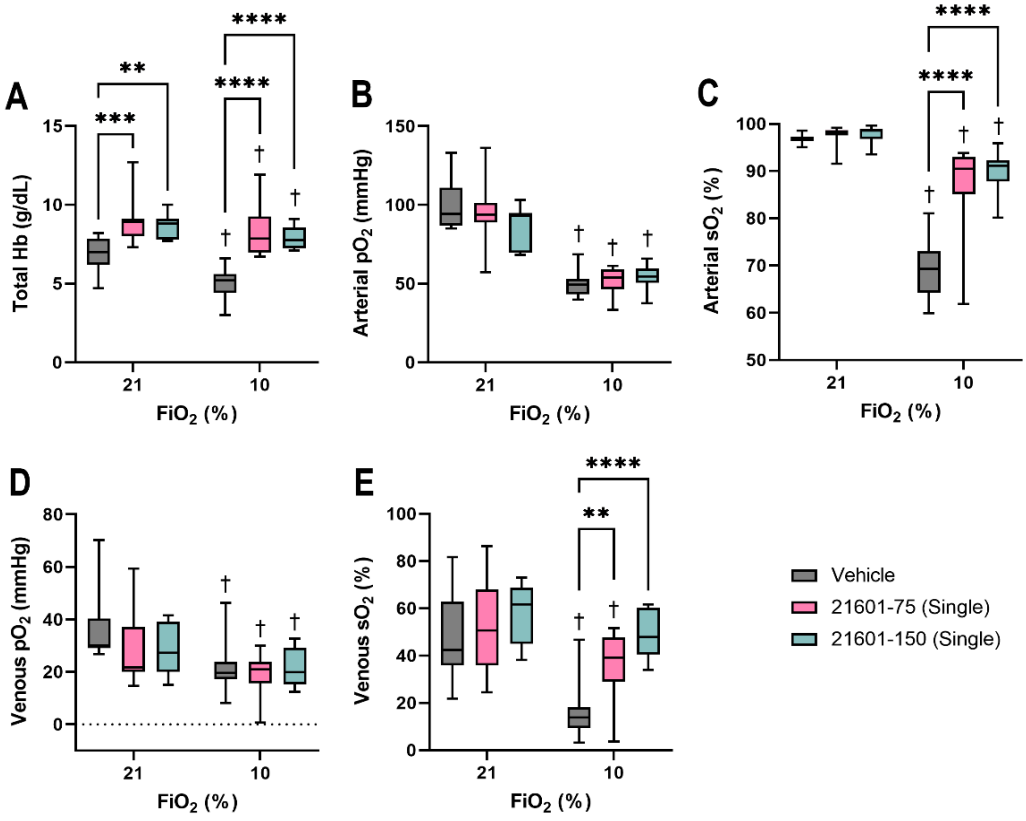

Figure 24: Effects of acute treatment of GBT21601 on Hb and oxygenation. A: A single dose of GBT21601 increases total Hb equally in both tested dose sizes. B: Acute treatment of GBT21601 did not have any significant impact on arterial pO_2. C: Arterial sO_2 is greatly increased with GBT21601 during 10% hypoxia, showing that GBT21601 greatly improves blood oxygenation during hypoxia with even a single dose. C: Venous pO_2 is unchanged by GBT21601 treatment. E: GBT21601 causes venous Hb-O_2 saturation to be much higher during hypoxia. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; pO_2, partial pressure of oxygen, sO_2, oxygen saturation of Hb.

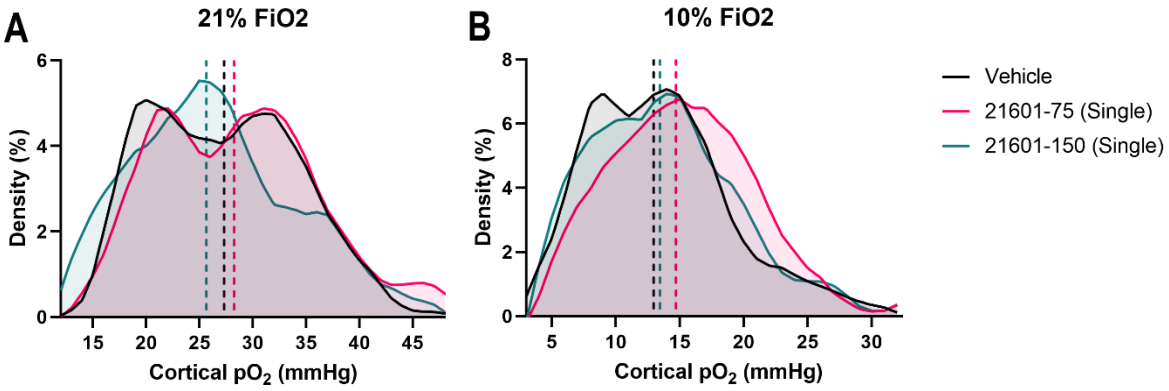

Figure 25: Density curve of cortical pO_2 levels for SCD at normoxia (A) and 10% hypoxia (B). Acute treatment of GBT21601 does not seem to affect oxygenation of high-O_2 consumption tissues such as the brain.

As with chronic treatment, a single dose of GBT21601 significantly improves oxygen delivery and extraction in SCD mice (Fig. 26). While the benefits in oxygen delivery are not as large as observed in the chronically-treated SCD mice, a single dose still imparts substantial benefits to oxygenation. The increased O_2 delivery during normoxia and hypoxia (Fig. 26A) allows tissues to consume greater amounts of O_2 (Fig. 26B). The decreased O_2 extraction ratio during hypoxia shown in Fig. 26C is reflective of decreased oxygen demand in tissues. The increased total Hb and O_2 delivery capacity allows tissues to consume more O_2 during both normoxia and hypoxia, reducing risk of RBC sickling and tissue damage. These results demonstrate that GBT21601 may be useful as an immediate treatment for hypoxia to improve tissue oxygenation and alleviate SCD-related complications.

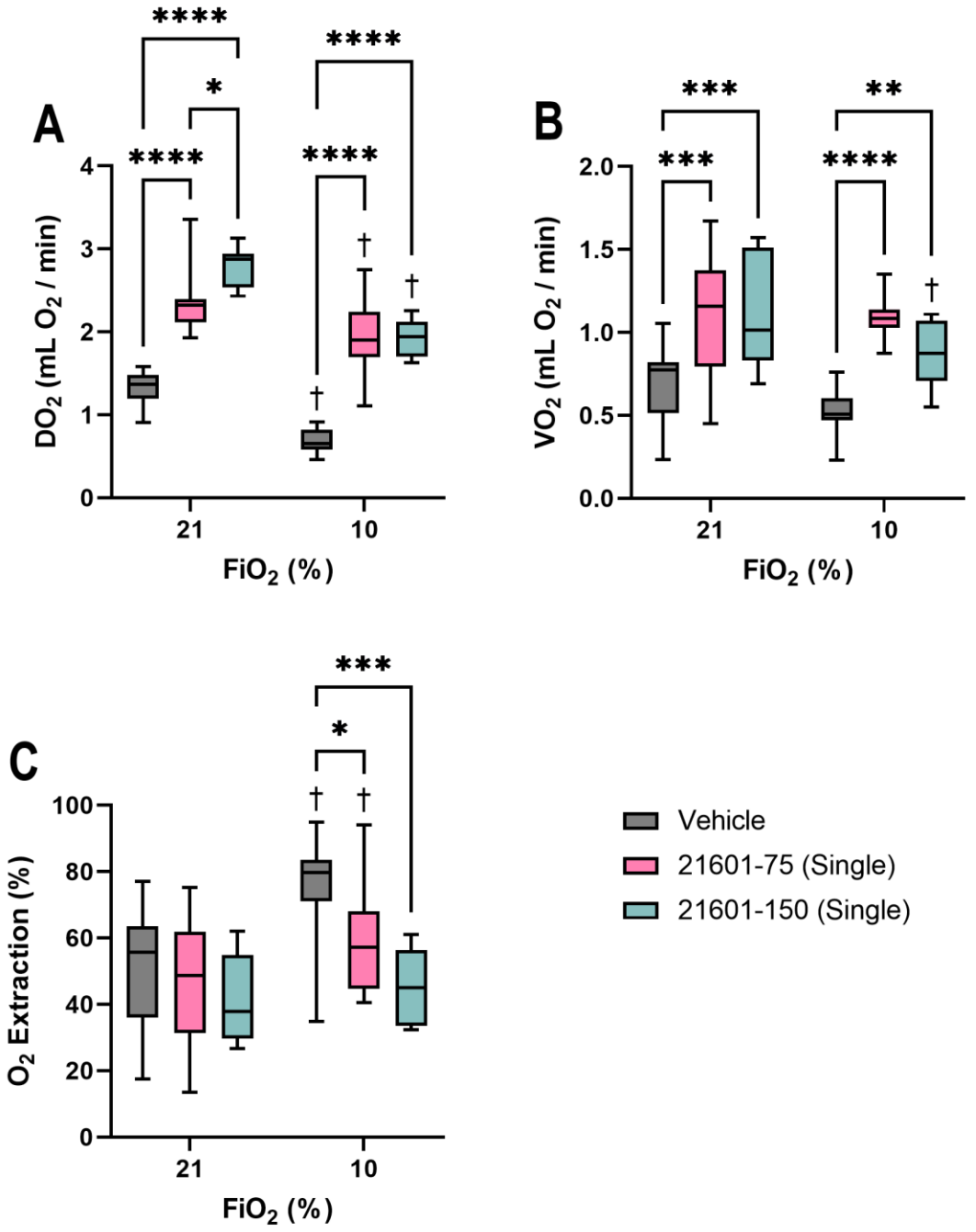

Figure 26: Acute dose treatments of GBT21601 improve O_2 delivery and consumption. A: O_2 delivery is increased two-fold with a single treatment of GBT21601. B: O_2 consumption is also improved with a single dose of GBT21601, both during normoxia and hypoxia. C: There is little difference in the O_2 extraction ratio at normoxia; however, at 10% hypoxia, the GBT21601-treated SCD mice demonstrate lower O_2 extraction ratios due to the comparatively larger increases in O_2 delivered compared to consumed. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; DO_2, oxygen delivery; VO_2, oxygen consumption.

2.2.2C Blood Acid-Base and Electrolyte Balance

The effects of acute treatment of GBT21601 in SCD mice is shown in Fig. 27. GBT21601 significantly increases both arterial and venous pH at 10% hypoxia compared to the vehicle-treated control (Figs. 27A and D). While the pH of the GBT21601-treated groups seems slightly higher at normoxia, there is no significant difference compared to the vehicle-treated mice, except the arterial pH measurements for the 75 mg/kg group. This increased pH is reflected in the decreased measurements for pCO_2 shown in Figs. 27B and E. GBT21601 significantly reduces pCO_2 at both 21% and 10% FiO_2. Figs. 27C and F show little difference between lactate and base excess between all three groups.

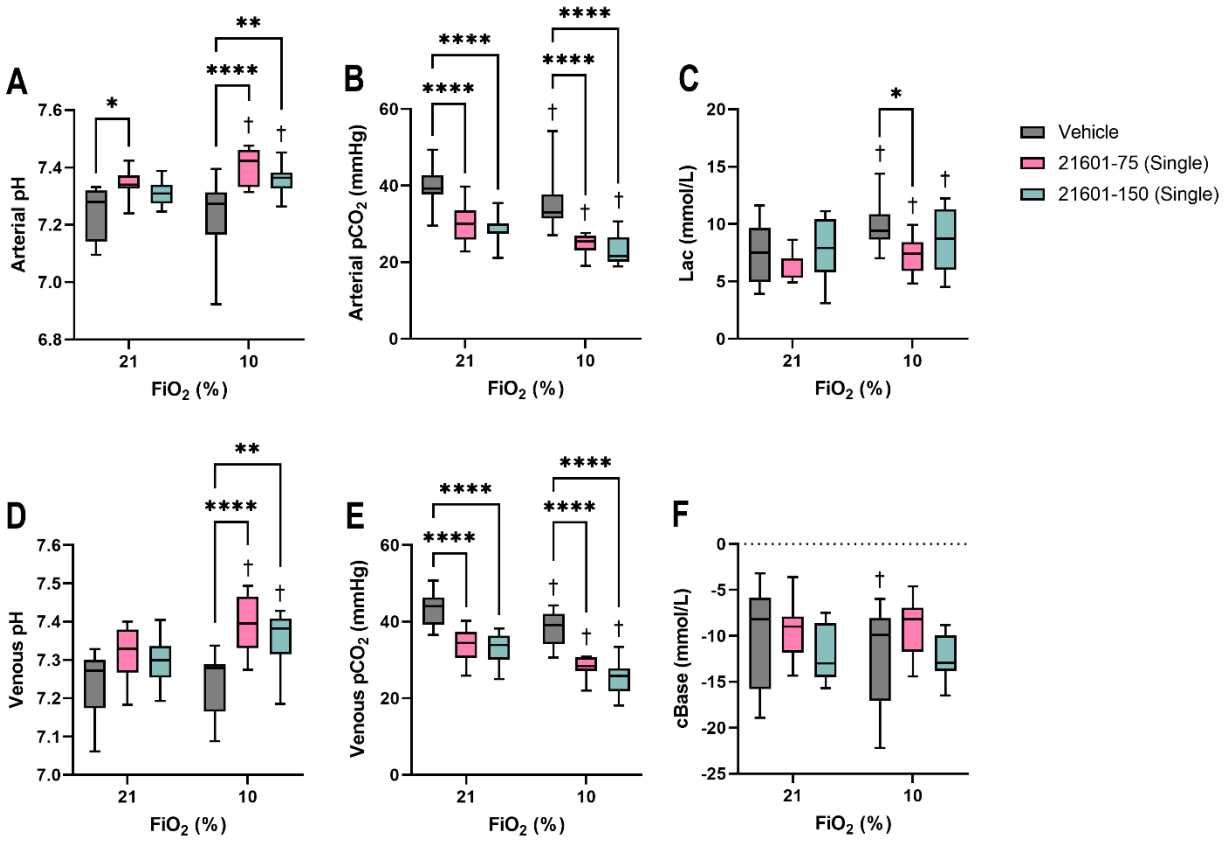

Figure 27: Effects of acute GBT21601 treatment on blood acid-base balance. A: GBT21601 increases arterial pH during hypoxia. B: Both doses of GBT21601 decrease arterial pCO_2 after a single dose, contributing to the lower acidity of blood. C: Lactate does not change much after acute GBT21601 treatment. The 75 mg/kg group has lower lactate during 10% hypoxia compared to both the vehicle-treated and 150 mg/kg-treated groups. D: Venous pH is not significantly impacted at normoxia, but SCD mice treated with GBT21601 have lower blood acidity during hypoxia. E: Venous pCO_2 is also significantly lower after a single treatment of GBT21601, again attributing to the lower observed blood acidity. F: Base excess shows little effect from GBT21601 treatment. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2, ‡P<0.05 vs. 15% FiO_2, and §P<0.05 vs. 10% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; pCO_2, partial pressure of carbon dioxide; lac, lactate; cBase, base excess.

Major electrolytes are shown in Fig. 28. Similar to the results from the chronic treatments, acute treatment of GBT21601 mostly affects potassium cation concentrations in blood, with increased potassium ion concentrations recorded during 10% hypoxia compared to the vehicle-treated control (Fig. 28A). No significant changes were found in sodium, calcium, or chloride ions (Figs. 28B, C, and D).

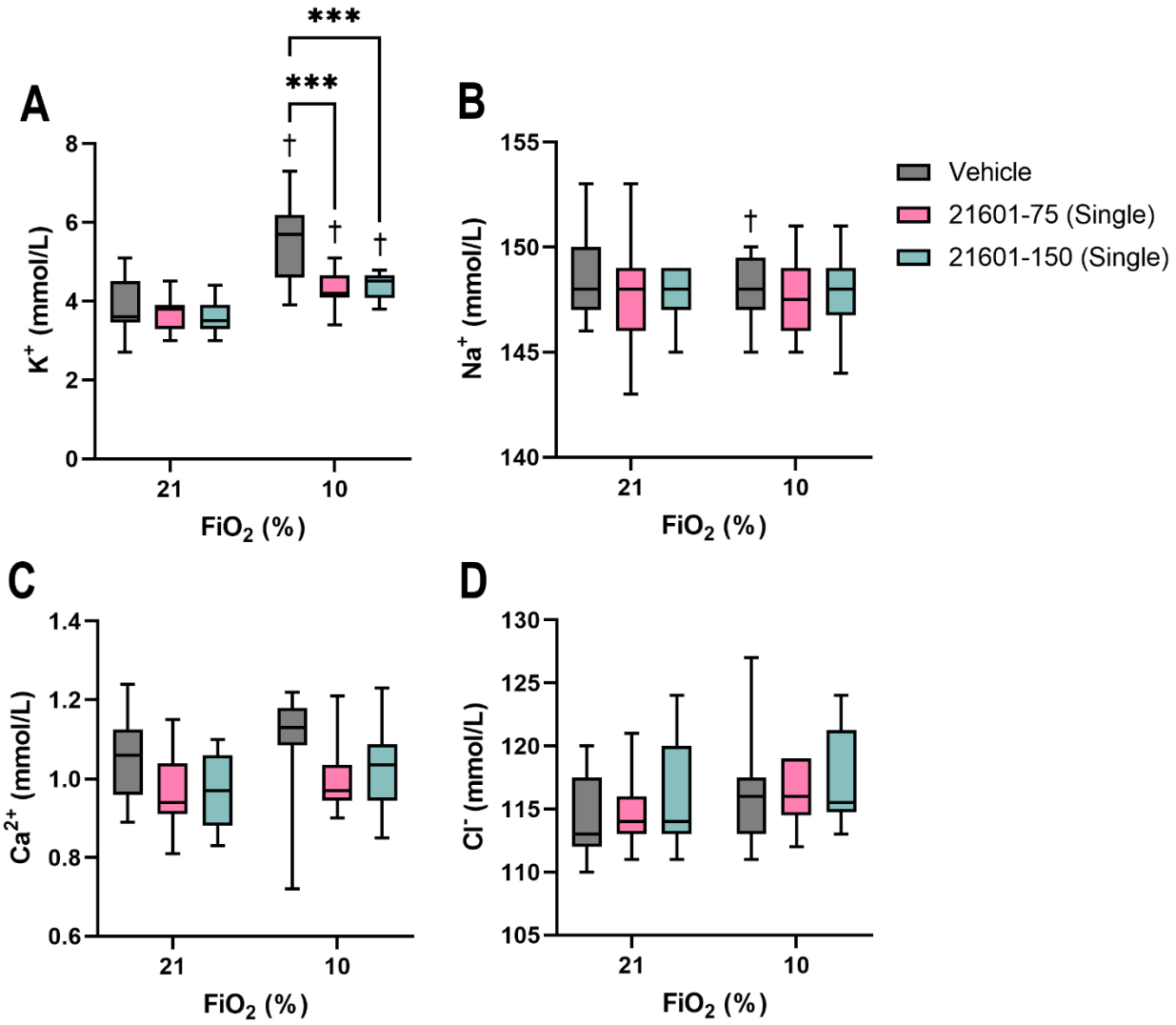

Figure 28: Major electrolytes are largely unaffected by acute treatment of GBT21601. Only potassium is significantly changed compared to the vehicle-treated SCD mice, in which the GBT21601-treated mice have significantly lower potassium ions during 10% hypoxia (A). *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2, ‡P<0.05 vs. 15% FiO_2, and §P<0.05 vs. 10% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2.

2.2.2D Effects on Cardiac MRI

GBT21601 improves cardiac function even with acute treatment (Fig. 29). Although the 75 mg/kg group displayed significantly lower HR during normoxia, no significant difference was found between the groups during hypoxia (Fig. 29A). GBT21601 significantly increases stroke

volume compared to the vehicle-treated mice, regardless of dose size (Fig. 29B). These changes are further reflected in the cardiac output of the GBT21601-treated mice (Fig. 29C). The SCD mice which received GBT21601 had significantly higher cardiac output both at normoxia and 10% hypoxia. At normoxia, there was a significant difference in CO between the 75 mg/kg and 150 mg/kg dosed mice, reflecting the lower HR in the 75 mg/kg group. It does not seem that the 150 mg/kg dose improves cardiac function over to the 75 kg/mg dose.

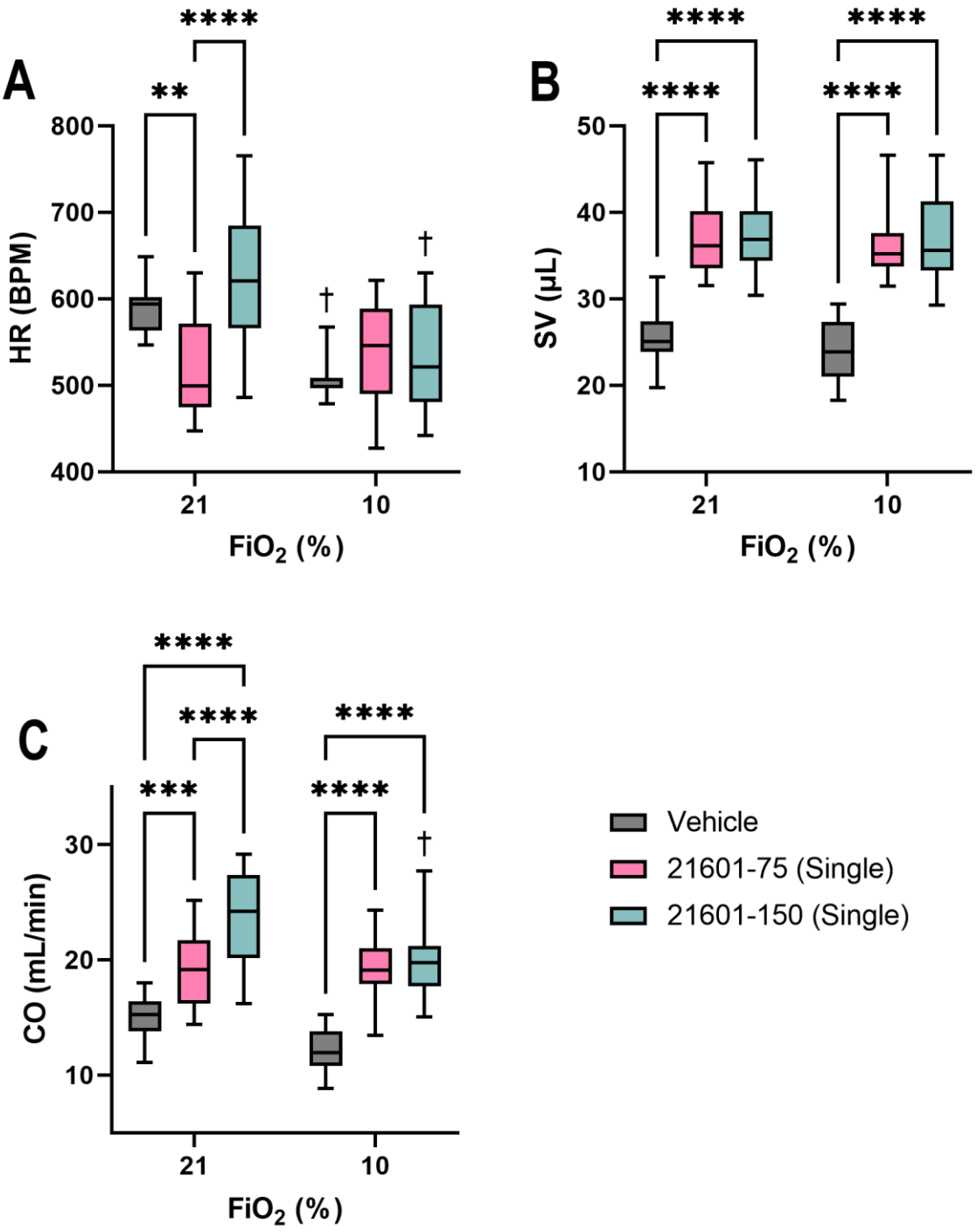

Figure 29: Cardiac function at normoxia and hypoxia following an acute treatment of GBT21601. A: The 75 mg/kg dosed group has a lowered HR at normoxia, but is unaffected by 10% hypoxia. B: GBT21601 causes a great increase in SV in SCD, both at normoxia and 10% hypoxia. C: GBT21601-treated SCD display much higher CO at normoxia and hypoxia, demonstrating cardioprotective properties of GBT21601 in treatment of SCD. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; HR, heart rate; BPM, beats per minute; SV, stroke volume; CO, cardiac output.

2.2.3 Cardiac Function in Chronic and Acute Treatment of GBT21601

Fig. 30 shows cardiac function of the GBT21601-treated SCD mice, comparing dose sizes at both acute and chronic treatment. Chronic treatment of 75 mg/kg GBT21601 maintains or improves the benefits in cardiac function as seen with a single dose. This is not the case with the larger dose size of 150 mg/kg. Instead, the 150 mg/kg group that received chronic treatment performed worse than their acutely-treated counterparts. When comparing the different groups at normoxia, the SCD mice receiving an acute 150 mg/kg dose treatment had significantly higher HR, and consequently CO, than the other groups but performed no differently than either 75 mg/kg dosed group during hypoxia. Additionally, the chronically-treated 150 mg/kg GBT21601 had the lowest HR, SV, and CO of all the GBT21601-treated mice. It seems that while a higher acute dose has greater effects on cardiac function during normoxia, some of the positive benefits of GBT21601 may be lost with chronic treatment of a high dose.

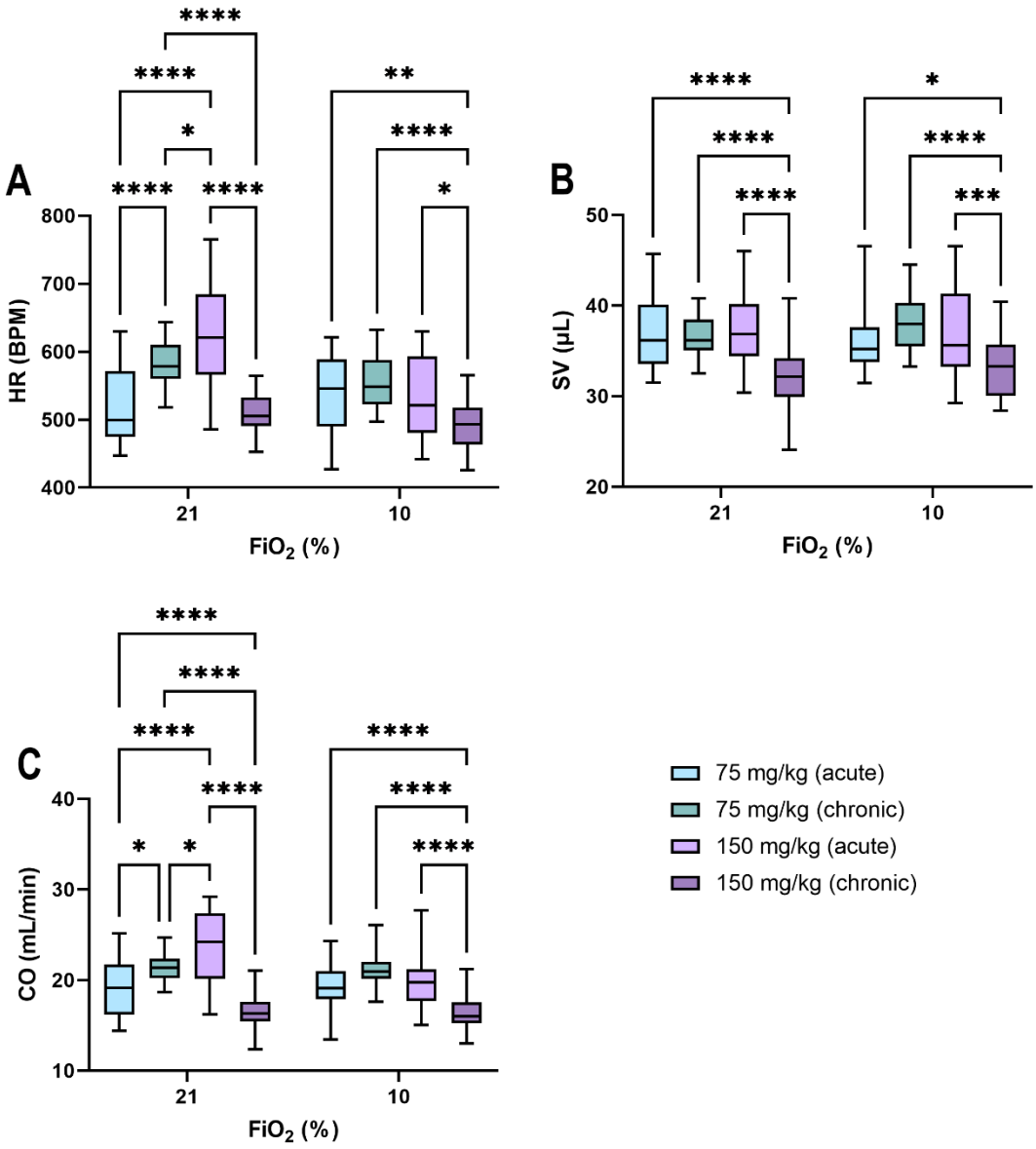

Figure 30: Cardiac function in GBT21601-treated SCD mice comparing both acute and chronic treatments of 75 and 150 mg/kg doses. A: Chronic treatment with 75 mg/kg further improves HR during normoxia compared to acute treatment, whereas chronic treatment with 150 mg/kg is unable to maintain the effects seen from a single dose. During hypoxia, the chronically-treated 150 mg/kg mice had significantly lower HR than all other GBT21601-treated mice. B: The chronic treatment of 150 mg/kg dose size imparts less of a benefit than all other GBT21601 treatments. C: Chronic treatment of 75 mg/kg improves CO at normoxia over a single dose, but decreases CO for the 150 mg/kg dosed mice. During hypoxia, the SCD mice receiving chronic treatment of GBT21601 have significantly lower CO than all other GBT21601-treated mice. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups. FiO_2, fraction of inspired O_2; HR, heart rate; BPM, beats per minute; SV, stroke volume; CO, cardiac output.

2.3 Discussion

Previously we studied GBT1118 to determine the safety of Hb-O_2 affinity modulating pharmaceuticals in high O_2-consumption tissues and tissues affected by SCD. We found that the drug-modulated increase in Hb-O_2 affinity does not negatively affect cortical tissue pO_2 and may have beneficial properties in a SCD model (34). In this study, our goal was to identify dose-dependent properties of such drugs. We used GBT21601, a newer formulation analog for voxelotor. In our previous studies, we treated SCD mice with 100 mg/kg of GBT1118, and in this study we used dose sizes of 75 and 150 mg/kg GBT21601 to determine if higher or lower doses have significantly different effects on cardiac and hematological parameters.

As expected, chronic treatment with GBT21601 increased total Hb present, similar to GBT1118. This change was significantly higher in the 150 mg/kg dosed group; however, there was no significant improvement in oxygenation and hypoxia tolerance compared to the 75 mg/kg dosed group. Chronically-treated GBT21601 SCD mice had higher O_2 delivery and consumption than both vehicle-treated and GBT1118-treated SCD mice. While GBT1118 already improved O_2 delivery, there was no significant difference found in O_2 consumption compared to vehicle-treated SCD mice. SCD mice treated with GBT21601 displayed even greater volumes of O_2 carried and delivered to tissues, as well as higher consumption of O_2, both during normoxia and hypoxia. This could indicate that GBT21601 may be more beneficial in preventing against hypoxia-induced tissue damage than GBT1118.

The increased Hb-O_2 affinity with GBT21601 improved both arterial and venous sO_2 at both normoxia and 10% hypoxia, showing the same positive benefits for SCD mice previously found with GBT1118. The newer formulation GBT21601 showed slightly different effects on blood pH, increasing pH compared to vehicle-treated controls where GBT1118 decreased blood

pH. The decrease in acidity caused by GBT21601 treatment is likely due to decreased pCO_2 as well as lactate. The lowered pCO_2 may imply that GBT21601 improves bulk ventilation in SCD mice compared to both vehicle-treated and GBT1118-treated SCD mice (35).

No additional benefit was found in sO_2 with chronic treatment of 150 mg/kg GBT21601 over 75 mg/kg. Additionally, no difference was found between the Hb saturation or hypoxia tolerance survival curves between the two dose sizes. When observing cardiac function, the SCD mice treated with a lower dose of GBT21601 demonstrated higher CO during both normoxia and 10% hypoxia. The 75 mg/kg dose size induced far less immune response than the 150 mg/kg dose, with fewer of platelets and WBCs. The higher presence of immune cells with the 150 mg/kg dose may suggest that high doses of GBT21601 negatively affect the immune system. The lower dose of GBT21601 shows similar benefits in tissue oxygenation and protection against hypoxia in SCD tissues with less stress on the immune system.

At normoxia, there is no difference in the change in blood P50 induced by chronic treatment of GBT21601. The 150 mg/kg dose only showed significant improvements over the 75 mg/kg dose during hypoxia, in which higher dosed mice displayed higher Hb-O_2 affinity. This difference is nonexistent when only an acute treatment is given. An acute dose over 75 mg/kg has no further effect on Hb-O_2 affinity modulation at either normoxia or hypoxia.

Although chronic treatment with 150 mg/kg displayed lower blood P50 during hypoxia than the 75 mg/kg dose, the lower dose may be better for long-term treatments. With chronic treatments of the 75 mg/kg dose, SCD mice displayed significantly higher Hb-O_2 affinity compared to vehicle-treated SCD mice. Additionally, when comparing cardiac function between acute and chronic treatments of GBT21601, continued treatment of 150 mg/kg decreasing benefits when compared to a single dose. This is not the case with the 75 mg/kg dose, which

retains or even slightly improves its effects on the heart with long-term use. Higher doses of GBT21601 may not maintain the same level of protection over long courses of treatment. It is unclear why this is the case with the 150 mg/kg dose, and more studies should be done to better understand the efficacy of different doses of GBT21601.

These studies face the same potential limitations as the GBT1118 studies. The results found here may not reflect human use due to previously discussed limitations with the murine SCD model presented. Additionally, these experiments used only male mice, and it is known that sex hormones affect Hb-O_2 affinity, with estrogens favoring a decrease and androgens favoring an increase in binding affinity (36). This may be due in part to the effect estrogens have on nitric oxide (NO). Estrogens have been found to increase NO concentration and are thought to play a role in the production of NO synthase (37, 38). NO is also known to affect Hb-O_2 affinity, and may improve Hb-O_2 affinity in SCD tissues (39, 40, 41). This may mean that female mice could display an improved response to SCD both with and without GBT1118 or GBT21601 treatment. Trials testing the use of steroid contraceptive treatments in humans with SCD have also suggested that some hormones may reduce SCD-related symptoms (42). It is unclear what the full effects of sex hormones on Hb-O_2 affinity and O_2 transport are.

2.4 Conclusion

Within a mouse model of SCD, chronic GBT1118 treatment increases in Hb-O_2 affinity and Hb, consequently improving O_2 delivery during both normoxia and hypoxia. GBT21601 shows the same benefits while also improving O_2 consumption. GBT21601 may also have greater benefits on cardiac function. In particular, when chronically-treated with 75 mg/kg of GBT21601, SCD mice exhibited higher HR, SV, and CO. This increased CO may improve oxygenation within high-consumption tissues and organs and may protect SCD tissues more

during hypoxia. Additionally, the similar performance but increased immune response of the 150 mg/kg dose suggests that high dose treatments of GBT21601 may have little benefit over lower doses. It is unclear why the higher dose treatment lost efficacy in cardioprotective properties with chronic treatment. Further studies should be done to better understand the dose-dependent properties and potential side effects of chronic treatment of GBT21601 for SCD.

Chapter 2: Exogenous Estradiol Treatments

Section 3: Sex as a Variable

3.1 Estrogens and the Cardiovascular System

Sex hormones are known to cause various differences in pathological and physiological responses. Cardiovascular diseases have been well-documented to differ significantly between men and women, as well as between pre- and post-menopausal women (43, 44, 45). Estrogens have protective effects in oxidative-stress mediated diseases and may directly affect regulatory mechanisms within the cardiovascular system (44, 45). In inflammatory models comparing male and female animals, estradiol treatments were found to attenuate NO increases and reduce expression of the TNF-α inflammatory cytokine produced by macrophages (46, 47).

The previous hypoxia model was repeated with female mice, comparing them to male mice. A septic shock model was also done to observe the role of estrogen in inflammatory response. In both models, two separate groups of female mice were observed, estradiol-treated and untreated females. For this study, wild type C57BL/6 mice were used in all groups.

3.2 Verification of Estrous Cycle Phase

Vaginal cells were obtained via lavage for 25 untreated females and 10 estradiol-treated females. The cell samples were then placed on slides, stained with crystal violet, and analyzed to determine what phase of the estrous cycle the mice were in. The results are shown in table 2, and visually in Fig. 31. About half of the untreated mice were in the estrus phase at 51%, 11% were in proestrus, 14% were at metestrus, and the remaining 22% were in diestrus. All of the estradiol-treated mice were found to be in the estrus phase. This was expected, because the elevated estradiol levels should have locked the treated mice in the estrus phase.

Table 2: Estrous phase of female mice determined by microscopy from cell slides obtained with vaginal lavage. The untreated mice are found in all phases of the estrous cycle, but all of the estradiol-treated mice remain in the estrus phase.

Estrous Phase	Untreated	Treated
Estrus	18	10
Proestrus	4	0
Metestrus	5	0
Diestrus	8	0
Total	35	10

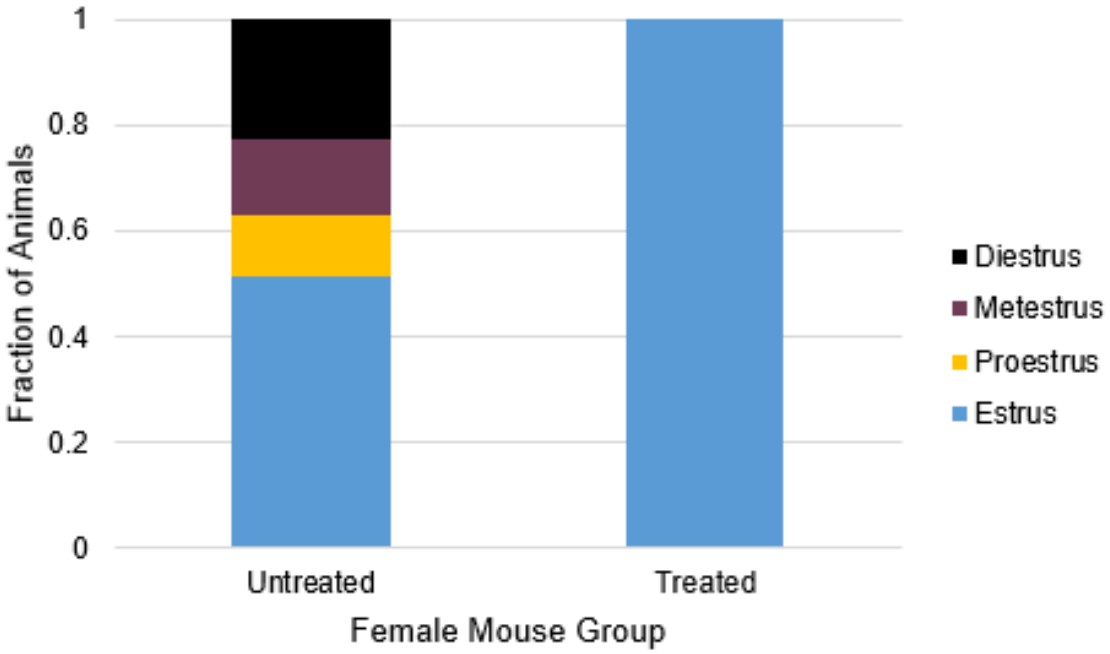

Figure 31: Estrous phase of female mice comparing estradiol-treated and untreated mice. Treatment with estradiol locks the mice into estrus.

Section 4: Exogenous Estradiol in a Hypoxia Model

4.1 Introduction

Following the results of our previous hypoxia tolerance tests, we then wanted to determine how hypoxia tolerance differs between male and female mice. Estradiol has previously been found to decrease HIF-1α (hypoxia-inducible factor 1-α) expression in rats (48). Additionally, estrogen has been shown to prevent hypoxia-induced vascular injury and hypertension (49, 50, 51, 52). Estrogen plays a direct role in hypoxia signaling pathways, protecting against tissue damage and disease pathogenesis. Due to this, we were interested in observing the effects of exogenous estrogen treatments on acute hypoxia tolerance and oxygenation.

4.2 Results

4.2.1 Tolerance to Hypoxia

When exposed to decreasing FiO_2, the three groups of mice displayed similar tolerance. The results of the tests are shown in Fig. 32. All groups had very similar survival curves, with no statistical significance was found with a log-rank test (Fig. 32A). No significant difference was found between MAP for each group within each timepoint, with all groups experiencing greatly depressed MAP at 10% and 5% FiO_2 (Fig. 32B). In Fig. 32C, we can see that HR stayed fairly consistent throughout the hypoxia tolerance trial. The male mice had higher HR than the female mice, but not the estradiol-treated female mice, at normoxia and 15% FiO_2. The male mice were also the only group to experience a significant decrease in HR during the test.

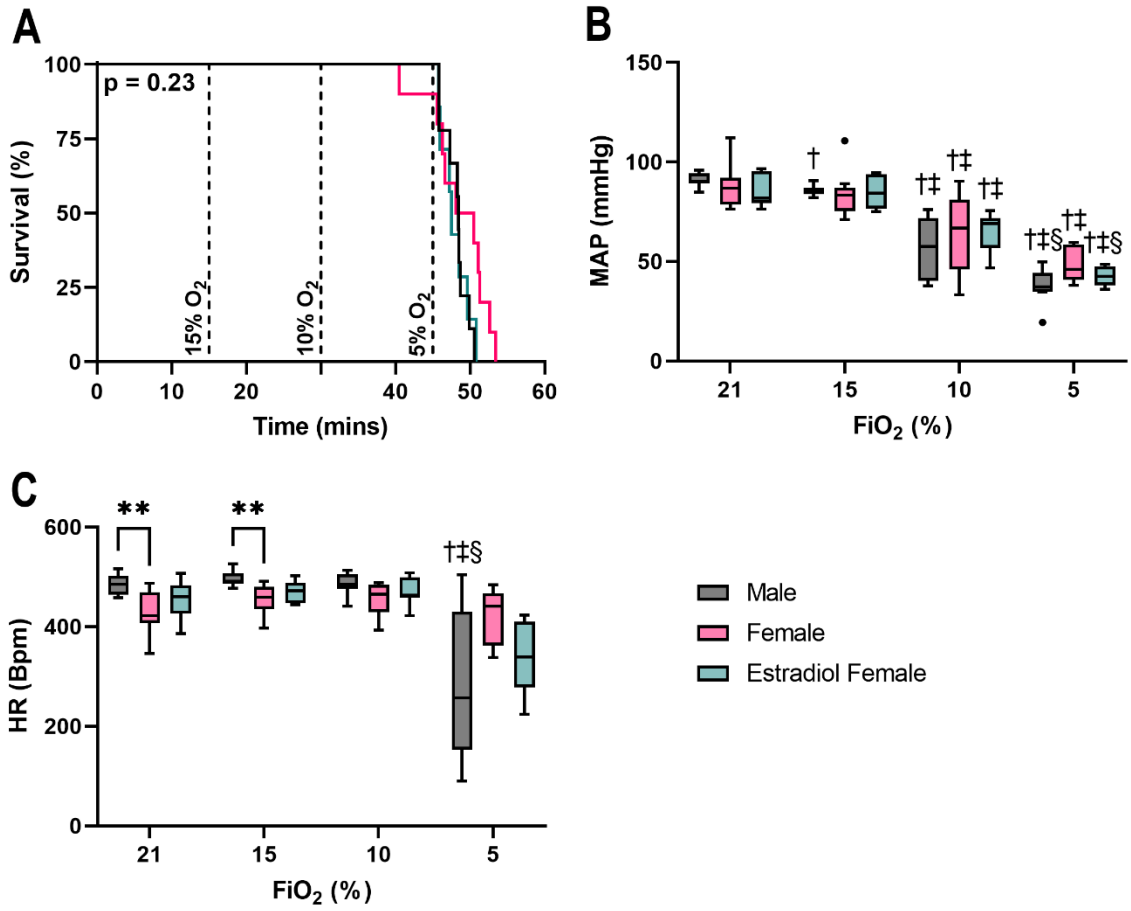

Figure 32: Hypoxia tolerance comparing male, female, and estradiol-treated female mice. A: No significant difference is found in the survival curves of the three groups. B: MAP decreases similarly between all groups as the severity of hypoxia is raised. At 10% and 5% FiO_2, all groups display significantly lower MAP than at normoxia and 15% FiO_2. At 5% FiO_2, the male and estradiol-treated female groups are also significantly lower than at 10%; however, no difference is found between the tree groups within any timepoint. C: HR in the male group is significantly higher than the female group at normoxia and 15% hypoxia. At severe 5% hypoxia, the male group displays significantly lower HR than previous timepoints. Survival P value was calculated via the log-rank test. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO2, ‡P<0.05 vs. 15% FiO2, and §P<0.05 vs. 10% FiO2 for two-way ANOVA with Šidák multiple comparisons test within each test group. MAP, mean arterial pressure; FiO_2, fraction of inspired O_2.

4.2.2 Blood Oxygenation

Estrogen does not seem to have much of an impact on blood oxygenation during either normoxia or acute 10% hypoxia. Both female and estradiol-treated female mice had similar

concentrations of total Hb as compared to male mice (Fig. 33A). Additionally, there was no observed difference in partial pressure of O_2 between groups (Fig. 33B). Estradiol-treated female mice displayed slightly higher blood oxygen saturation during 10% hypoxia compared to male mice (Fig. 33C). This difference is likely minimal, however, as no significant difference in found in blood P50 between the groups at 10% hypoxia (Fig. 33D).

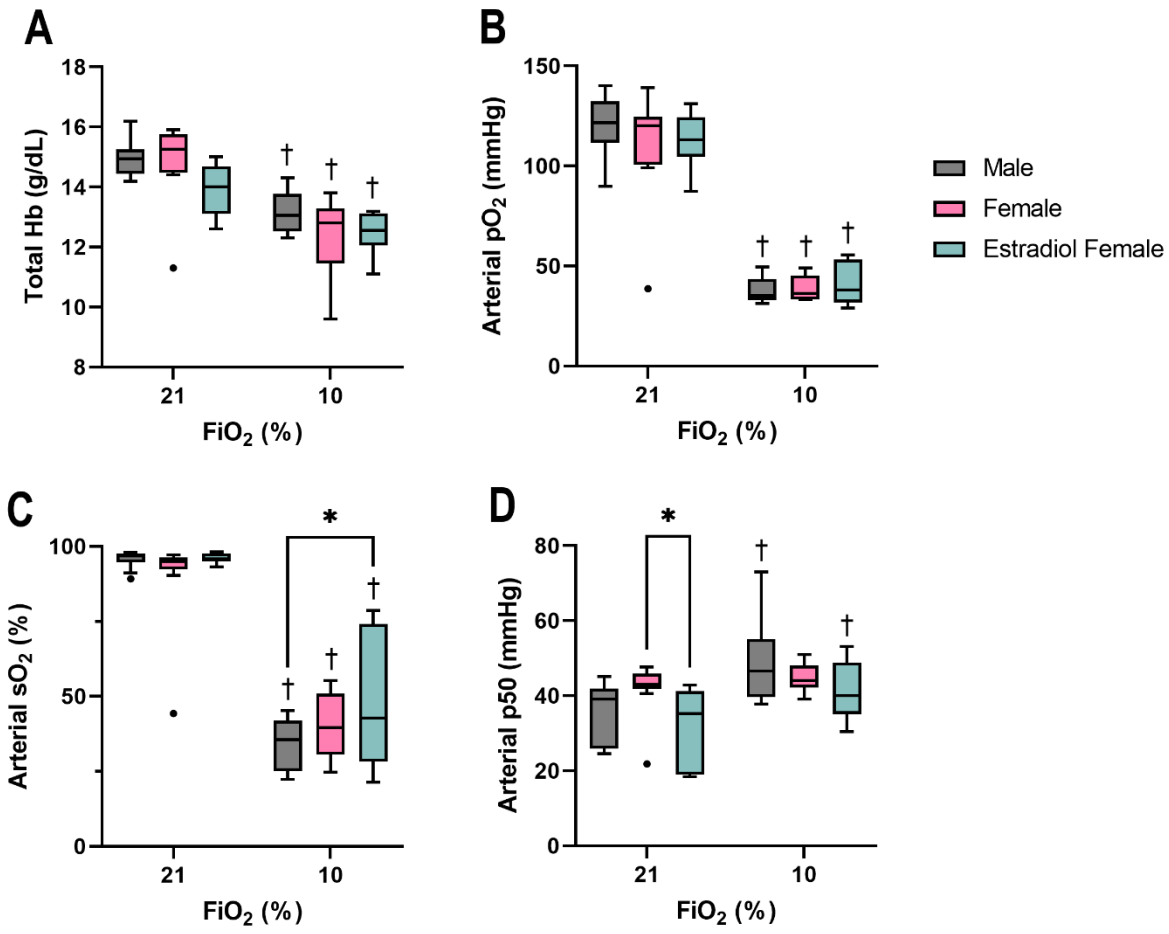

Figure 33: Estrogen does not seem to have a large effect on blood oxygenation. A: No significant difference in total Hb was found between the groups. B: All groups had similar arterial pO_2 measurements and experienced similar decreases in pO_2 during 10% hypoxia. C: The estradiol-treated female mice retained significantly higher arterial blood O_2 saturation than the male mice during hypoxia. The difference between the two female groups, however, was not significant. D: The non-treated female mice had higher blood P50 during normoxia than the estradiol-treated mice, and were the only group that did not experience a significant increase in blood P50 during hypoxia. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; pO_2, partial pressure of oxygen, sO_2, oxygen saturation of Hb.

4.2.3 Blood Acid-Base Balance

Estrogen seems to have little effect on acid-base balance of blood both during normoxia and acute 10% hypoxia. Fig. 34 compares the acid-base balance between the three groups. As seen in Fig. 34A, no significant difference in pH is visible between the groups at normoxia or hypoxia. The male mice did experience a significant drop in blood pH during 10% hypoxia that was not observed in either group of female mice. When comparing the partial pressure of CO_2 between the groups, the estradiol-treated female mice tended to have lower values (Fig. 34B). This difference is significant compared to non-treated female mice at normoxia, as well as compared to male mice during acute 10% hypoxia. It is unclear if this is due to increased bulk ventilation, and more testing should be done to determine the cause. Figs. 34C and D show no difference in lactate concentration or base excess between the different groups of mice at either normoxia or acute hypoxia.

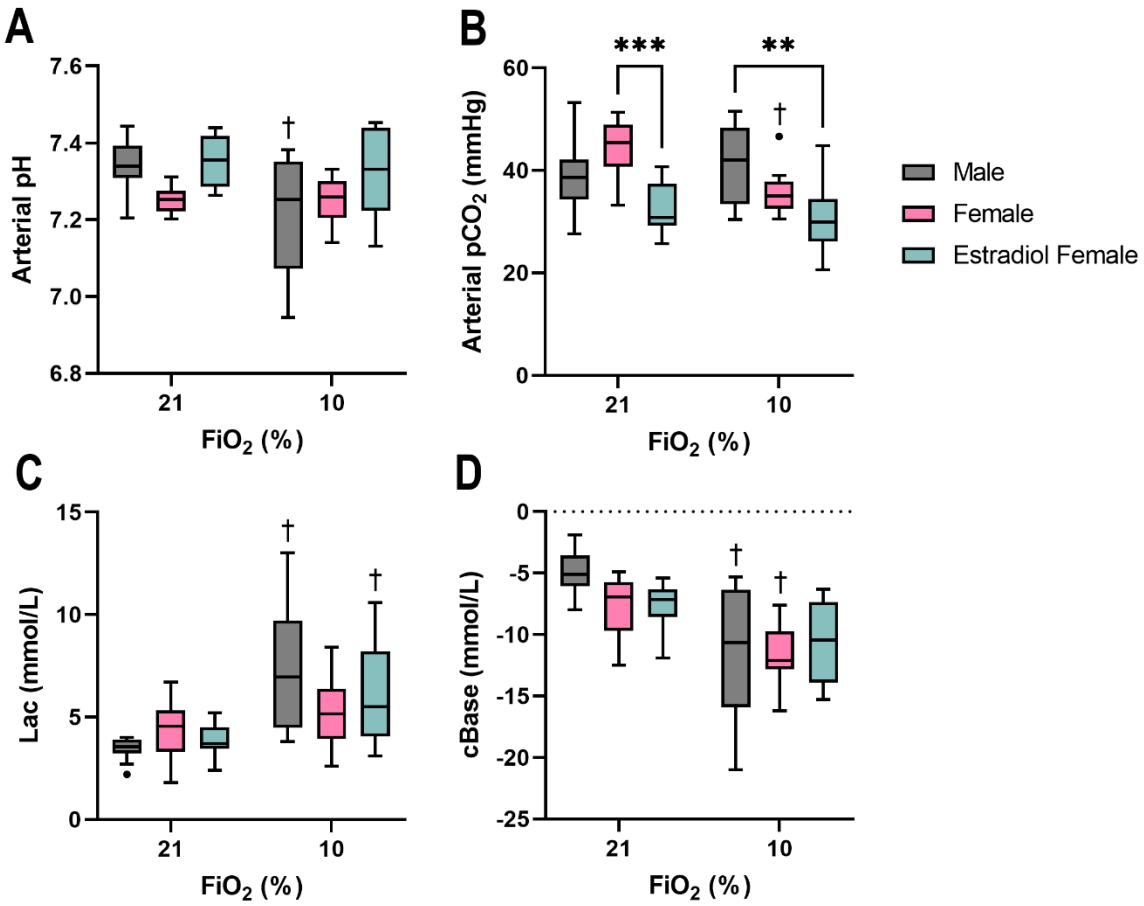

Figure 34: Blood acid-base balance comparing male, female, and estradiol-treated female mice. A: All groups show similar arterial pH measurements. B: Estradiol-treated female mice have significantly lower pCO_2 during normoxia than their untreated counterparts. At 10% hypoxia, estradiol-treated female mice have significantly less pCO_2 than male mice. C: No significant difference in lactate was found between the three groups. D: All groups show similar values for base excess. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P< 0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; pCO_2, partial pressure of carbon dioxide; lac, lactate; cBase, base excess.

4.2.4 Blood Electrolyte Balance

The impact of sex and estradiol treatment is shown in Fig. 35. All groups had similar potassium and sodium concentrations under normal conditions (Fig. 35A and B). Sodium ions were unchanged during acute hypoxia, while potassium ion concentrations rose for the male and estradiol-treated female mice. The non-treated female mice were the only group to not

experience a significant increase in free potassium ions during hypoxia. In Fig. 35C, calcium ion concentrations are unchanged during acute hypoxia but are lower in both female groups compared to the male mice. Chloride ion concentrations are shown in Fig. 35D, with the male and untreated female mice experiencing elevated concentrations during 10% hypoxia. The estradiol-treated female mice showed no significant change. The male mice displayed significantly lower chloride ion concentrations than the estradiol-treated female mice at normoxia and the untreated female mice during 10% hypoxia.

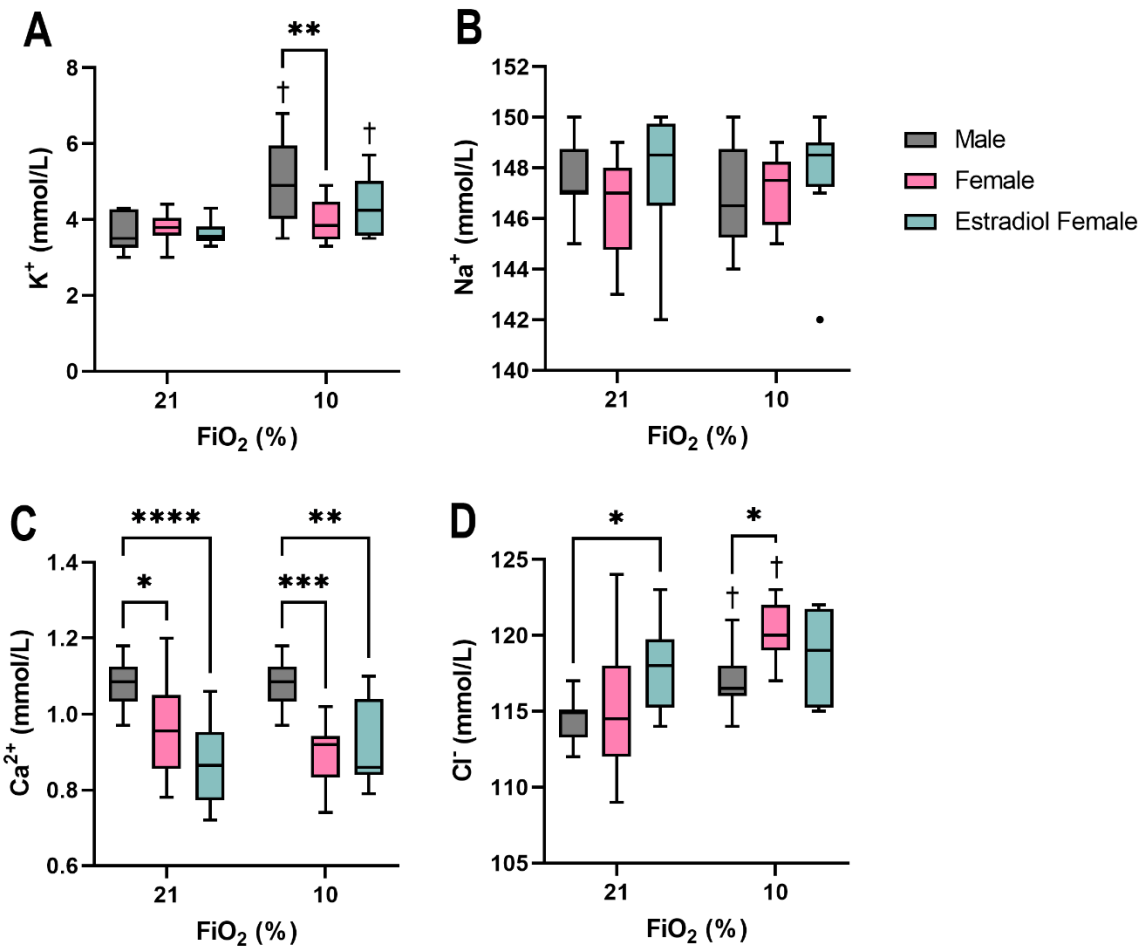

Figure 35: Major electrolytes in blood comparing male, female, and estradiol-treated female mice. A: Potassium ions are consistent through the three groups at normoxia; however, at 10% hypoxia, the male group has a significantly higher concentration of free ions than the untreated female mice. B: Sodium ion concentrations are unaffected by either sex hormones or 10% hypoxia. C: Calcium ion concentrations are unchanged during 10% hypoxia; however, the male mice have a significantly higher concentration than both untreated and estradiol-treated female mice. D: Estradiol-treated female mice do not experience an increase in chloride ion concentration at hypoxia. The male mice have a significantly lower concentration than the estradiol-treated female mice at normoxia, and the untreated female mice at hypoxia. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2.

4.2.5 Cardiac Function During Acute Hypoxia

When examining cardiac function during our acute 10% hypoxia tests for blood gas analysis, we observed significant differences between the groups not found during the hypoxia tolerance tests (Figs. 32 and 36). All three groups showed similar MAP under normal conditions; however, the male mice experience the largest drop in MAP during hypoxia. Both groups of female mice displayed significantly higher MAP (Fig. 36A). The estradiol-treated female mice were observed to have even higher MAP than the non-treated female mice during hypoxia. It seems that estradiol may attenuate decreases in MAP due to acute hypoxia. Estradiol-treated female mice also displayed significantly higher HR than both other groups during hypoxia (Fig. 36B). This may indicate that estrogen is protective during sharper decreases in O_2. More tests should be done, including MRI analysis of cardiac function to confirm as this was not apparent in our previous hypoxia tolerance tests (Fig. 32).

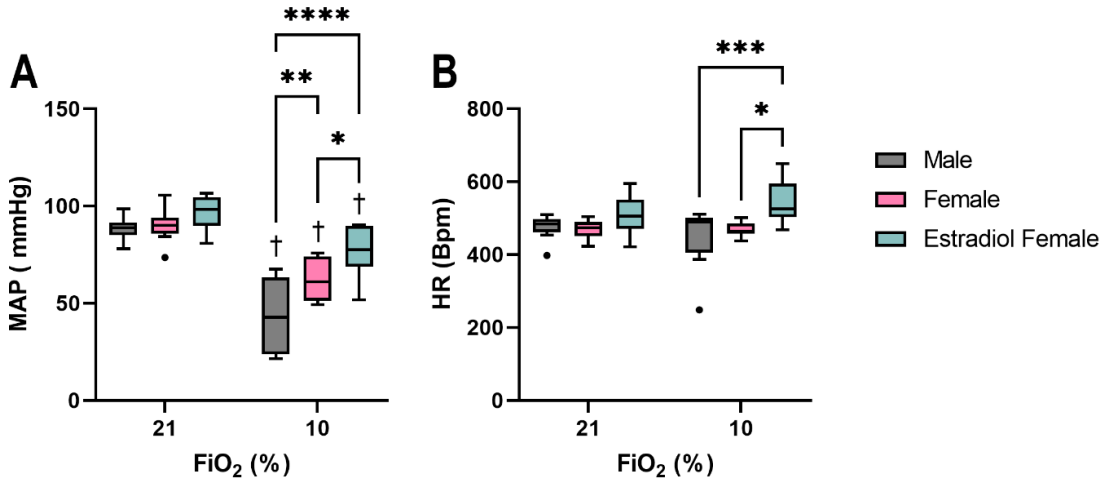

Figure 36: MAP and HR during blood gas analysis tests. Chronic exogenous estradiol treatment may improve cardiac function during hypoxia. A: All groups have similar MAP at normoxia. Estrogens seem to attenuate drop in MAP during 10% hypoxia, with the untreated female mice have significantly higher MAP than the male mice, and the estradiol-treated female mice having significantly higher MAP than both other groups at 10% hypoxia. B: The female mice that received exogeneous estradiol have significantly higher HR than both other groups during 10% hypoxia. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs. 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. FiO_2, fraction of inspired O_2; MAP, mean arterial pressure; HR, heart rate.

4.3 Discussion

Estradiol does not seem to affect survival under hypoxia conditions. All groups of mice showed similar tolerance to moderate and severe hypoxia, with most mice surviving until 5% oxygen. Upon reaching severely low FiO_2, all groups show rapid drops in survival. Estradiol may, however, blunt some of the effects of acute hypoxia. Female mice that received exogenous estradiol treatments showed higher blood oxygen saturation than male mice during acute 10% hypoxia (Fig. 33C). Additionally, the estradiol-treated mice demonstrated higher MAP and HR during acute hypoxia (Fig. 36). Although there was no difference in acute hypoxia survival between groups, these small changes could lead to improved long-term outcomes in chronically low O_2 conditions. The improved blood sO_2, MAP, and HR during acute 10% hypoxia may play a role in the previously observed protective effects of estrogen during chronic hypoxia (53, 54).

Hypoxia normally causes the transcription factor HIF-1α to be up-regulated, which in turn regulates many physiological responses to low oxygen supply. Estradiol, however, has been shown to reduce HIF-1α expression in previous studies (55, 56). If estradiol interferes with HIF-1α expression, it could explain why the estradiol-treated mice displayed some differences during hypoxia. Further testing should be done to confirm the effects of exogenous estradiol treatment on cardiac function as we did not observe the same effects in all of our tests. MRI analysis should also be done in future tests to better understand the effects of estradiol on stroke volume and cardiac output.

Estrogen is known to affect blood pressure regulation and is thought to have protective effects in the cardiovascular system, reducing the effects of hypertension (57, 58, 59). Estrogen is known to induce NO production, and NO inhibition has repeatedly been shown to increase blood pressure (60, 61, 62, 63). This could be a pathway in which estrogen decreases blood

pressure; however, it is unclear why we observed an attenuation of MAP loss with estradiol treatment. In our acute 10% hypoxia test, we observed female mice having the least drop in MAP compared to normoxia, with exogenous estradiol treatment further attenuating the effects of hypoxia on MAP. This may suggest that estrogens not only reduce hypertension but also help maintain consistent MAP close to normal values. It is unclear what pathways play the greatest role.

In examining the electrolyte balance in blood, we observed that male mice had significantly higher concentrations of calcium ions than both female groups. This seems to be consistent with studies that find that estrogen replacement therapy in postmenopausal women improves calcium absorption, decreasing blood serum calcium concentration (64, 65, 66). Exogenous estradiol treatment may also prevent increases in serum anion concentration during hypoxia, as we observed a significant mean increase in chloride ions within all groups except for the estradiol-treated female mice during 10% hypoxia. Although estradiol is known to affect ion channel expression and activity in cell cultures (66), there does not seem to be many widespread differences between male and female mice in regulation of serum ion concentrations.

4.4 Conclusion

Estradiol may improve cardiac function and blood O_2 saturation during acute 10% hypoxia. In general, there does not seem to be a large immediate difference between male and female mice during acute hypoxia. Exogenous estradiol treatment does not seem to have a large immediate effect during acute hypoxia either. Although estrogen has been found to have beneficial effects during chronic and intermittent hypoxia, the cardioprotective effects of estradiol may be less clear during short timespans. We recommend that more studies be done to further investigate the role of estrogen in hypoxia-induced pathways.

Section 5: Exogenous Estradiol in a Septic Shock Model

5.1 Introduction

We examined the effects of estrogens in a septic shock model to determine their role in inflammatory response. We also wanted to determine if exogenous estradiol treatments have any effects on inflammation. For this model, we treated experimental mice with 10mg LPS (lipopolysaccharide) per kg of bodyweight and obtained RBC and plasma samples over the course of 6 hours for use in an untargeted metabolomics analysis.

5.2 Results

5.2.1 Filtering for Important Metabolites

Our initial metabolomics panel consisted of 117 metabolites, which were then analyzed with two-way ANOVA and post-hoc multiple comparisons tests. From that original set, we found 25 metabolites in which the estradiol-treated female mice significantly differed from both other groups during at least one timepoint. Of these 25 metabolites, 17 had significant differences in only a single timepoint. 15 of these were observed at baseline, 1 at four hours, and 1 at six hours post LPS exposure. Of the remaining 8 metabolites, the estradiol-treated female mice were found to be significantly different than both other groups at two or more timepoints. Most of these differences were observed during baseline or one-hour post-injection. All the metabolites that were significantly different in the estradiol-treated female mice at more than one timepoint were related to lipid biosynthesis or fatty acid metabolism.

5.2.2 Essential Amino Acids

Fig. 37 shows two essential amino acids, L-serine and L-cystine, that were significantly impacted by exogenous estradiol treatment. In both of these metabolites, LPS exposure causes an increase over time compared to male control mice. This increase over time is relatively slower in L-serine, with no significant increases in any LPS-treated group until 4 hours post-injection (Fig. 37A). The estradiol-treated mice have a significantly higher amount of L-serine present in their blood at baseline than both other groups. At later stages, no significant difference between groups can be observed in L-serine levels. Fig. 37B shows the impact of estradiol treatment on L-cystine during septic shock. All groups are similar during baseline to 2 hours post-injection. The estradiol-treated group temporarily rises much faster than the other groups and is significantly higher at the 4-hour timepoint. All groups are seen again to behave similarly to each other at 6 hours.

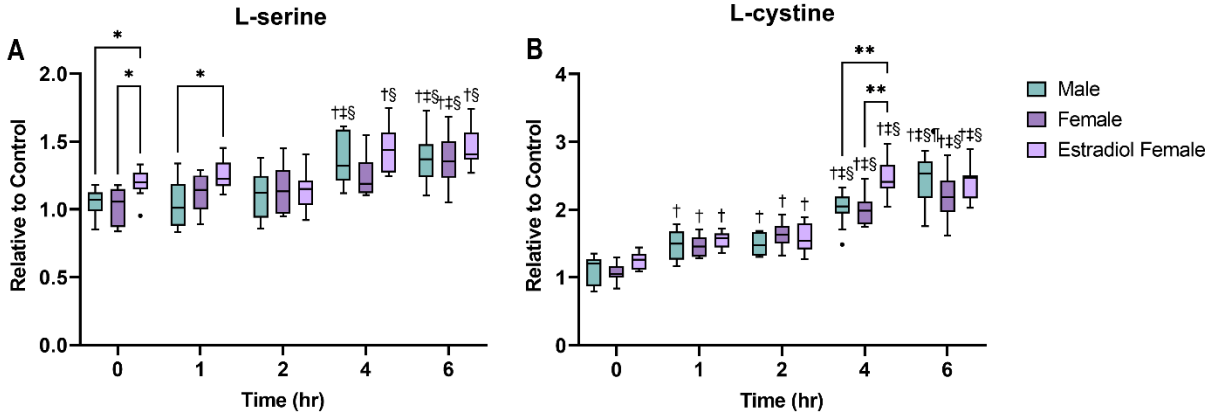

Figure 37: Essential amino acids that were significantly impacted by exogenous estradiol treatment shown over the course of 6 hours following LPS exposure. A: L-serine is significantly raised by estradiol-treatment at baseline compare to both male and untreated female mice. L-serine in estradiol-treated female mice remains significantly higher than male mice an hour after exposure to LPS. B: L-cystine is significantly higher in estradiol-treated female mice compared to both male and untreated female mice at 4 hours post exposure to LPS. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

5.2.3 Urea Cycle and Sulfur Metabolism

Metabolites involved in the urea cycle and sulfur metabolism are shown in Fig. 38. L-citrulline has a central role in the urea cycle and NO synthase inhibition (67). At baseline, estradiol-treated female mice display significantly higher amounts than both male and untreated female mice (Fig. 38A). All mice display a rise in L-citrulline as sepsis progresses over the 6-hour period. Figs. 38B and C show two metabolites involved in sulfur metabolism. Taurine can be found in high concentrations in the liver and WBCs, while L-methionine S-oxide may be a marker of proper liver health (68, 69). Taurine substantially rises within hours of exposure to LPS for all groups and is only significantly different between groups at baseline (Fig. 38B). L-methionine S-oxide increases over time only in the male and untreated female mice (Fig 38C). The estradiol-treated female mice display significantly higher levels of L-methionine S-oxide at baseline display no significant change over the course of 6 hours after LPS exposure.

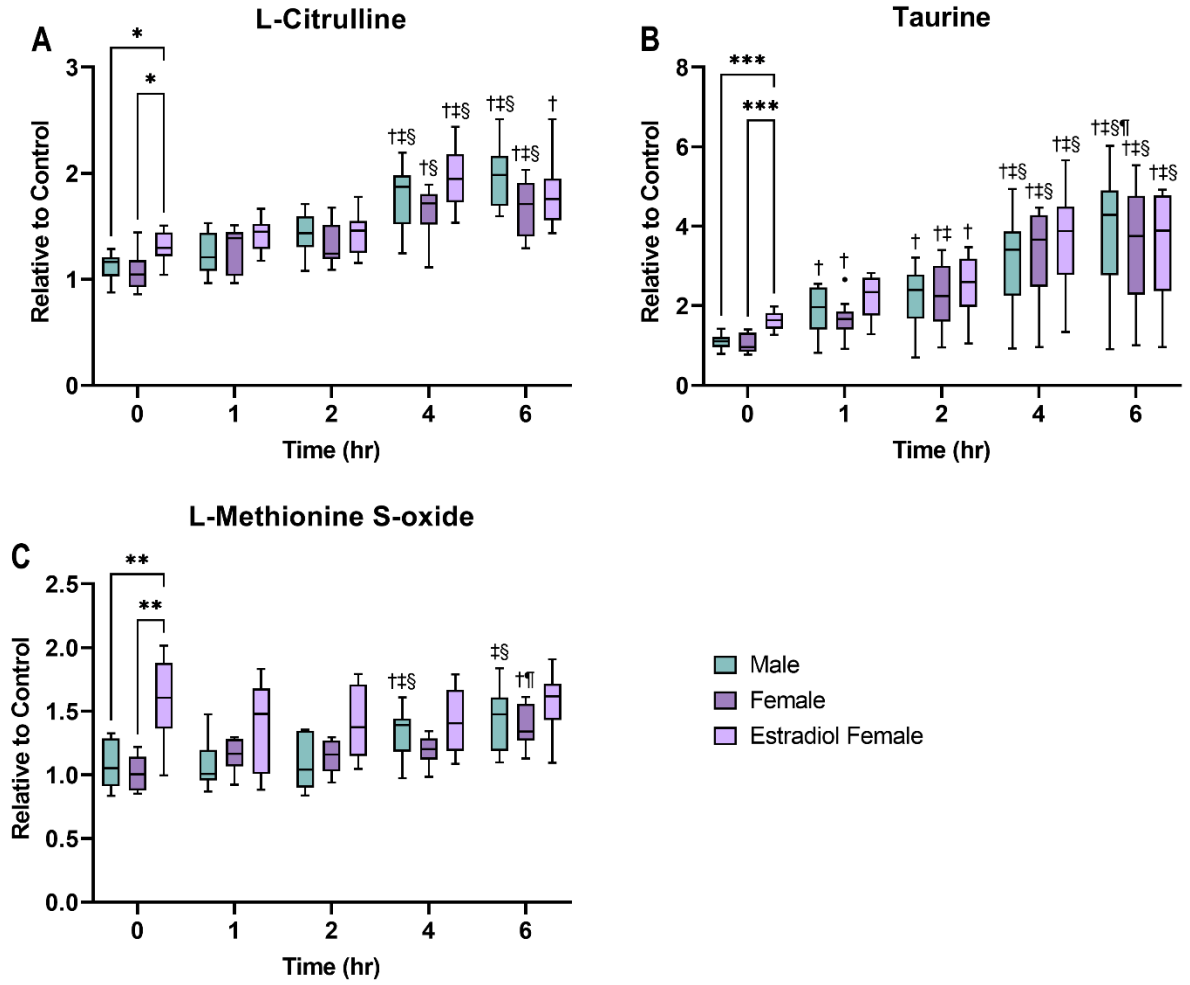

Figure 38: Metabolites involved in the urea cycle and sulfur metabolism that were significantly impacted by exogenous estradiol treatment. A: L-citrulline is significantly higher at baseline in estradiol-treated female mice compared to both male and untreated female mice. All groups experience an increase in mean concentration at 4 and 6 hours post LPS exposure; however, the estradiol-treated females seem to recover a little towards baseline at the 6hr timepoint. B: Taurine is significantly increased at baseline with exogenous estradiol treatment. C: L-Methionine is also significantly higher at baseline with exogenous estradiol treatment. Although it is higher at baseline, no change is observed in the estradiol-treated group following exposure to LPS. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

5.2.4 Indole and Tryptophan

Indole and tryptophan metabolism-related metabolites are shown in Fig. 39. 3-methyleneoxindole shows no significant change over time following LPS exposure, but the

estradiol-treated female mice have a significantly higher baseline measurements than either other group (Fig. 39A). Kynurenine rises significantly in the male and untreated female mice at 4 hours post-LPS exposure (Fig 39B). This may be related to the role kynurenine plays in vasodilation during inflammatory response (70). The estradiol-treated mice did not experience a significant increase in kynurenine over the course of the 6 hours but started with significantly higher baseline measurements than the male and untreated female mice.

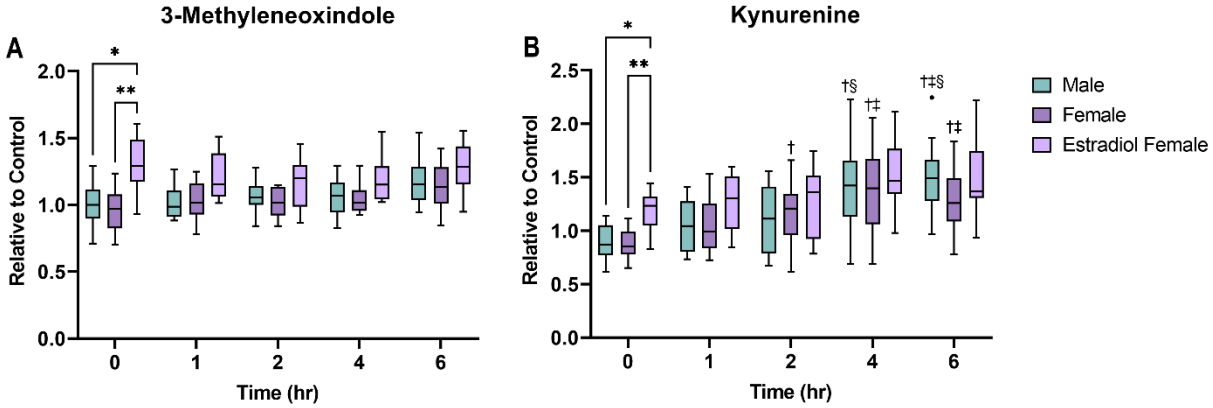

Figure 39: Metabolites involved with indole and tryptophan processes that were significantly affected by exogenous estradiol treatment. A: 3-Methyleneoxindole is significantly increased in estradiol-treated female mice at baseline. There is no significant change within each group between timepoints. B: Kynurenine is significantly higher at baseline in estradiol-treated female mice. Male and untreated female mice show significant increases following LPS exposure, but no significant change is observed in the estradiol-treated mice. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

5.2.5 Glycerophospholipid and Sphingolipid Biosynthesis

Fig. 39A shows the impact of exogenous estradiol treatment and LPS-induced inflammation on sphingosine 1-phosphate, a metabolite involved in glycerophospholipid biosynthesis. Sphingosine 1-phosphate does not seem to be affected by LPS, with only the estradiol-treated female mice showing a significant change from baseline at 4 and 6 hours post-LPS injection. In Fig. 39B, sphinganine 1-phosphate, a metabolite involved in sphingolipid

biosynthesis, similarly shows minimal change over time after LPS treatment. The estradiol-treated female mice tended to have significantly higher amounts of both metabolites, which could indicate that the exogenous estradiol treatment is inducing greater amounts of lipid synthesis.

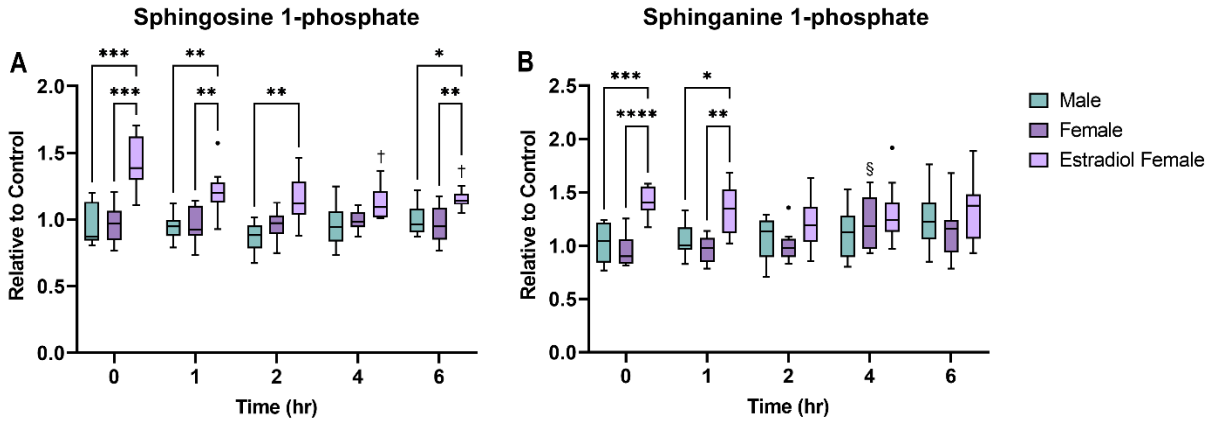

Figure 40: Metabolites involved in glycerophospholipid and sphingolipid biosynthesis that were significantly impacted by exogenous estradiol treatment. A: Sphingosine 1-phosphate is significantly higher in estradiol-treated female mice than male mice at baseline, 1, 2, and 6 hours post LPS exposure, and significantly higher than untreated female mice at baseline, 1, and 6 hours post LPS exposure. LPS exposure had no significant impact on male and untreated female groups, but the levels of sphingosine 1-phosphate can be observed to decrease over time after exposure to LPS in the estradiol-treated mice. B: Sphinganine 1-phosphate is significantly increased by estradiol treatment at baseline at 1 hour post LPS exposure. Septic shock does not seem to have much of an effect on Sphinganine 1-phosphate. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

5.2.6 Carnitine and Fatty Acid Metabolism

Figs. 41 and 42 show metabolites related to carnitine and fatty acid metabolism. In all of the metabolites shown, the estradiol-treated female mice measure significantly higher at baseline, indicating increased fatty acid metabolism. In Fig. 41A, L-carnitine is shown to be unaffected by LPS stimulation, but the estradiol-treated mice have significantly higher amounts than both other groups at over half of the timepoints. L-carnitine aids in fatty acid entry into the mitochondria for

use as an energy source (71). The elevated levels of L-carnitine may suggest that exogenous estradiol treatment promotes the use of fatty acids as an energy source. Acetyl-L-carnitine, L-octanoylcarnitine, decanoyl-L-carnitine, decenoyl-L-carnitine, and dodecanoyl-L-carnitine (Figs. 41B, 42B, C, D, and E) also show little significant change over time from LPS stimulation. Propionyl-L-carnitine, butanoyl-L-carnitine, hydroxybutyrylcarnitine, isovalerylcarnitine, and tiglylcarnitine all experience large increases with LPS stimulation (Figs. 41C, D, E, F, and 42A). Between groups, the largest significant differences are at baseline or 1-hour post-injection in the estradiol-treated female mice.

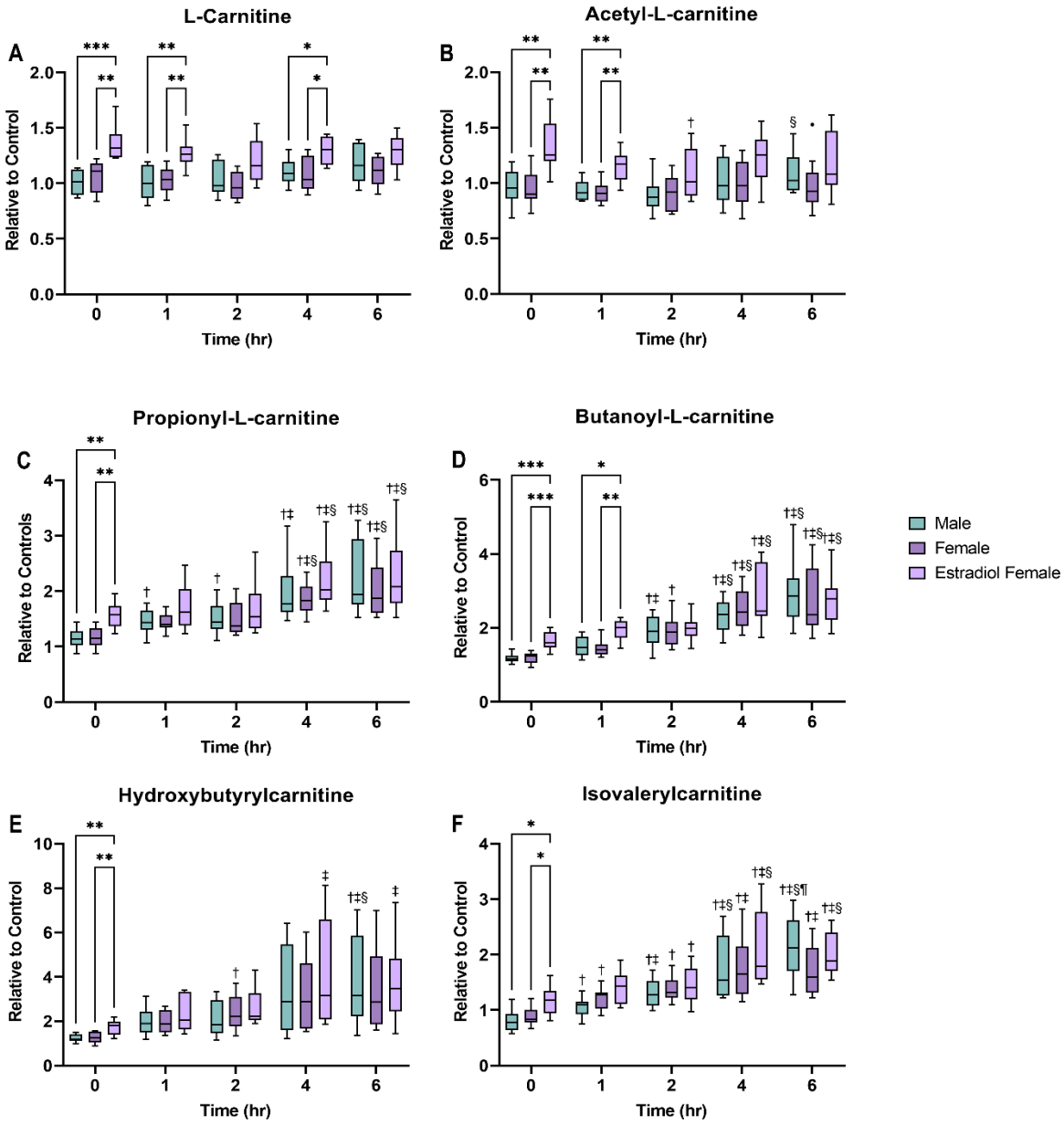

Figure 41: Metabolites involved in carnitine and fatty acid metabolism that were significantly affected by exogenous estradiol treatment (1 of 2). A: L-Carnitine is significantly increased in estradiol-treated mice at baseline, 1, and 4 hours post LPS exposure. There is no significant impact of septic shock on L-Carnitine. B: Acetyl-L-carnitine is significantly elevated by treatment with estradiol at baseline and 1 hour post LPS exposure. Septic shock seems to have minimal significant impact on acetyl-L-carnitine. C: Propionyl-L-carnitine is significantly elevated in estradiol-treated female mice at baseline. All groups see significant increases over time after injection of LPS. D: Butanoyl-L-carnitine is significantly increased by estradiol treatment at baseline and 1 hour post LPS injection. All groups experience significant increases over time following exposure to LPS. E: Hydroxybutyrylcarnitine is significantly increased by estradiol treatment at baseline. F: Isovalerylcarnitine is significantly elevated at baseline with exogenous estradiol treatment. All groups experience increases over time following LPS exposure. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

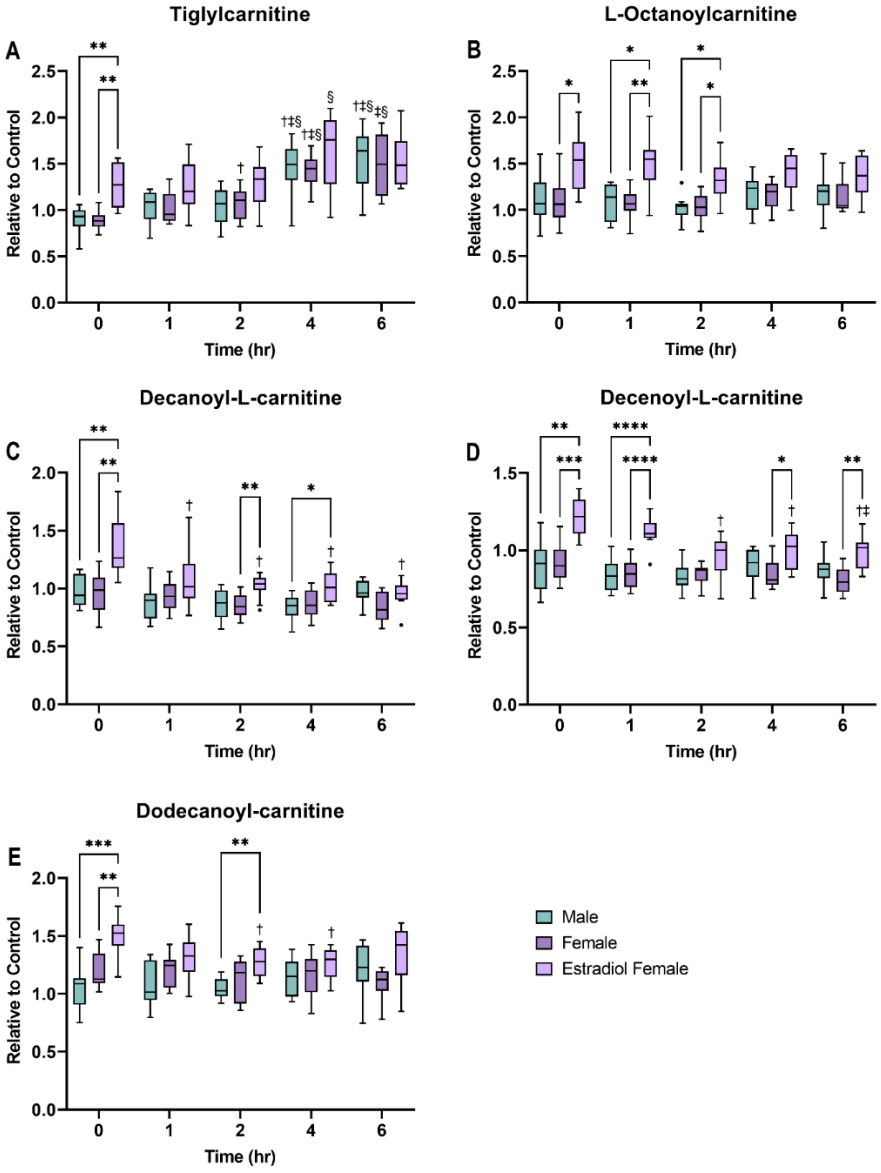

Figure 42: Metabolites involved in carnitine and fatty acid metabolism that were significantly affected by exogenous estradiol treatment (2 of 2). A: Tiglylcarnitine is increased at baseline by estradiol. Following LPS exposure, the other groups experience an increase over time as well. B: L-Octanoylcarnitine is significantly higher in estradiol-treated female mice than untreated female mice at baseline, 1, and 2 hours post LPS exposure, and male mice at 1 and 2 hours post LPS exposure. Septic shock does not seem to affect L-octanoylcarnitine. C: Decanoyl-L-carnitine is increased significantly with estradiol treatment at baseline, and lowers over time after exposure to LPS. D: Decenoyl-L-carnitine is significantly higher in estradiol-treated female mice than both male and untreated female mice at baseline and 1 hour post LPS exposure. The estradiol-treated female mice also have significantly higher decenoyl-L-carnitine than their untreated female counterparts at 4 and 6 hours post LPS exposure. Only the estradiol-treated female group experiences a significant change over time during septic shock. E: Dodecanoyl-carnitine is significantly elevated in the estradiol-treated female mice compared to both other groups at baseline, and compared to male mice at 2 hours post LPS injection. There is no change over time within the male and untreated female groups and a slight decrease and return in the estradiol-treated female group. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

5.2.7 Fatty Acids

Fatty acid levels are shown in Figs. 43 and 44. In Fig. 43, we see two saturated fatty acids, octanoic acid and decanoic acid. Octanoic acid is significantly higher in estradiol-treated mice compared to both other groups at baseline. Estradiol-treated female mice continued to demonstrate significantly higher levels than males at 1 and 2 hours following LPS injection (Fig. 43A). The estradiol-treated female mice are the only group that do not experience significant increase over time from LPS stimulation. Decanoic acid experiences no such change over time due to LPS stimulation (Fig. 43B). Fig 44A shows tetradecenoic acid, a mono-unsaturated fatty acid. Again, the estradiol-treated female mice display significantly higher levels than both other groups at baseline and continue to demonstrate significantly higher levels than the male mice at 1 and 4 hours post LPS exposure. There is some significant decrease of tetradecenoic with LPS stimulation in both female groups that is not observed in the male group. This is not the case with octadecenoic acid, another mono-unsaturated fatty acid. All groups have significant decreases at nearly every timepoint following introduction of LPS (Fig. 44B). Although the estradiol-treated female mice have higher mean values at every timepoint, the only significant difference between the groups is at 6 hours following LPS exposure. Fig. 44C shows dodecanedioic acid, a poly-unsaturated fatty acid. The estradiol-treated female mice have significantly higher levels at baseline than both other groups. Only the male mice display a significant change over time following LPS stimulation.

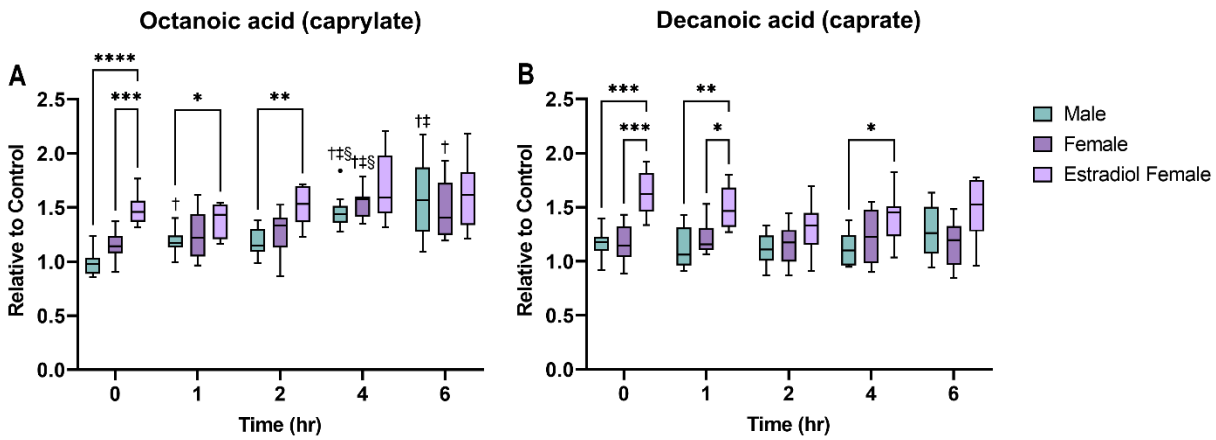

Figure 43: Saturated fatty acids that were significantly impacted by exogenous treatment of estradiol. A: Octanoic acid (caprylate) is significantly increased with estradiol treatment at baseline. Estradiol-treated female mice are also significantly higher than male mice at 1 and 2 hours post LPS exposure. Estradiol-treated mice show no significant change or increase over time during septic shock. B: Decanoic acid (caprate) is significantly elevated in the estradiol-treated female group compared to both other groups at baseline and 1 hour post LPS exposure, as well as compared to male mice at 4 hours post exposure. There is no significant change over time due to LPS. *P <0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P < 0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

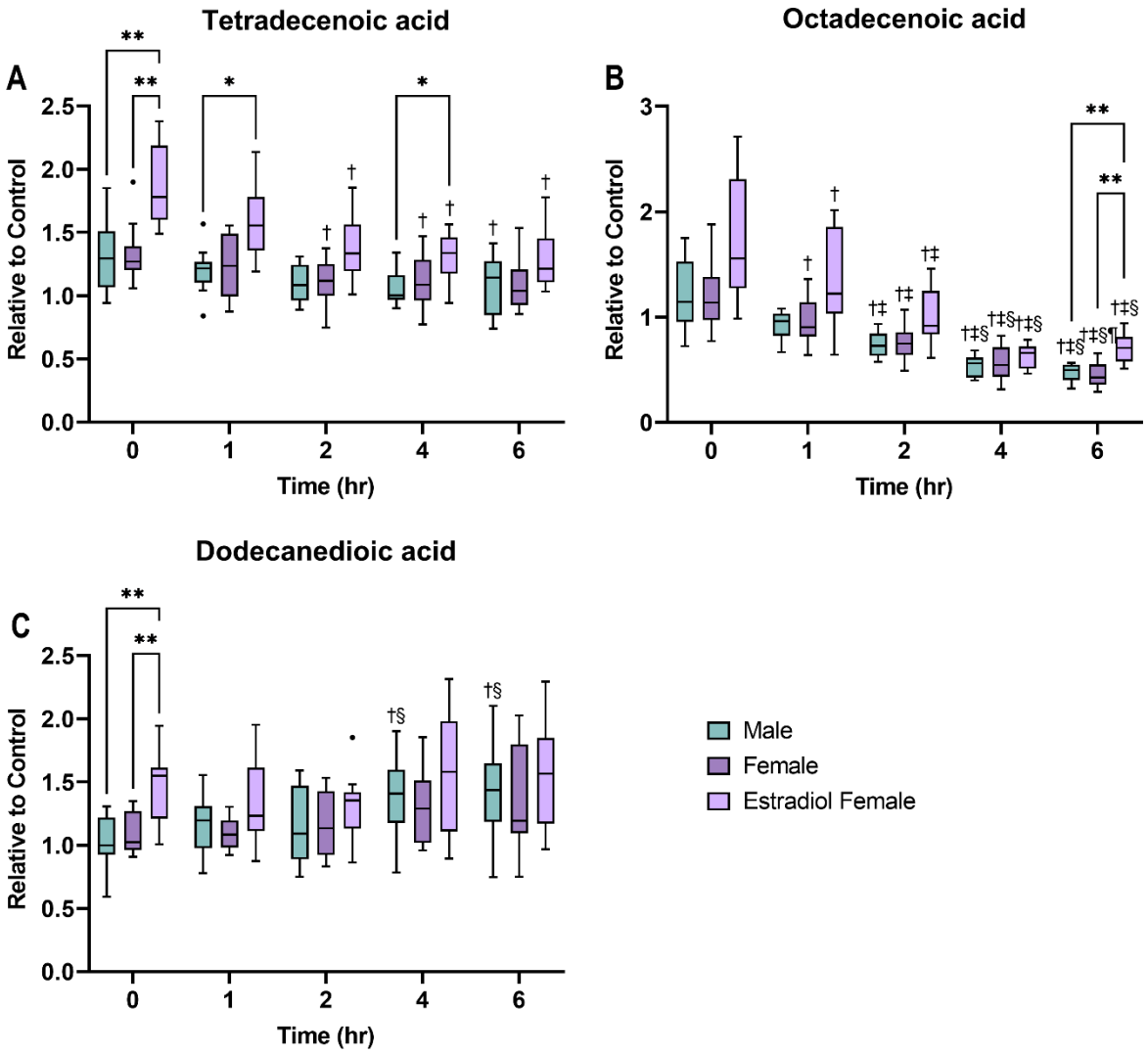

Figure 44: Unsaturated fatty acids that were significantly affected by exogenous treatment of estradiol. Tetradecenoic acid, a monounsaturated fatty acid, is significantly elevated by estradiol at baseline compared to both other groups. Estradiol-treated mice are also significantly higher than male mice at 1 and 4 hours post LPS exposure. There is some decrease over time shown in all groups. B: Octadecenoic acid, another monounsaturated fatty acid, decreases over time in all groups after injection of LPS. The estradiol-treated female mice display the highest mean values of all groups, and retain significantly higher amounts than both other groups at 6 hours. C: Dodecanedioic acid, a poly-unsaturated fatty acid, is significantly higher at baseline in the estradiol-treated female mice. Only the male group experiences a significant increase in mean over time. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for two-way ANOVA with Tukey's multiple comparisons test between test groups, †P<0.05 vs 0hr, ‡P<0.05 vs 1hr, §P<0.05 vs 2hr, ¶P<0.05 vs 4hr. for two-way ANOVA with Šidák multiple comparisons test within each test group. All metabolites normalized to male control mice.

5.2.8 Trends in Lipid Biosynthesis and Metabolism

Heatmaps of the average values of 42 metabolites related to lipid biosynthesis or carnitine and fatty acid metabolism are shown in Figs. 45-47. In Fig. 45, the metabolites are shown at baseline normalized to the male control mice. Although not all the metabolites had significant p-values, the estradiol-treated female mice tended to have higher average levels than the other groups. Additionally, we can see that the mean measurements of the fatty acids in the estradiol-treated group tended to be higher, indicating that the estradiol treatment may increase production of fatty acids. Fig. 46 shows the same metabolites at 6 hours post-LPS stimulation normalized to the male control mice. Some of the carnitines trend upwards and nearly all of the fatty acids trend downwards in the LPS-treated mice. Fig. 47 shows the same metabolites at 6 hours comparing only the LPS-treated mice normalized to the male experimental group. The male and untreated female mice have similar mean values of all the metabolites. However, the estradiol-treated female mice had higher averages of all of these metabolites. This is especially important with the fatty acids, listed in the bottom 17 rows, which decreased with LPS exposure. Although all groups tended to decrease fatty acid levels following LPS stimulation, the estradiol-treated female mice started with higher averages and therefore had the highest values at the 6-hour timepoint.

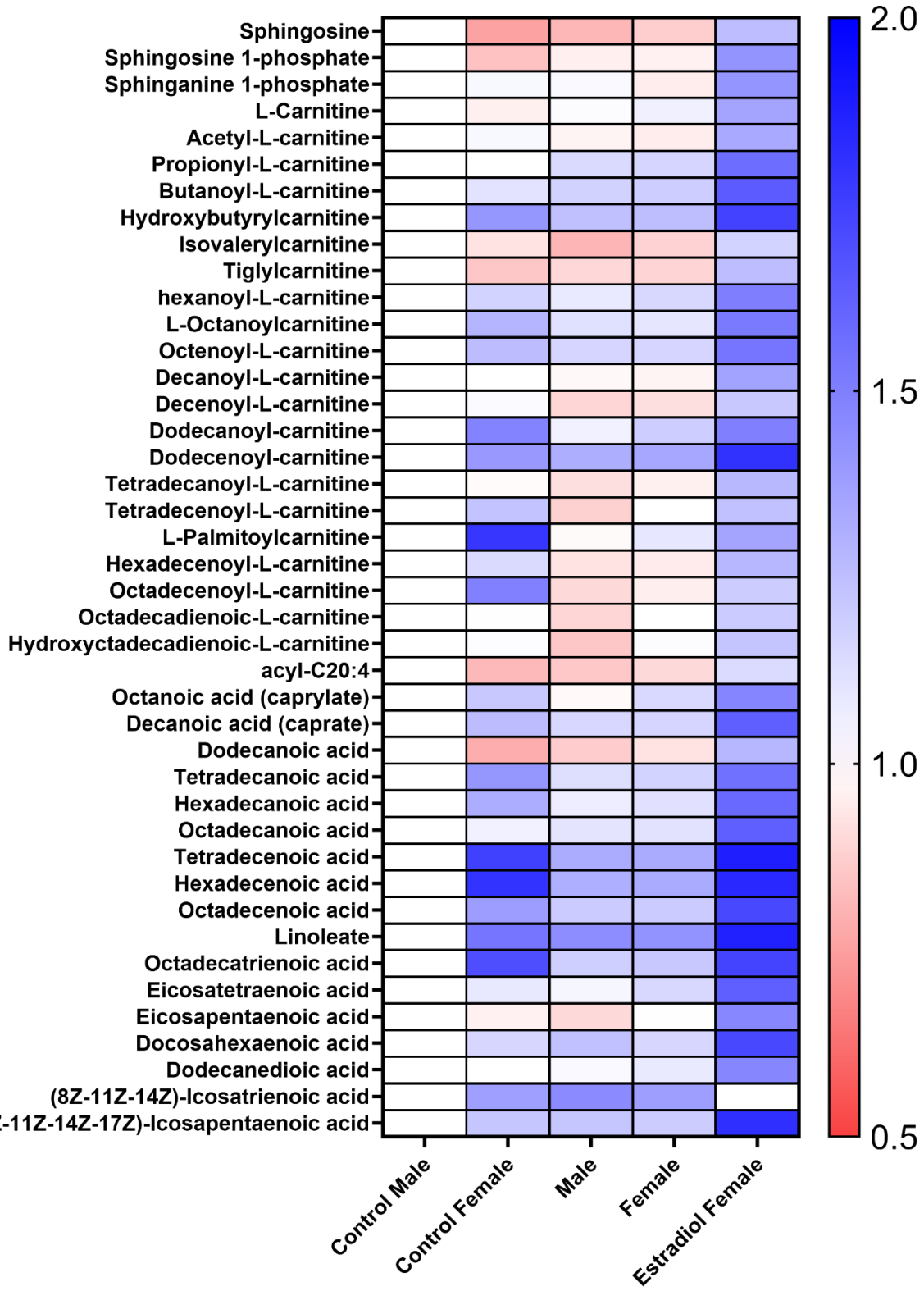

Figure 45: Heatmap of metabolites related to lipid synthesis and fatty acid metabolism at baseline, normalized to control male mice. Significance not shown.

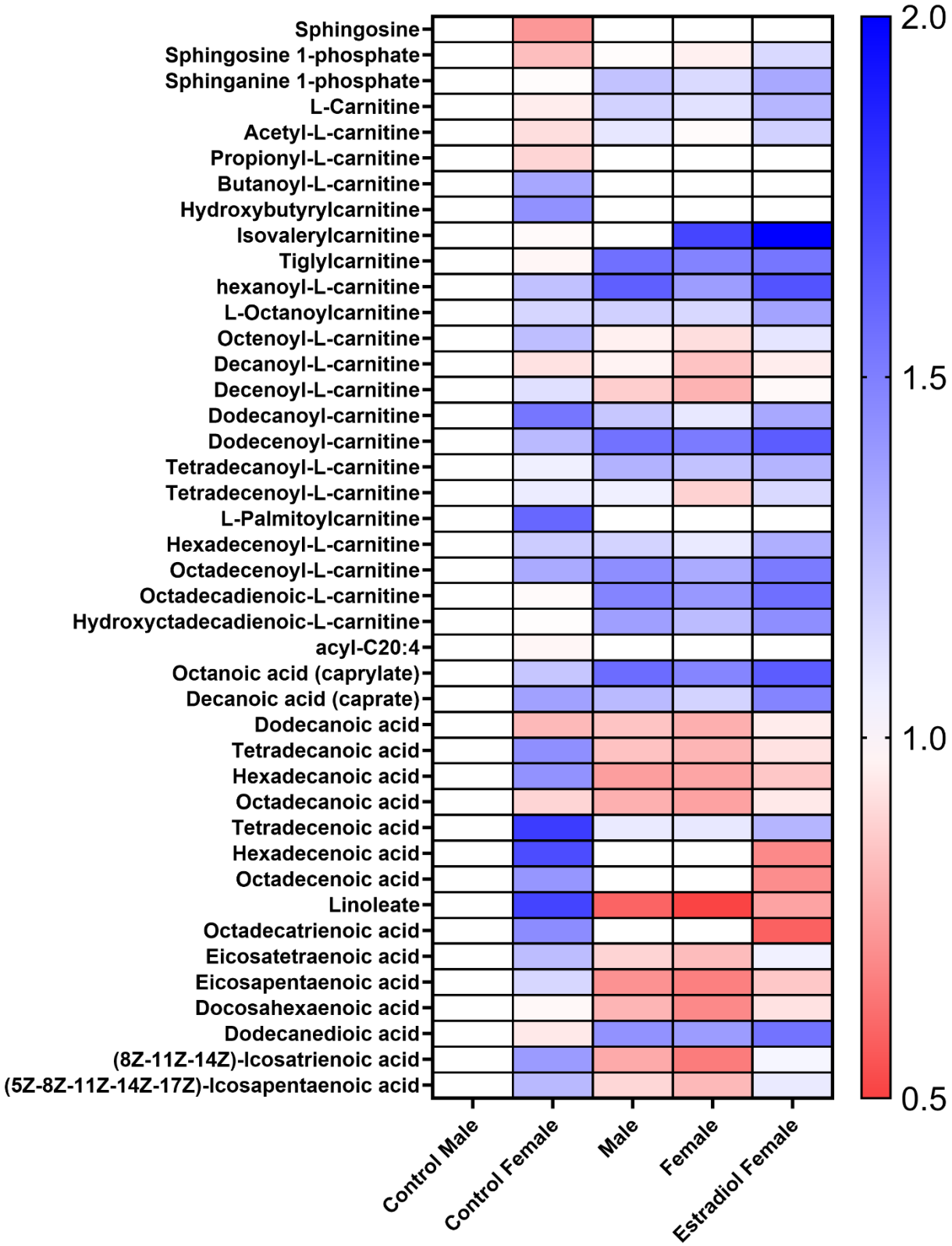

Figure 46: Heatmap of metabolites related to lipid synthesis and fatty acid metabolism at 6 hours post-LPS stimulation, normalized to control male mice. Significances not shown.

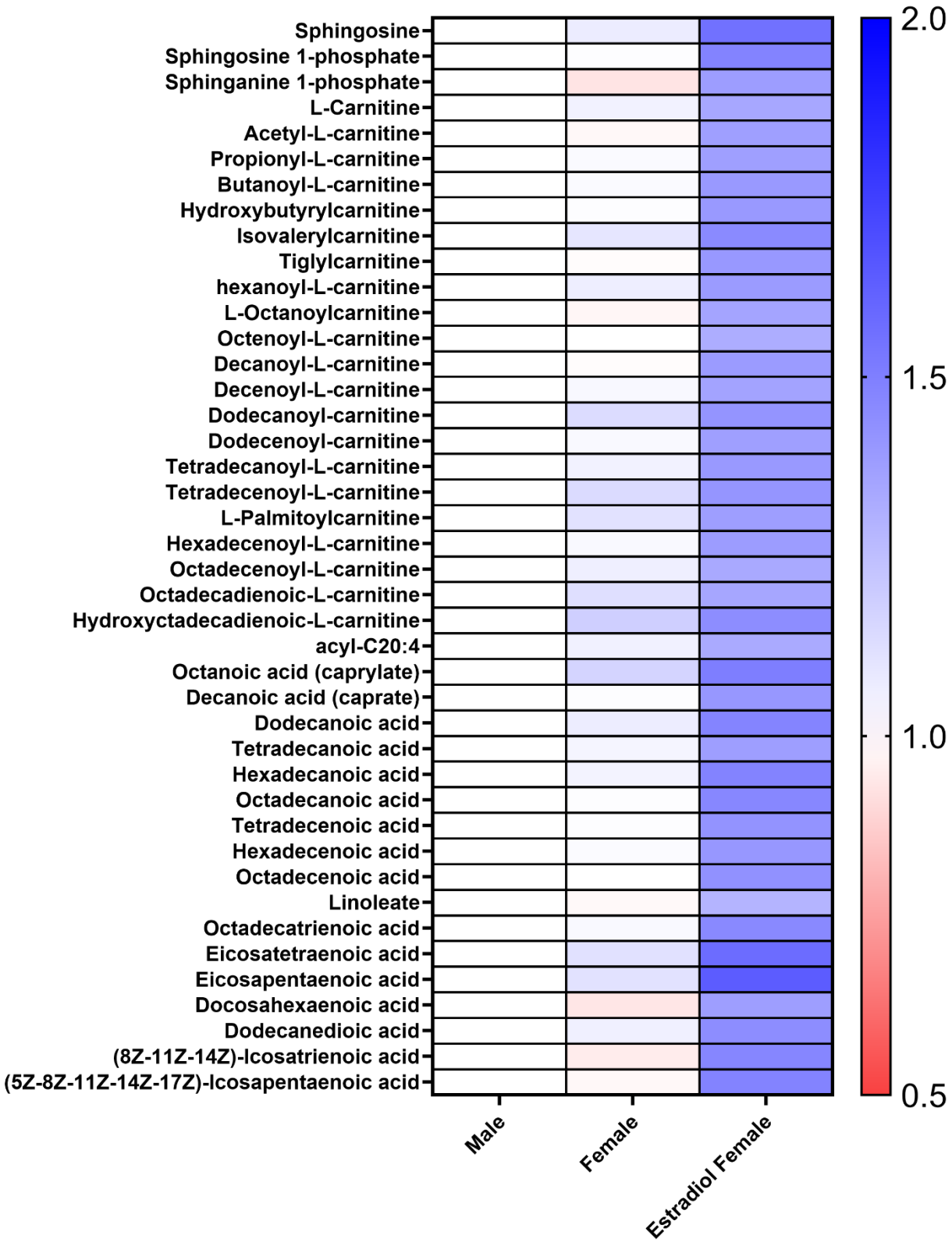

Figure 47: Heatmap of lipid biosynthesis and fatty acid related metabolites showing only the experimental groups that received LPS stimulation. Groups were normalized against male group. Significance not shown.

5.3 Discussion

Exogenous estradiol treatments most strongly impact lipid biosynthesis and metabolism. Estradiol-treated female mice tended to have higher amounts of metabolites related to fatty acid metabolism, indicating that exogenous estradiol influences pathways in which fatty acids are synthesized and metabolized. It is unclear what effects estradiol has on inflammatory response to LPS; however, there may be some protective effects from estradiol pre-treatment. Fatty acids tended to decrease with LPS exposure, corroborated by previous studies that found depressed levels of fatty acids during septic shock (72). Pre-treatment of estradiol increases baseline levels fatty acids and metabolites involved in lipid synthesis, potentially acting as a buffer during sepsis. This could help to attenuate some negative inflammatory effects as well.

One interesting observation in this study is the increase of L-citrulline following LPS exposure. In a case study of septic shock in human adults, L-citrulline was seen to decrease in the first day of sepsis (73). This could reflect inherent differences in our model, as we observed a much shorter timeframe within mice.

L-carnitines have previously studied for use in sepsis treatment. The results seem promising, especially in patients who have lower levels of ketone bodies (74). It is suggested that loss of mitochondrial function is related to the severity of sepsis and that L-carnitine treatments can improve outcomes for some patients (74). Additionally, another study found that dietary fat content affected the survival and prognosis of mice during sepsis. A diet rich in poly-unsaturated fats and omega-3 fatty acids increased survival (75). In our tests, estradiol affected the number of fatty acids present in blood plasma. Pre-treatment with estradiol increased the number of fatty acids at baseline compared to control groups and provided a buffer for the decrease in fatty acids caused by sepsis. This could suggest that estradiol may improve survival during sepsis. Estradiol,

however, has shown to suppress LPL and fat uptake by adipocytes (76, 77). This could indicate that estradiol reduces the positive effect of a poly-unsaturated fat-rich diet on survival in septic shock. Further studies should be done to determine the effect of estradiol on sepsis when combined with specialized diets.

During sepsis, multiple inflammatory and immune pathways are triggered. Of these NF-κB, TNF-α, and C5a being of most interest (78). Previous studies have found that estradiol attenuates LPS-induced inflammation, with estradiol reducing TNF-α expression both in vitro and in vivo (46, 47). Estradiol is believed to reduce TNF-α by suppressing the NF-κB pathway, while also inhibiting IL-6 by outcompeting binding on NF-κB (79). Previous septic shock studies have found that estrogen plays a role in inflammatory response, with male mice displaying higher markers of inflammation compared to female mice (80). This difference could be due to IL-1ra, which was found to be more abundant in the plasma female rats following LPS injections (81).

Estradiol pre-treatment may also promote increases in metabolites that have beneficial effects during inflammation. For example, sphingosine 1-phosphate, which was significantly elevated in estradiol-treated mice has previously been shown to inhibit some T-cell processes and reduce IL-8 secretion (82). Piperine, an alkaloid found in blood serum and black pepper, was previously shown to suppress IL-8 secretion following LPS exposure and may attenuate inflammatory response (83). This could suggest that sphingosine 1-phosphate may also be able to attenuate inflammatory response by suppressing IL-8 secretion from T-cells using the same pathways. It is unclear, however, if the elevated levels of sphingosine 1-phosphate induced by the exogenous estradiol treatment are sufficient to provide significant suppression of IL-8 or anti-inflammatory response. This should be further investigated with a cytokine panel.

5.4 Conclusion

It is not abundantly clear from our metabolomics analysis how much inflammatory response is affected by exogenous estradiol treatments. It seems, however, that male and untreated female mice display similar metabolomic responses both at baseline and with LPS stimulation. Estradiol treatments show significant metabolomic changes that could potentially provide protective or beneficial effects during sepsis. It is unclear, however, if the changes caused by estradiol pre-treatment have a significant impact on inflammatory response and septic shock survival. Further survival tests and cytokine studies may shed light on this issue.

Section 6: Materials and Methods

6.1 GBT1118 and GBT21601 Dosing

GBT1118 was prepared in a 0.5% methylcellulose/phosphate buffer of pH 7.4/0.01% Tween-80 at 10 mg/mL. SCD mice were then treated with 100 mg/kg GBT1118 PO twice a day for either 14 or 24 days. All terminal procedures were done within an hour of the final dose. All control mice were treated with 10mL/kg vehicle PO following the same schedule as the GBT1118-treated mice (34).

GBT21601 was similarly prepared, with SCD mice being treated with either 75 mg/kg or 150 mg/kg GBT21601 twice a day for either 14 or 24 days. All terminal procedures were done within one hour of the final dose.

6.2 Hypoxia Model

Mice were randomly assigned to either the hypoxia survival tolerance protocol or the hypoxia blood gas analysis protocol. O_2 concentrations were measured using a calibrated O_2 sensor (Maxtec, Valley City, UT), with the inhaled air being adjusted to be within 0.5% of the target O_2 concentration (34).

6.2.1 Hypoxia Tolerance and Survival

Mice were subjected to normoxia (21% FiO_2) for 15 min, following which they were exposed to hypoxic conditions at 5% decreases for 15 minutes each (normoxia, 15%, 10%, and 5%). Hypoxia tolerance and survival was defined as the ability of the animal to maintain MAP above 35mmHg and HR above half of the baseline measurement at normoxia for 1 min. This follows previous studies to maintain humane treatment of the animals (21, 23, 24). Once these

criteria were no longer sustained by the mouse, they were immediately removed from hypoxia and euthanized (34).

6.2.2 Hypoxia Blood Gas Analysis

Mice were exposed to normoxia (21% FiO_2) and hypoxia (10% FiO_2) for 15 min each, after which blood samples were taken from each the arterial and venous catheter lines using microhematocrit tubes. These samples were then measured with a blood gas analyzer (ABL-90 Flex, Radiometer, Denmark) to determine blood gas content, total Hb, lactate, and electrolytes (34).

6.2.3 Animal Inclusion

Animals were checked to ensure they fit suitable criteria for the experiments. At baseline (normoxia) MAP must be above 65 mmHg and HR must be above 300 BPM (34).

6.3 Exogenous Estradiol Dosing

Animals were anesthetized and then had an estradiol pellet implanted under their skin behind their shoulders. The mice were then allowed to recover for 3 weeks before being used in experimentation in the septic shock model.

6.3.1 Verification of Estrous Phase

To verify the hormone stage of the estradiol-treated female mice, vaginal lavage with saline was applied to both treated and untreated female mice at baseline to obtain cell samples. The samples were then placed on glass slides and allowed to dry, after which the slides were stained with crystal violet. Top slides were then placed with glycerol and studied under microscopy to determine what phase of their estrous cycle the mice are in at baseline.

6.4 Septic Shock Model

A baseline measurement blood sample was obtained from the mice at the start of experimentation. The mice were then given an intraperitoneal (IP) injection of 2mg/mL diluted LPS with the injection volume calculated to be 10mg/kg LPS by bodyweight. Mice were anesthetized briefly with 2% vol. inhaled isoflurane and placed on a heated pad while obtaining blood samples at baseline, 1hr, 2hr, 4hr, and 6hr post-injection. Blood samples were taken from the tail vein, unless unable due to poor blood flow as experiment progressed. If enough blood could not be obtained from the tail vein, the mice were sampled using a facial vein instead. Samples were then centrifuged following which RBC and plasma samples were obtained for metabolomics analysis. The mice were euthanized via cervical dislocation immediately following collection of the final blood sample. Following euthanasia, tissue samples were obtained for the brain, heart, lung, liver, kidney and plasma.

Both estradiol-treated and untreated female mice had a vaginal lavage applied to obtain cell samples to determine the phase of the hormonal cycle of the mice.

6.5 Arterial and Venous Catheterization

Mice were anesthetized using 5%/vol. inhaled isoflurane which was reduced to 2%/vol. during surgery (Drägerwerk, AG, Lübeck, Germany). The neck of the animals was then shaved and cleaned with 70% ethanol and betadine to prepare the surgical site. The mice were then placed on a heated platform for surgery. The carotid artery and jugular veins were then catheterized with catheters filled with heparinized saline (30 IU/mL) to prevent clotting in the lines or at the vessel entry sites. During surgery, animals freely breathed 40% O_2 during

anesthesia. Afterward catherization, isoflurane was reduced again to 1.5% to maintain anesthesia during measurements (34).

6.6 Statistical Analysis

For Section 1:

Results are presented as Tukey's box-and-whisker plots. Unless otherwise noted, data in the text are presented as means ± SD. All animals included in the analysis passed Grubbs test to confirm closeness for all measured parameters at baseline as inclusion criteria. Data analysis between groups and time points was performed via two-way analysis of variance (ANOVA), with Tukey's post hoc test when appropriate. Survival analysis was performed by the Kaplan–Meier log-rank test. Analyses between only two groups were performed with Welch's unpaired t test. All data analysis was performed in R, version 3.6.3. Before experiments were initiated, sample sizes were calculated based on a = 0.05, and power = 0.9 to detect differences between primary end points (MAP, HR, SaO_2). Results were considered statistically significant if $P < 0.05$ (34).

For Sections 2-5:

Results are presented min-max box-and-whisker plots in sections 2 and 3. Results are shown as Tukey's box-and-whisker plots in sections 4 and 5. Data analysis within a single timepoint (comparing animal groups) was done using two-way ANOVA with Tukey's post-hoc multiple comparisons tests, unless otherwise noted. Data analysis within a single animal group (comparing timepoints) was done using two-way ANOVA with Šidák's post-hoc multiple comparisons tests. Survival analysis was done using a log-rank test to compare animal groups.

Data analysis for section 2.2.1E for CBC analysis (Figs. 16-18) was done with one-way ANOVA using Tukey's post-hoc multiple comparisons tests to compare different animal groups.

6.7 Animal Model

The mice used for the SCD model were male 8-12 week old homozygous HbSS-Townes transgenic SCD mice [B6; 129-Hbatm1(HBA)Tow Hbbtm2(HBG1, HBB)Tow/Hbbtm3(HBG1,HBB)Tow/J] obtained from Jackson Laboratory (Bar Harbor, ME). The HbSS-Townes mice have a C57BL/6J background, and are transgenic with both human α and β (β^A and β^S) globin genes knocked in.

The WT mice used were male C57BL/6J 8-12 weeks old, obtained from Jackson Laboratory (Bar Harbor, ME).

The female mice used were C57BL/6J female 8-12 weeks old, obtained from Jackson Laboratory (Bar Harbor, ME).

6.8 Use of Animals

All mice studies were approved in accordance to the UC San Diego Institutional Animal Use and Care Committee, protocol no. S11306.

6.9 Notable Restrictions

Due to time limitations in order to abide by mandated shutdowns due to COVID-19 in March 2020, the estradiol-treated mice used in the hypoxia experiments were only able to recover for 1 week following implantation of the estradiol pellet. Additionally, vaginal lavage and cell-staining was not done for any of the female animals in the hypoxia experiments. Fewer animals were used in the estradiol-treated female groups than the initially planned n=10 (hypoxia

survival n=7; hypoxia blood gas n=8). These changes, however, should not have a significant effect on the results of the experiments.

Section 7: References

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