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Novel drug and exercise therapies to restore metabolic function in children with severe burns

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Novel drug and exercise therapies to restore metabolic function in children with severe burns

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Dedication

This work is dedicated to all the patients and their families for graciously volunteering their time and well-being.

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Novel drug and exercise therapies to restore metabolic function in children with severe burns

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Severe burns encompassing over 30% of the total body surface area result in a pathophysiological stress response characterized by increased cardiac workload, elevated energy expenditure, altered muscle metabolism, a severe loss in lean body mass, and impaired physical function. This stress response can extend well beyond wound healing, which can negatively impact an individual's quality of life and their ability to reintegrate into society. Clinical and translational research in the past few decades has vastly improved the outcomes of patients who have suffered severe burns. However, continued research is needed to answer more questions to help us further understand severe burn trauma and how to improve medical care and rehabilitation. In the studies described within this dissertation, novel methods to measure the perturbations in skeletal muscle metabolism were employed, including stable isotope approached to quantify muscle protein turnover and high-resolution respirometry (HRR) to assay muscle mitochondrial function. Additionally, we were able to use these methods to test the efficacy of a combined pharmacological treatment of a testosterone analog and β-blockade in severely burned pediatric patients during their acute hospitalization period. Following their discharge, we were able to test the effectiveness of this new pharmacological approach combined with a hospital-based exercise program in restoring metabolic and physical function. Our results shows for the first time, that β-blockade and testosterone therapy can significantly reduce cardiac workload, decrease resting energy expenditure, and restore skeletal muscle mass and function in children recovering from severe burns. Our study is limited to a small sample size, but our results have tremendous implications in clinical treatment of patients with severe burns. Future multi-center clinical trials involving men and women of all ages are now needed to validate the utility of the combined drug therapy in restoring physiological function in burn survivors.

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3RM	3 Repetition-Maximum
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BPM	Beats Per Minute
CoA	Coenzyme A
CS	Citrate Synthase
DC	Discharge
DEXA	Dual Energy X-ray Absorptiometry
DTNB	Dithiobis Nitrobenzoic acid
EX	Exercise
FA	Fatty Acid
FBR	Fractional Breakdown Rate
FFA	Free Fatty Acid
FSR	Fractional Synthesis Rate
GCMS	Gas Chromatography Mass Spectrometry
Gly	Glycerol
GSBS	Graduate School of Biomedical Science
HR	Heart Rate
IGF-1	Insulin Growth Factor 1
IL-6	Interleukin-6

IL-7	Interleukin-7
IL-8	Interleukin-8
LMI	Lean Mass Index
Nm	Newton meter
OX	Oxandrolone
OXEX	Oxandrolone and exercise
Oxphos	Oxidate Phosphorylation
Oxprop	Oxandrolone and Propranolol
PARP	Poly (ADP-Ribose) Polymerase
PE	Post Exercise
Phe	Phenylalanine
P-NMR	Phosphorus-31 Nuclear Magnetic Resonance
PPARa	Peroxisome Proliferator-Activated Receptor alpha
PROPEX	Propranolol and exercise
Ra	Rate of Appearance
Rd	Rate of Disappearance
REE	Resting Energy Expenditure
rhGH	Recombinant Human Growth Hormone
RHR	Resting Heart Rate
RPE	Rate of Perceived Exertion
SD	Standard Deviation
SE	Standard Error
TBSA	Total Body Surface Area

TCA	Tricarboxylic Acid
TG	Triglyceride
TNF-a	Tumor Necrosis Factor alpha
UCP1	Uncoupling Protein 1
UCP2	Uncoupling Protein 2
UTMB	University of Texas Medical Branch
VCO_2	Carbion dioxide production
VE	Ventilator Equivalence
VO2	Oxygen uptake
W	Watts

CHAPTER 1

Introduction

SEVERE BURN INJURY: A PUBLIC HEALTH ISSUE

Severe burn injury results in disability and mortality. In 2004, 11 million people around the world with severe burn injury required medical care[1]. This ranked fourth in all injuries, topping the incidence rate of tuberculosis and HIV infection, combined. In the United States, more than half a million burn injuries occur annually[2]. Of these cases, approximately 40,000 require hospitalization and 3,400 results in death. Advances in burn care over the last three decades have vastly improved the morbidity and mortality of severe burn injury[3].

The cost of care for burn injury is significant due to prolonged length of stay, multiple surgical procedures, intensive care, wound care, and extended periods of rehabilitation. The mean cost of burn care in high-income countries was $88,218, ranging from $704 to $717,306[4]. This figure was higher than non-burn trauma patients at $17,245 and acute surgery patients at $26,468[5]. The percentage of total body surface area (TBSA) burned was shown to be a strong predictor of health care cost. One percentage TBSA equated to $4,097, up to 80% TBSA[5]. Higher burns beyond 80% did not fit this relationship probably due to high mortality rates.

Despite the major advancements in burn care, burn survivors still endure a prolonged metabolic stress response. This stress response warrants further investigation and continued improvement of the quality of burn care.

PATHOPHYSIOLOGY OF BURN HYPERMETABOLISM

Severe burn injury over 30% of the total body surface area (TBSA) results in a hypermetabolic and hyperinflammatory effect that may persist up to three years after injury[6]. The initial "ebb" phase occurs with the first 48 hours after injury is associated with *hypometabolism* and *decreased* cardiac output. Subsequently, the prolonged "flow" phase is characterized by a hyperdynamic circulation and *hypermetabolism*. Post-burn hypermetabolism can be attributed to altered levels of catecholamines, pro- and anti-inflammatory cytokines, and corticosteroids[7]. These altered levels can be from 10- to 20-fold increases in major burns compared to non-burned individuals[6,8].

Hypermetabolism can be measured by calculating the patient's resting energy expenditure (REE) from O_2, consumption and CO_2 production. REE is elevated up to 140% of the predicted value by the Harris-Benedict equation for three years after burn injury with males exhibiting higher REE than females[9]. This elevated REE is accompanied by increased cardiac workload where the heart rates of burned patients at rest exceed non-burned healthy values by 1.6 times[10], and myocardial oxygen consumption exceeds the values of a trained adult[11].

Increased REE in burn patients can be attributed, at least in part, to increased glucose, lipid, and amino acid metabolism. Previous work by led by Wolfe and Yu and their colleagues utilizing stable isotope tracers have shown altered ATP consuming pathways. Approximately 57% of the increase in energy expenditure can be explained by altered whole-body protein synthesis, gluconeogenesis, urea production, and substrate cycles[12]. For example, profound increases in the triglyceride-fatty acid and glycolytic-gluconeogenic cycles are increased in burn patients by 450% and 250%, respectively[13]. The liver and skeletal muscle top the list of organs shown to exhibit increased oxygen utilization as a result of burn injury.

Hyperglycemia with concomitant hyperinsulinemia are hallmarks of the stress response to burns, resulting from impaired central and peripheral insulin sensitivity[14,15]. As a result of severe burn, gluconeogenesis is increased and accounts for approximately 11% of the hypermetabolic response[12]. Increased glucose production led to increased glucose to delivery to peripheral tissues; however, it was not matched by an increase in glucose oxidation[3]. A study utilizing stable isotopes demonstrated that increasing glucose feeding from 5 to 8 mg/kg per minute in burned pediatric patients did not increase glucose utilization for energy production[16]. Therefore, the increase in glucose production and delivery is almost entirely directed to the wound where it is likely utilized by fibroblasts, endothelial, and inflammatory cells by anaerobic metabolism[17,18]. As a byproduct of anaerobic metabolism, lactate is produced and recycled to the liver for glucose production[3]. Additionally, alanine is also broken down from skeletal muscle to contribute to gluconeogenesis[3]. Hyperglycemia and insulin resistance present shortly after burn injury and can persist through recovery and long-term rehabilitation[6].

More recent studies have suggested that increased REE in burn patients may be attributed to altered mitochondrial function. By analyzing fat tissue biopsies of burned patients, Sidossis and colleagues found that subcutaneous white adipose tissue can adopt a brown fat phenotype by a process called "browning"[19]. The browning of white fat is due to the chronic increased adrenergic stimulation induced by severe burn injury. Characteristics of brown fat compared to white fat include increased mitochondrial density and size, increased uncoupling protein-1, and multilocular lipid droplets[20-22]. Brown fat in humans have been shown to contribute to non-shivering thermogenesis and can possibly explain part of the purpose for increased whole-body lipolysis, triglyceride-free fatty acid cycling, and fat oxidation[23-25]. This thermoregulatory response may be beneficial for severely burned patients since heat loss and the inability to properly thermoregulate is commonly observed[26].

IMPACT OF BURN INJURY ON SKELETAL MUSCLE

Skeletal muscle is the main storage depot for amino acids. Therefore, the increased demand for amino acids by other organs and tissues following severe burn trauma comes at the cost of losing lean muscle mass. Following severe burn injury, the increased demand for amino acids for skin wound healing augments the breakdown of amino acids in skeletal muscle[27,28]. Therefore, burn injury results in chronic muscle catabolism, which is influenced by REE, weight, age, the male sex, and delays in surgical treatment[29]. Presumably, some of those correlations exist because the more muscle mass someone has the more amino acids are available to breakdown and utilize elsewhere. Utilizing stable isotopes of amino acids, Hart et al. showed that this exaggerated protein turnover and loss of lean mass was seen up to 9 months post-injury[30]. Unlike the potential benefits of substantial fat loss, the erosion of skeletal muscle mass led to further complications and ill-effects. Chang et al. noted that when 10% of lean body mass is lost the immune system is comprimized. Additionally, a 20% loss in lean mass is associated with impaired wound healing. A 30% loss in lean mass results in pneumonia and mortality is increased to 50%, and death is expected when there is a 40% loss of lean mass[31].

MITOCHONDRIAL DYSFUNCTION IN SEVERE BURNS

The impact of burn injury on mitochondrial function has not been as extensively studied as hypermetabolism and muscle wasting. However, this area has gained traction with the surge in investigations on brown fat[24,32-34], browning of white fat or “beige” fat[19,35-37], and the role that mitochondria uncoupling proteins play in it[22,38-40].

As previously mentioned, severe burn injuries increase the demand for ATP. This has a great implication on mitochondrial function and the electron transport chain where the constant requirement for ATP production is necessary[40]. Although ATP demand is increased, animal studies analyzing mitochondrial gene expression showed down-regulation of the TCA cycle and oxidative phosphorylation[41]. Moreover, that same study showed that citrate synthase activity, commonly used as a proxy of mitochondrial density, was also down-regulated. After burn injury, mitochondrial cytochrome C levels were reduced with increased concentration of cytosolic cytochrome C indicating damaged mitochondrial membrane damage[42]. Utilizing permeabilized skeletal muscle myofibers, Cree and colleagues found diminished maximal coupled mitochondrial respiration with no alteration in uncoupled respiratory capacity[43]. This indicates a shift in energy utilization showing that a larger percentage of mitochondrial respiration going towards uncoupling without ATP production. The authors suggested that this is a wasted mechanism that does not contribute to wound healing, but other investigators may argue this may be a protective mechanism from reactive oxygen species[44,45] or provide necessary thermoregulation[46,47]. Nonetheless, the compounded effects of damaged mitochondria and diminished mitochondrial abundance and oxidative capacity may result in the deficit of ATP production in muscles of severely burned individuals, particularly during muscular contraction.

ADVANCEMENTS IN BURN CARE

A major goal of acute burn care is promote wound healing and to prevent chronic infections and the development of sepsis, which was considered to be the leading cause of death after burn injury[48]. It has also been shown that septic patients exhibit greater muscle catabolism and hypermetabolism compared to non-septic patients with the same degree of burn injury[29]. Reduced mortality after severe burns is partly attributable to early

excision and grafting of burn wounds early after injury[49,50]. Excision of burn wounds within 24 to 48 hours after injury was shown to decrease blood loss, mitigate the inflammatory and catabolic response, decrease the length of stay, and decrease mortality rate in burned children and young adults[51-57]. For example, early excision of large burn wounds burns that cover at least 50% TBSA will decrease the metabolic rate by 40% if it is done within three days rather than one-week post injury[29]. Stable isotope studies also show that patients who underwent early excision within three days post-injury, combined aggressive feeding, showed less net protein loss than those who had burn excision at 10 days post-injury with enteral feeding[56]. Removal of burned tissue also reduces the proliferation of inflammatory mediators to surrounding tissues such as IL-6, IL-8, TNF-α, and lipopolysaccharide[58]. This decrease in inflammatory mediators was also associated with decreased REE. The effect of these inflammatory mediators on hypermetabolism has a profound impact in burn care since it was shown that IL-6, IL-7, and IL-10 in particular, may be implicated as a predictor of mortality in burn victims[59]. Allografts, xenografts, skin substitutes may be utilized for patients with extensively large burns and limited donor sites[60].

The hypermetabolic response to burns functions, at least partially, to compensate for the heat loss by water loss through evaporation and absence of intact skin at the burn site[3]. It was suggested that a central physiological mechanism resets in body temperature to 2°C above normal[3]. Multiple studies showed the impact that environmental factors can have on thermoregulation in severely burned individuals. Wilmore et al. showed that hypermetabolism can be reduced by raising the ambient room temperature to 33°C[26]. This simple method of setting environmental temperature between 28 and 33°C can reduce the resting energy expenditure for patients with severe burns over 40% TBSA. Caldwell et al. found that the removal of burn wound dressing and exposure to thermoneutral temperature at 28°C for four hours led to an increased rate of heat loss of 27 watts per square meter of body surface area when compared to normal values[61]. In this

study, they suggested that increased heat production in burned victims was a response to an increase in heat loss and not vice versa. Additionally, they suggested that using a patient's comfort level to the ambient temperature may reduce hypermetabolism[62].

NUTRITIONAL INTERVENTION

Patients with severe burns over 40% TBSA have been observed to lose up to 25% of their admission body weight three weeks after injury[63]. Therefore, aggressive nutritional support must be implemented by enteral or parental feeding, especially when the burn injury limits the ability of burn patients to consume food ad libitum. There is little data available regarding the impact of nutrition on morbidity and mortality after burn injury. Nutritional guidelines are available; however, these guidelines do not guarantee specific benefits. Some suggest that 65 kcal per kg or 150% of the REE, or 2.5 g of protein per kg of body weight will provide the support needed to promote anabolism and physical activity[64]. Wolfe and colleagues suggest that protein intake over 3 g/kg/day is required to provide the amino acids necessary to promote protein synthesis and healing of burn injury to 50% of the body[65,66]. They even suggested if the amino acid requirement of other tissues, such as liver and immune cells are to be considered, then the protein recommendation for burned individuals can be as high as 4 g/kg/day, which is approximately four times the intake of healthy adults. Determining the nutritional requirements of burn victims must be approached with caution. Researchers found that parenteral nutrition combined with maximally tolerated enteral feeding led to decreased liver function, diminished immune response, and increased mortality[67,68]. The estimated nutritional requirement can be determined by measuring REE by indirect calorimetry, then provide nutrition at 1.4 times the measured REE[69]. Providing nutrition at 1.2 times REE showed a 10% reduction in lean body mass. However, increasing that amount to 1.4 times REE showed maintenance of body weight with an increase of fat mass rather than

lean body mass[70]. Further investigation found that high carbohydrate (82% carbohydrate, 15% protein, 3% fat) diet stimulates protein synthesis, promoted muscle anabolism, and improves insulin production over an isocaloric-isoprotein high fat (42% carbohydrate, 14% protein, 44% fat) diet[71]. This may seem counterintuitive to feed a high carbohydrate diet to hyperglycemic patients. But in severely burned patients, carbohydrates may provide a better energy source for wound recovery and muscle preservation than fat.

Some investigators suggest that supplements may offer additional support in improving burn recovery by combating infection and protection against reactive oxygen species. Some supplements that have been studied include glutamine[72-75], trace elements (copper, selenium, zinc)[76-78], arginine[79,80], and vitamins D and E[81].

Despite the absence of a consensus for nutritional support across burn centers, the approach taken by each institution is similar. In 2013, the European Society for Clinical and Nutrition and Metabolism released their recommendation for nutritional therapy for severe burn injury[82]: enteral feeding should be provided early along with trace element and vitamin supplements, increase protein intake (1.5 – 2 g/kg in adults, 3 g/kg in children), and limit glucose delivery to 55% of energy or 5 g/kg/h by infusion. Similarly in 2016, the American Society for Parental and Enteral Nutrition (ASPEN) and the Society for Critical Care Medicine (SCCM) recommended early enteral nutrition, protein intake between 1.5 – 2 g/kg, and that antioxidant vitamins and trace elements may improve outcome in critically ill patients with severe burns[83].

PHARMACOLOGICAL INTERVENTIONS

Multiple pharmacological interventions have been studied with an aim to mitigate the stress response to burns and/or promote rehabilitation. Recombinant human growth hormone (rhGH) has been shown to accelerate wound healing and improve the quality of wound healing without increased scarring[84-86]. Also, burned children who received rhGH

showed improvement in immune response, height, lean body mass, bone mineral content and total body weight over burned children who received the same standard of care without rhGH[87-90]. However, the adverse effects of rhGH, particularly hyperglycemia, limit its use as part of the standard of burn care[91]. Furthermore, a multicenter study in critically ill non-burned adults showed that rhGH increased morbidity and mortality[92].

The positive effects of rhGH were thought to be mediated in part by insulin-like growth factor-1 (IGF-1). Indeed, protein oxidation was decreased, and glucose uptake was increased in patients receiving IGF-1, but hypoglycemia was also present in these patients[93]. It appears that the combining IGF-1 and its principal binding protein (IGFBP-3) results in improved protein metabolism without heightened risk of hypoglycemia[94]. However, consideration for further use of IGF-1 with IGFBP-3 requires further safety and efficacy studies.

Intensive insulin therapy has been employed to alleviate hyperglycemia in burned patients. In addition to improving glucose control, patients receiving intensive insulin therapy also showed increased donor site protein synthesis[95]. Insulin was suggested to have anabolic properties; therefore, it was shown to improve protein synthesis in cross-leg studies utilizing stable isotope tracers of amino acids[96,97]. When combined with a high carbohydrate – high protein diet, patients receiving intensive insulin therapy exhibited improved lean body mass, bone mass, decreased muscle wasting, and shorter hospital length of stay than those patients who did not receive insulin therapy[98]. Despite the beneficial effects seen with insulin therapy, it is technically challenging to monitor and maintain euglycemia in critically injured patients. Additionally, the risk of hypoglycemia may be a significant barrier to universal use of intensive insulin therapy in the burn ICU.

The use of metformin to improve blood glucose has been studied in burned patients, and has been considered a safer approach over insulin because it does not carry the same risk of inducing hypoglycemia. Commonly known as Glucophage, it is a biguanide prescribed to treat hyperglycemia in type II diabetic patients. In severely

burned patients, metformin can augment peripheral insulin sensitivity and inhibit central gluconeogenesis[99]. It was also shown to improve muscle protein synthesis and thereby improve net protein balance[100]. A large concern for the utilization of metformin is the potential side effect of inducing lactic acidosis. Eight severely burned adults who were treated with metformin showed increased plasma lactate concentration, but no substantial change in arterial pH[100]. The association of lactic acidosis with metformin use in severe burn injury remains unclear. Metformin may provide clinical value for critically ill burned patients, but further investigation with close patient monitoring is needed.

Another approach to control blood glucose is the use of fenofibrate. It is a peroxisome proliferator-activated receptor alpha (PPARα) agonist that can reduce plasma glucose absent risk of hypoglycemia. Cree and colleagues found that two weeks of fenofibrate treatment in severely burned children improved plasma glucose by improving insulin sensitivity[101]. This same team also showed improved skeletal muscle mitochondrial function and increased whole body fat oxidation with fenofibrate treatment[102]. The evidence on the efficacy of fenofibrate for severe burn injury is quite limited. Additional research to further support the previous studies may have promising outlook on the use of fenofibrate to treat severe burn trauma.

BETA-BLOCKADE TREATMENT FOR HYPERMETABOLISM

Severe burns result in a substantial increase of catecholamines which results in increased myocardial oxygen consumption, myocardial contractility, and hyperdynamic blood flow[6,103]. This action is mediated by catecholamine signaling through β-adrenergic receptors[104]. Propranolol is a non-selective β-adrenergic receptor blocker that has been extensively studied to ameliorate the hypermetabolic response to severe burn injury. Its mechanism of action is to block the effect of the burn-induced surge in catecholamine on β-adrenergic receptors thereby decreasing the cardiac work load, heart rate, and rate

pressure product[105]. Early investigations on short-term, low-dose propranolol use at 0.5 – 2 mg/kg/day reduced tachycardia and cardiac workload but had no effect on REE, cardiac output, plasma glucose or free fatty acids[103,105]. It was also found that there was a dose-dependent effect where patients would start propranolol treatment at 1 mg/kg/day but must increase to 4 mg/kg/day within ten days to sustain the effects on cardiac work[106]. It was suggested that it was safe to use propranolol at a dose to reduce heart rate by 20% of the admission value in severely burned patients while effectively reducing cardiac work and hypermetabolism[11]. A large randomized controlled trial confirmed the safety and efficacy of long-term (one year) propranolol use at 4 mg/kg/day without risk of hypotension[104].

Propranolol also reduces lipolysis seen in severely burned individuals, thereby inhibiting ectopic fat accretion in tissues and organs, such as the liver[107,108]. This effect is mediated particularly by inhibiting β2-adrenergic receptors[109] since the reduction in lipolysis was not found when burned patients were administered metoprolol tartrate, a selective β1-blocker[109]. Patients who were treated with propranolol to reduce their heart rate by 12 – 15% also showed a reduction in liver size. One year use of propranolol in severely burned children over 30% TBSA showed lowered plasma triglycerides and prevention of central fat accumulation[104].

Studies utilizing animal and human muscle biopsies found that the nuclear enzyme poly(ADP-ribose) polymerase (PARP), an enzyme that promotes cellular necrosis, was activated chronically after severe burn injury[110]. Patients who were treated with propranolol showed suppression of PARP activation suggesting that there is an anti-catabolic effect. Indeed, a randomized controlled trial with severely burned pediatric patients found an 82% increase in skeletal muscle protein net balance with propranolol treatment versus a 27% decrease in protein net balance in patients who did not receive propranolol[111].

Additionally, propranolol treatment improved burn wound healing, decreased recovery time, and reduced the hospital length of stay[112]. The benefits of propranolol treatment did not increase the risk of infection or sepsis[113]. A systematic review and meta-analysis from nine randomized controlled trials and one nonrandomized controlled trial found that propranolol decreased REE, improved peripheral lean mass, and improved insulin resistance without adverse effects[114]. Although propranolol is currently not standard treatment for burn care, there is a large body of evidence to support that supposition.

TESTOSTERONE THERAPY

Severe burns results in an increased circulating levels of testosterone within the 10 days of injury, followed by prolonged downregulation that persists up to three years which may contribute to skeletal muscle loss[6]. Anabolic agents, such as testosterone enanthate, were used to improve protein synthesis in severely burned hypogonadal men[115]. However, oxandrolone is considered safer than testosterone due to its low virilization making it the preferred method for women and children[116-118]. By stimulating androgen receptors in skeletal muscles, oxandrolone stimulates protein synthesis and anabolism. Indeed, stable isotope infusion studies show increased protein synthesis and greater amino acid utilization in skeletal muscle when treated with oxandrolone[119,120]. Additional gene studies with patients who received oral administration of oxandrolone at 0.1 mg/kg twice a day showed upregulation of genes associated with myogenesis and muscle tissue regeneration, and suppression of genes involved in apoptosis and trauma-induced cell death[121,122]. Many studies support the benefits on oxandrolone to improve body composition, maintain lean body mass, increase bone mineral density and content, shorten the length of stay, and reduce mortality[123-126]. These benefits of oxandrolone continue to be seen five years after injury with few side effects, even when treatment has

stopped[127]. Along with its low side effects, easy oral administration, and relatively low cost, oxandrolone will continue to play a key role in improving burn care[128].

REHABILITATIVE EXERCISE

Extensive burn wounds, immobilized limbs, and prolonged bed rest during inpatient hospitalization greatly diminishes the functional capacity of severely burned individuals. Indeed adults with large burns who exhibit lower muscular strength and endurance than non-burned adults[129,130]. This problem persisted one year after burn injury, long after the wound has healed showing insufficient recovery and rehabilitation. This was also seen in severely burned children who had lower lean body mass and lowered leg strength and muscular performance compared to non-burned children[131].

Suman and colleagues showed that a standard hospital rehabilitation exercise program improved muscle strength and power, and lean body mass compared to a standard outpatient rehabilitation program that did not include exercise[132]. A 12-week resistance training program for children over seven years old with over 50% TBSA showed greater improvement in upper- and lower-limb muscle strength compared to an age-matched group receiving only the standard of care without exercise[133]. Improvements were also seen in pulmonary function in these patients who participated in a 12-week exercise rehabilitation program[134].

The combination of propranolol and exercise therapy was utilized to rehabilitate severely burned children. These patients began propranolol treatment during their acute care, and continued their treatment while participating in resistive and aerobic exercise for 12 weeks. Improvements in muscle mass, strength, and cardiorespiratory fitness were improved after exercise training in both patients who took propranolol and those who did not[135]. In this study, children who took propranolol showed significantly greater increases in cardiorespiratory fitness after exercise than children who did not take propranolol.

The use of oxandrolone and exercise was also investigated to determine if additional benefits would be seen with oxandrolone administration. Indeed, all pediatric patients who exercised showed improvement in muscle strength, lean body mass, and cardiopulmonary capacity[136]. However, children who received oxandrolone showed greater increase in lean body mass after exercise than children who did not receive oxandrolone and exercised and children who took oxandrolone but did not exercise[136].

In addition to the physiological benefits of rehabilitative exercise, patients also reported improved psychosocial functioning and improved quality of life[137,138]. Hypermetabolism was not exacerbated, and hyperthermia did not occur in burned patients when participating in exercise[139-141]. In a recent survey of 103 physical and occupational therapists, 81% reported that no hospital-based exercise program was available[142]. With the evidence supporting the benefits of exercise, a hospital-based exercise program should be strongly considered as standard of rehabilitation from burn injury to improve the physical and social functioning of burned victims.

SUMMARY

A recent review on burns found that improved burn care drastically improved the survival of victims of severe burn injury. From 1980 to 1986, victims with massive burns covering 60% TBSA had a 32% mortality rate which was reduced to 18% from 1987 – 2011[143]. Pharmacological, nutritional, environmental, and surgical therapies greatly reduced some of the burn-induced hypermetabolic and hypercatabolic stress response. However, these conditions persist at abnormal levels in burn victims. Thus, continued progress by researchers and clinicians conducting basic science and clinical and translational research is needed to further elucidate and the pathophysiology of severe burn injury, and to develop new treatment strategies.

Propranolol is one of the most extensively studied drugs that to have a substantial effect on reducing post-burn hypermetabolism and hypercatabolism. Thus far, it has been shown to be safe for long-term use in burned children and adults[104]. Oxandrolone has also showed promising results to improve protein metabolism and increase lean body mass for pediatric and adult burn patients with very few side effects[117,125]. It is low cost and, as an oral drug, very easy for patients to administer and comply with treatment. Long-term outcomes after five years showed that oxandrolone treatment is safe and effective for burn survivors[144]. However, the combined use of these effective treatments has yet to be explored.

A hospital-based exercise program has been beneficial for the long-term recovery for burn survivors to improve lean body mass and restore skeletal muscle function[132]. Aside from the beneficial results in clinical studies, an exercise program can provide a sense of well-being and assist in re-integrating burned survivors back into society as independently functioning individuals. Oxandrolone and propranolol have individually been combined with exercise therapy to show additional benefits in lean body mass and cardiopulmonary fitness, respectively[135,136]. However, the combined use of oxandrolone and propranolol with exercise therapy is still unknown.

Combination therapies to take advantage of the individual benefits that each drug can provide have largely been unexplored. Through their different modes of action, anti-catabolic properties with β-blockade and anabolic action with oxandrolone have the potential to have a combined effect that can further improve patient outcome. In this study, we explored the effect of combined oxandrolone and propranolol therapy during the acute inpatient recovery and outpatient exercise rehabilitation. Our central hypothesis is that severely burned pediatric patients receiving oxandrolone and propranolol will show improved outcomes compared to patients who only received the standard of care. To test our hypothesis, we set the following specific aims:

Specific Aim 1: *To determine protein turnover in severely burned children from their early acute care until one-year post injury*. We will utilize a novel bolus injection method with stable isotope tracers of phenylalanine. We will quantify the whole body skeletal muscle fractional synthesis (FSR) and breakdown (FBR) rates by the precursor to product method. We hypothesize that protein turnover will be chronically elevated long-term, particularly by an exaggerated FBR.

Specific Aim 2: *To determine long-term skeletal muscle mitochondrial function in severely burned children*. We will utilize our novel method of measuring skeletal muscle mitochondrial respiratory capacity by high-resolution respirometry as an indicator of skeletal muscle quality. Muscle biopsies will be collected from severely burned children from their early acute care until one-year post injury. We hypothesize that altered mitochondrial function will persists long-term after burn injury.

Specific Aim 3: *To determine the efficacy of oxandrolone and propranolol therapy during inpatient care after severe burns*. Severely burned children will be randomized to receive standard of care or standard of care with oxandrolone and propranolol. We will determine the benefits of these combined drugs on skeletal muscle protein turnover, lipid metabolism, lean body mass, and skeletal muscle mitochondrial function. We hypothesize that patients receiving oxandrolone and propranolol will show reduced protein turnover and lipolysis, improved body composition and mitochondrial function over patients receiving standard of care alone.

Specific Aim 4: *To determine the effectiveness of oxandrolone and propranolol on muscle mass and function after a 6-week exercise program*. After their discharge, all patients enrolled in the study will participate in a 6-week exercise program. We will measure their muscle strength and cardiorespiratory fitness as well as body composition. We hypothesize that children taking oxandrolone and propranolol with show greater improvements than children who participated in exercise only without drugs.

We anticipate that propranolol will exert its effect in the reduction hypermetabolism eliciting a reduction in protein and lipid kinetics. Additionally, we also anticipate the anabolic effect of oxandrolone on improving muscle mass and function. This is the first study to provide physiological evidence of the effect of combined oxandrolone and propranolol treatment on burn-induced hypermetabolism.

CHAPTER 2

Skeletal muscle protein turnover in severely burned children

INTRODUCTION

Severe burns (>30% of the total body surface area [TBSA]) result in a pathophysiological stress response that lasts at least two years post injury[145]. Inflammation and chronic adrenergic stress following burn injury are thought to contribute to increased amino acid turnover in skeletal muscle[146-148]. Increased skeletal muscle protein breakdown and synthesis are hallmarks of the acute stress response to burns. However, increased muscle protein synthesis does not match the increase in muscle protein breakdown after burn injury, leading to chronic amino acid loss [94,149-151]. This discordance in skeletal muscle proteolysis and accretion is considered the principal culprit underlying skeletal muscle cachexia in burn survivors.

Our group has previously reported that amino acid loss across the leg remains elevated in burn survivors for as long as nine months after the burn [152]. In that study, the rates of protein synthesis and breakdown in skeletal muscle were determined indirectly via cross-leg arterial venous balance studies. Notably, the necessity for arterial and venous catheters, prolonged infusion times to reach isotopic steady states (>3 h), and the measurement of blood flow cross-leg makes these studies invasive and technically challenging to perform, particularly in the outpatient setting. In recent years, our group has developed methodologies to determine skeletal muscle fractional synthesis rate (FSR) and fractional breakdown rate (FBR) during non-steady state conditions using bolus injections of two isotopically labeled phenylalanine tracers[153]. This permits the determination of FSR and FBR in a study lasting 60 minutes, without the need for central lines and blood flow measurements.

In the current study, we used this novel method for determining skeletal muscle FSR and FBR to investigate the long-term effect of burns on protein turnover in skeletal muscle. We hypothesized that elevated skeletal muscle protein turnover, primarily increased FBR, would persist in burn victims long into their convalescence.

MATERIALS AND METHODS

Patients

This study was approved by the Institutional Review Board at the University of Texas Medical Branch (Galveston, TX). Informed consent was obtained from each patient's parent or guardian prior to enrollment in the study. Between July 2008 and March 2014, 42 subjects who met the inclusion criteria were enrolled in this study. Inclusion criteria were as follows: children over the age of 1 year but less than 18 years, children with burns covering more than 30% of the TBSA who were admitted to Shriners Hospitals for Children—Galveston for acute burn care within three days of injury, and children able to participate in stable isotopic studies.

Upon arrival at the hospital, all patients received standard burn care, which included fluid resuscitation and total burn wound excision within 48 h of admission. Sequential surgical procedures for wound grafting were performed as needed until the burn wounds were healed. A constant infusion of insulin was administered intravenously to patients when patient's blood glucose concentration exceeded 200 mg/dl, in accordance with standard clinical practice.

Patients were intubated for operations and immediately extubated after. Ventilator settings for those who remained intubated followed ARDS-NET recommendations [154]. Any occurrence of sepsis was recorded and aggressively treated with the administration of antibiotic/antimicrobial agents. Patients received up to five days of bed rest after excision and grafting procedures. Afterward, patients ambulated daily and received occupational and physical therapy as part of their rehabilitation.

Each patient received enteral nutrition via nasoduodenal tube during their acute hospitalization until they were able to consume food voluntarily. Patients received Vivonex® TEN (Sandoz Nutritional Corp., Minneapolis, MN), composed of 82%

carbohydrate, 15% protein, and 3% fat. Daily caloric intake was calculated using the Galveston formula, which delivers 1,500 kcal/m^2 TBSA burned (for burn hypermetabolism) + 1,500 kcal/m^2 TBSA (maintenance) [155,156]. The target caloric intake equaled 1.4 times the patients' resting energy expenditure, as measured by indirect calorimetry. This feeding regimen was started at admission and continued at a constant rate until the wounds were healed. Total caloric intake remained constant during hospitalization, and this protocol was carried through the infusion studies. Once discharged, patients received nutritional drinks (Boost, Nestle Health Care Nutrition) to ensure that caloric intake was 1.4 times the measured resting energy expenditure.

For outpatient infusion studies, nutritional support was provided with 10% Travasol® (amino acid) solution (Clintec Nutrition, Deerfield, IL). A primed dose of 0.45 ml/kg was followed by a continuous infusion at 1.35 ml/kg/h. This primed dose was administered one hour prior to the first bolus injection followed by the constant infusion given through the duration of the study. The 10% Travasol® solution contained 100 mg/ml of amino acids composed of leucine (7.3 mg/ml), isoleucine (6 mg/ml), lysine (5.8 mg/ml), valine (5.8 mg/ml), phenylalanine (5.6 mg/ml), methionine (4 mg/ml), tryptophan (1.8 mg/ml), alanine (20.7 mg/ml), arginine (11.5 mg/ml), glycine (10.3 mg/ml), proline (6.8 mg/ml), serine (5 mg/ml), and lysine (0.4 mg/ml).

Stable isotope infusion procedure

Skeletal muscle protein kinetics were measured using a bolus tracer injection method (*Fig. 1*). This method was developed to less invasive and time intensive for patients than arterial-venous dilution approaches, while providing comparable quantitative data on protein turnover rates. Our group has previously found that this method produces FSR and FBR values similar to the muscle protein synthesis and muscle protein breakdown values derived from arterial-venous balance methodologies [153,157].

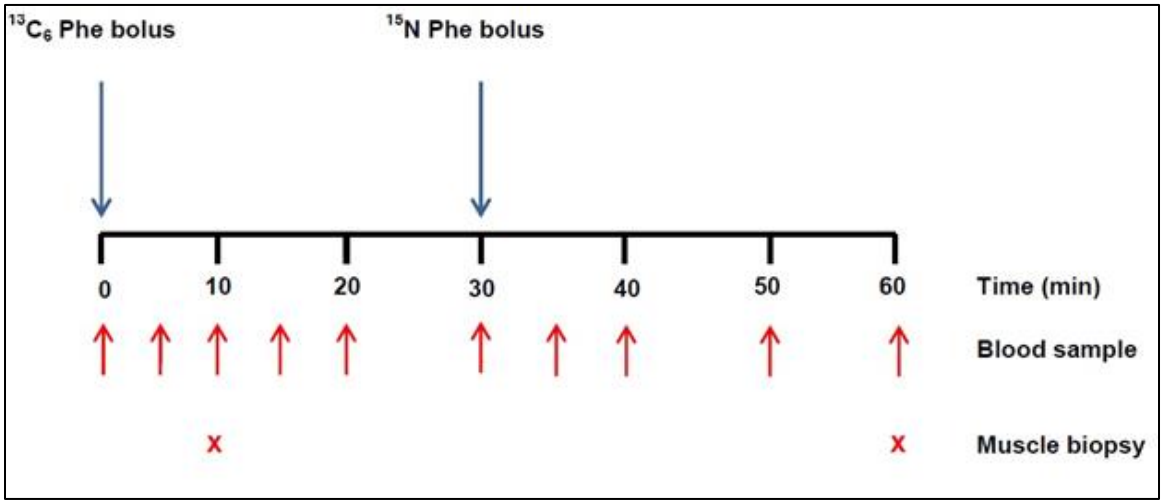


Figure 1. Time line of the stable isotope study using the bolus injection method.
A bolus of isotopically labeled $^{13}C_6$ Phe was injected at 0 minutes followed by ^{15}N Phe at 30 minutes. Approximately 1 ml of blood was sampled at times indicated in red arrows. Muscle biopsies from the m. vastus lateralis was collected at 10 and 60 minutes indicated by X. Phe, phenylalanine.

Blood samples were collected prior to the bolus tracer injection to determine background enrichment. A bolus injection of L-[ring-$^{13}C_6$]Phe (5.56 mg/kg) and L-[^{15}N]Phe (5.40 mg/kg) in 3 ml of 0.45% saline were injected intravenously at 0 and 30 min, respectively. Blood samples (~1.0 ml) were collected at 5, 10, 15, 20, 29, 35, 40, 50, and 60 min following the injection of the L-[ring-$^{13}C_6$] Phe tracer. Muscle samples (~100 mg total) were obtained from the *m. vastus lateralis* at 10 and 60 min using a suction-adapted Bergström needle[158]. Muscle samples were washed in ice-cold saline to remove visible blood, frozen in liquid nitrogen, and stored at -80°C for later processing. Blood samples were centrifuged at 3,000 rpm for 20 min. Plasma (~0.5 ml) was aliquoted into cryotubes and stored at -20°C for later processing.

Sample analysis

For measurement of Phe enrichment in blood, 500 μl of plasma was pipetted into a glass tube containing the same volume of 15% sulfasalicylic acid. These samples were centrifuged at 3,000 rpm for 10 min. Supernatants were loaded into solid phase extraction columns to separate Phe. Next, Phe was eluted using 1 M ammonia hydroxide and dried overnight in a speedvac. Phe enrichment was then determined in tert-butyldimethylsilyl derivatives by gas chromatography-mass spectrometry [159].

For measurement of Phe concentration and enrichment in muscle samples, an internal standard containing 3 μmol/l of L-[ring-$^{13}C_9$,^{15}N] Phe was added to ~30 mg of muscle. The muscle sample was homogenized twice in 10% perchloric acid. Samples were centrifuged at 3,000 rpm for 10 min. The supernatant was collected to measure free intracellular (unbound) amino acid[159]. Enrichment of protein-bound Phe was determined by thoroughly washing muscle pellets with saline and alcohol, drying them overnight at 50°C [160], and hydrolyzing the dry protein pellets by an overnight incubation in 6 N HCl at 110°C. The bound protein hydrolyzate and intracellular supernatant were processed by the same method as blood samples. Isotopic enrichments in blood and muscle samples were measured on an Agilent 6890 gas chromatograph-mass spectrometer. Ions were selectively monitored at mass-to-charge (m/z) ratios of 336, 337, 340, 342, and 346 for phenylalanine enrichment. Isotopic enrichments were expressed as tracer-to-tracee ratio. Muscle concentrations of free intracellular phenylalanine were calculated from the internal standard.

Calculations

Muscle FSR was calculated according to the precursor-product method[161]. The value for the precursor was the area under the decay curve for intracellular phenylalanine

enrichment, and the product was the change in bound enrichment over time (Eq. 1). FBR was calculated from the decay in intracellular and plasma enrichment E^{153}, where the ratio of bound to free intracellular phenylalanine is represented by Q_M/T. The amount of free phenylalanine in the muscle sample was measured and normalized to micromoles of free phenylalanine per gram of muscle. It has previously been shown that one gram of dry muscle protein contains 150 μmol of phenylalanine[162]. The content of protein-bound phenylalanine in one gram of muscle was calculated by [(250 μmol/g) × (percent dry protein in muscle)].

$$FSR = \frac{EB(t2)-EB(t1)}{\int_{t0}^{t1} EM(t)\Delta t} \quad (1)$$

$$FBR = \frac{[EM(t2)-EM(t1)]\cdot \int_{t2}^{t3}[EP(t)-EM(t)]\Delta t-[EM(t3)-EM(t2)]\cdot \int_{t1}^{t2}[EP(t)-EM(t)]\Delta t}{\int_{t2}^{t3} EM(t)\Delta t \cdot \int_{t1}^{t2} EP(t)\Delta t- \int_{t1}^{t2} EM(t)\Delta t\cdot \int_{t2}^{t3} EP(t)\Delta t} \cdot \left(\frac{QM}{T}\right) \quad (2)$$

EB, bound protein enrichment; EM, enrichment of muscle intracellular pool, EP, plasma enchrichment; t, time; QM, muscle intracellular concentration of Phe; T, bound protein concentration of Phe. Phe, phenylalanine.

Statistical analysis

A mixed multiple analysis of variance related each outcome (FSR, FBR, and net balance) to the time points (2 wk, 4 wk, 6 mo, 12 mo) and the potentially prognostic covariates of age and burned TBSA, blocking on subject. Differences among time points were assessed by Tukey-adjusted contrasts. An analysis of variance was performed with a compound symmetry correlation structure to compensate for repeated measures and with weighting per time point to compensate for heterogeneity of variance. Values for burned patient outcomes at each time point were compared to values from healthy subjects by student's *t*-test. Statistical analyses were performed using R statistical software (R Core Team, 2013, version 3.1.1). A 95% level of confidence was assumed. Values are reported as mean ± SE. Statistical significance was accepted when $p \leq 0.05$.

RESULTS

Muscle protein turnover was analyzed in 42 severely burned pediatric patients (30 male, 12 female) in the fed state. Their mean age was 7.1 ± 4.8 years with a burn injury of 50 ± 14% TBSA. Subject characteristics are shown in Table 1.

Table 1. Subject characteristics

n	42
male/female	30/12
Age (y)	7.1 ± 4.8
TBSA (%)	50 ± 14
3rd degree (%)	34 ± 23

Values are mean ± SD. TBSA, total body surface area.

Skeletal muscle FSR was 0.11 ± 0.02%/h at 2 weeks, 0.19 ± 0.03 %/h at 4 weeks, 0.11 ± 0.02 %/h at 6 months, and 0.14 ± 0.02 %/h at 12 months. No significant differences were detected among any time points (*Fig. 2A*). However, FSR values at 2 weeks, 4 weeks, and 12 months were al higher than muscle protein FSR previously reported in non-burned adults by Phillips *et al.* (0.07 ± 0.01 %/h, $p \leq 0.05$) [163], who used labeled phenylalanine tracers to measure protein turnover in 19 healthy untrained young men who were also in the fed state. At 6 months post-burn, FSR tended to be higher than healthy control, but it was not statistically significant ($p = 0.07$). Similar to FSR, FBR values did not significantly differ at 2 weeks (0.18 ± 0.02 %/h), 4 weeks (0.28 ± 0.03 %/h), 6 weeks (0.18 ± 0.02 %/h), or 12 months (0.25 ± 0.05 %/h) (*Fig. 2B*). These values were 4- to 6-fold higher than those seen in healthy adults (0.05 ± 0.01 %/h, $p < 0.01$) as previously reported[164].

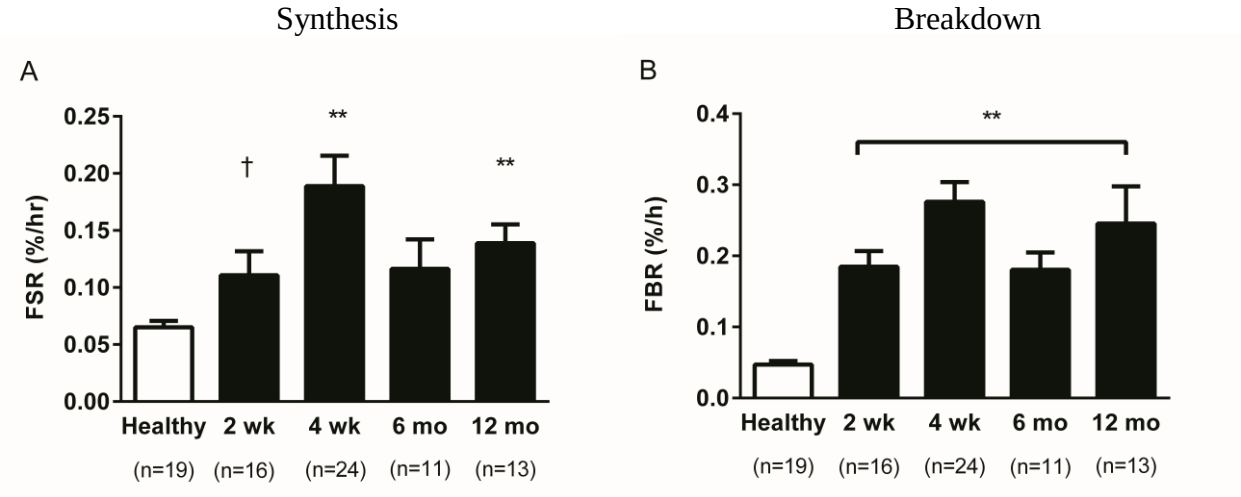


Figure 2. Protein turnover in pediatric burn patients and healthy adults.

Fractional synthesis rate (FSR) (A) and fractional breakdown rate (FBR) (B) were elevated in burn patients (black bars) compared to healthy adult (white bars). †p=0.05, *p<0.05, and **p<0.01 vs. healthy. Data from healthy men was from Phillips et al.[164].

Net protein balance was determined by calculating the difference between FSR and FBR. We observed a negative net protein balance from acute hospitalization to 12 months post burn, as elevation of muscle FBR was greater than that of FSR (*Fig. 3*). The net protein balance in burn patients was -0.07 ± 0.02 %/h at 2 weeks, -0.09 ± 0.04 %/h at 4 weeks, -0.06 ± 0.04 %/h at 6 months, and -0.11 ± 0.05 %/h at 12 months. In contrast, healthy, non-burned adults in the fed state showed a net positive protein balance (0.02 ± 0.01 %/h, $p < 0.05$ vs. burn all values).

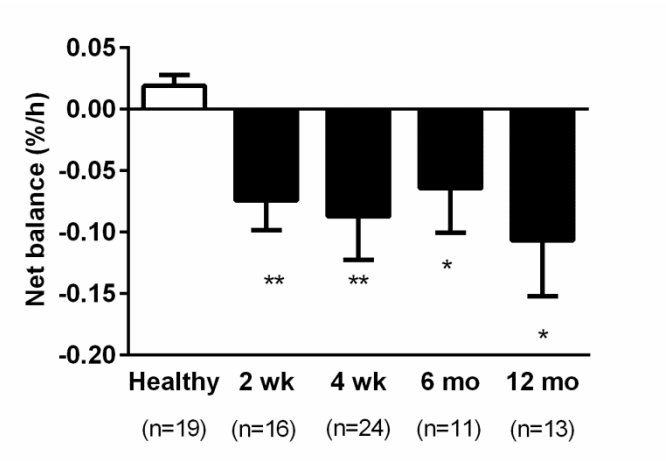


Figure 3. Net protein balance in pediatric burn patients and healthy adults.

Net balance was calculated as the difference of FBR from FSR. *$p<0.05$ and **$p<0.01$ vs. healthy.

DISCUSSION

Severe burns induce acute, concurrent elevations in muscle protein synthesis and breakdown, with the rate of breakdown outpacing synthesis to induce loss of skeletal muscle proteins. Whether this imbalance in skeletal muscle protein metabolism persists after closure of burn wounds remains unclear. Using a novel stable isotope approach, we found that, in skeletal muscle, derangements in protein metabolism persist for up to one year after burns. In particular, our results indicate that increased muscle protein turnover following burn injury is attributable to elevated FBR. What we found particularly interesting was the similar increasing and decreasing temporal change in both FSR and FBR following burn injury, indicating that FBR likely drives FSR, as suggest previously[165]. However, the magnitude of the increase in FBR was consistently greater than that of FSR, which likely leads to a chronic loss of skeletal muscle proteins over time in burn survivors.

Consumption of protein or intravenous amino acid infusion results in hyperaminoacidemia. This increases availability of amino acids within myocytes, where protein synthesis incorporates some of these amino acids into bound proteins such as cellular organelles and myofibrillar proteins. Free amino acids arising from protein catabolism also augment intracellular amino acid concentration. When breakdown occurs at greatly elevated rates, as seen in burned patients, the intracellular free amino acid pool becomes saturated, stimulating protein synthesis. Our group has previously shown that intravenous amino acid infusion does not further stimulate protein synthesis in children with massive burns [166], suggesting that the inward flow of amino acids from feeding does not significantly augment the availability of free intracellular amino acids. Perhaps this is because burn victims have intracellular amino acid concentrations in skeletal muscle that are several fold higher than those seen in healthy individuals.

Intracellular amino acid cycling (see schematic of process in *Fig. 3*), where amino acids derived from breakdown are resynthesized into proteins, is known to increase in burn victims [148,166]. Therefore, we suggest that FBR drives FSR in skeletal muscle of burn vicitms. While this could be viewed as energetically wasteful and contributing to hypermetabolism in burn survivors, it may be somewhat protective by preventing unchecked amino acid loss from skeletal muscle.

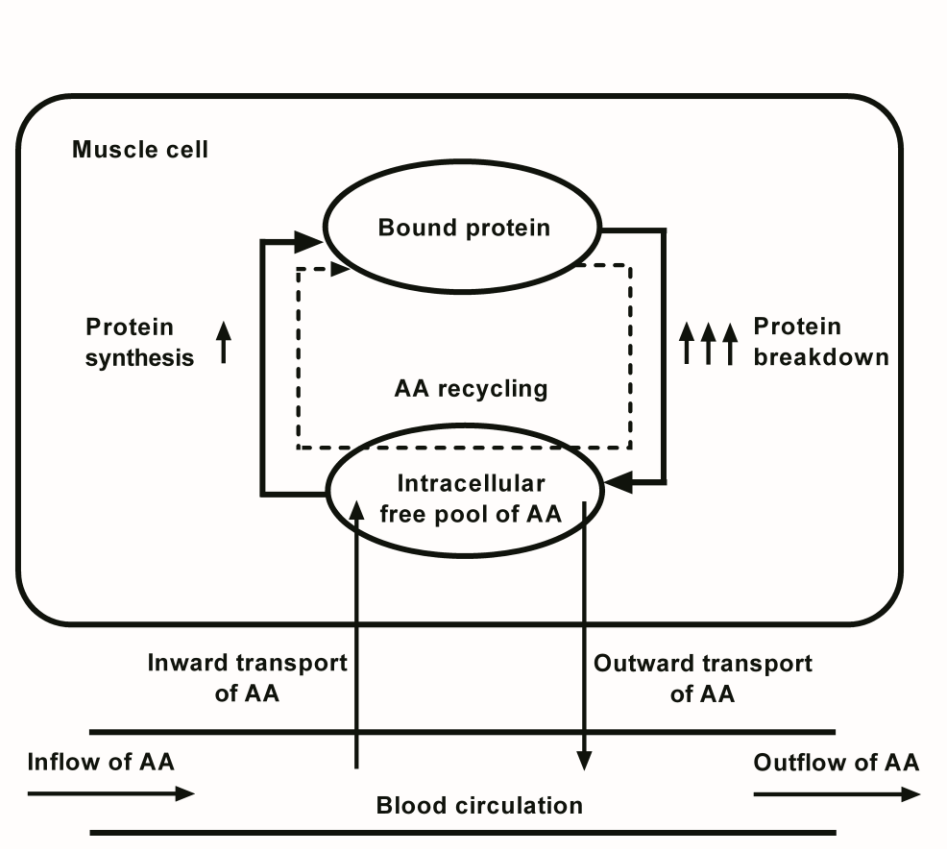


Figure 4. Schematic of amino acid (AA) recycling.

Elevated protein breakdown stimulates release of AAs from bound protein. This increases the availability free intracellular AAs, driving incorporation of the same AAs back into bound protein. The intracellular free AA pool is largely augmented by protein breakdown and not affected by influx of AA from exogenous sources. Diagram is adapted from Diaz et al. [167]. AA, amino acid.

Our current findings are consistent with our previous protein kinetic data obtained from healthy and severely burned adults[168]. In this previous study, muscle protein breakdown was 83% greater in burn victims than in healthy individuals. Similarly, muscle protein synthesis was 50% greater in burn patients than in healthy adults, most likely due to the significantly higher intracellular amino acid concentration in muscle of burn patients[165]. Moreover, leucine and lycine kinetics were similarly perturbed as phenylalanine kinetics in burn victims. In this previous study, our group used the three-compartmental model measuring arterio-venous differences in amino acid concentrations to calculate muscle protein breakdown and synthesis. As in the current study, skeletal muscle amino acid losses after burn could be explained by an increase in muscle protein breakdown that was not accompanied by an elevation in muscle protein synthesis of similar magnitude. The ability of the bolus injection method to identify the same burn-related disruption in skeletal muscle amino acid kinetics as more contemporary constant infusion approaches suggests that this method is a valid means of determining protein turnover in skeletal muscle after burns.

We have previously determined skeletal muscle amino acid kinetics in pediatric patients for up to 1 year after burns using arterial-venous balance methods[152]. We demonstrated that, consistent with our current findings, the rate of amino acid appearance in the femoral vein (a marker of skeletal muscle protein breakdown) was elevated for 9 months post injury. Moreover, amino acid appearance in the femoral vein occurred at a greater rate than amino acid disappearance from the femoral artery (an index of muscle protein synthesis), resulting in a negative protein balance across the leg. Our current study, which involves direct measurement of skeletal muscle FSR and FBR, provides further support for the notion that muscle cachexia in burn victims is the result of marked elevations in proteolysis.

In recent decades, advances in the standard of clinical care for burn victims have significantly increased survival. However, our current data show that these patients

experience profound alterations in skeletal muscle protein metabolism for as long as one year following burn injury (*Fig. 2*). This chronic muscle proteolysis contributes to the profound cachexia seen in burn survivors, which delays recovery and negatively affects quality of life. Therapeutic strategies aimed at improving skeletal muscle protein balance and thus mass, will likely be efficacious in reducing morbidity in burn victims.

Previous studies have shown that a number of pharmacological interventions increase muscle protein synthesis in burn victims[169-171], leading to a better match between the rates of muscle protein breakdown and muscle protein synthesis. Diaz and colleagues recently reviewed the effects of oxandrolone and propranolol on skeletal muscle protein turnover in burn victims[167]. It was noted that most pharmacological interventions increase the efficiency of muscle protein synthesis but have little effect on protein breakdown. Given that excessive muscle protein breakdown is the overriding phenotype of the massively burned patients, interventions to reduce catabolism of muscle protein may be more useful in preserving lean mass in burn victims.

One limitation of the current study is that muscle protein kinetics in burned children was unable to be compared to those in healthy non-burned children due to ethical issues involved in performing invasive studies in otherwise healthy children. However, we were able to compare data from burned children to data from healthy non-burned young men[163], which are likely similar to healthy children. This particular study was preferred for our healthy control comparison because their FSR and FBR were measured while subjects were in the fed state, which would eliminate any assumptions we would have to make if they were fasted. Although their methods were slightly different, previous studies show that there were no significant differences between the bolus injection and constant infusion method for quantification of muscle protein kinetics[157,161]. An additional weakness was that the current study was a cohort study. A total of 42 patients were included in the study, but each individual was not included in each time point. On several occasions in the acute period post injury patients could not to

be studied due to clinical issues, conflicts, or the patients early discharge, while other patients were lost to follow up at 6 and 12 months. Not all 42 patients were represented equally in all four groups. However, our mixed analyses of covariance were blocked on subjects to compensate for repeated measures, which should help compensate for this problem. Future prospective studies are warranted to study the same patients over time. With that said, the stable isotope bolus injection method used here allows skeletal muscle protein turnover to be determined in a study lasting less than 2 h, without requiring the catheterization of the femoral artery and vein or the measurement of blood flow. This provides a less invasive and time-consuming approach for quantifying skeletal muscle amino acid kinetics in clinical populations. Further, these future studies can investigate the cellular pathways responsible for this deviation in protein turnover we observed in severely burned patients.

In conclusion, we demonstrated that protein turnover remains elevated for up to one year following severe burn injury. Chronically elevated skeletal muscle catabolism is not matched by a similar increase in muscle protein synthesis, causing persistent loss of skeletal muscle amino acids. We suggest that chronically elevated FBR is responsible for muscle cachexia observed in burn survivors. Investigation of novel therapies that blunt skeletal muscle proteolysis to improve protein net balance will likely hasten the recovery of burn survivors.

CHAPTER 3

Altered mitochondrial function in severe burn injury

INTRODUCTION

Severe burn injury covering over 30% total body surface area (TBSA) results in a prolonged hypermetabolic and hypercatabolic stress response[6,29,30]. This stress response is characterized by increased resting energy expenditure (REE), elevated heart rate, and an elevated rate of the loss of muscle mass[172]. If left unmanaged, these stress responses increase morbidity and mortality in burned victims[173].

The metabolic rate of humans is determined by the amount of oxygen required to support physiological functions, such as substrate oxidation by ATP production and thermoregulation. The overwhelming majority (90%) of whole-body oxygen consumption occurs within mitochondria[174]. Yu et al. suggest that approximately 50% of the increased energy requirement is due to ATP turnover. Therefore, mitochondria may play a significant role in burn-induced hypermetabolism. However, this cannot be fully explained just by increased oxidative phosphorylation. The biochemical understanding of mitochondrial biogenesis in burn hypermetabolism is not fully understood.

Our colleagues previously showed that coupled mitochondrial respiration (ATP-producing) in skeletal muscle is lower in burned children compared to healthy non-burned children[43]. However, they also found that uncoupled respiration (non-ATP-producing) remain unchanged indicating that a larger portion of energy requirement is directed to thermogenesis rather than ATP production. This hypothesis was supported by previous studies showing that the hypermetabolic response is affected by occlusive wound dressings and elevations in ambient temperature[26,61,62]. This indicates that altered skeletal muscle mitochondrial function may be an adaptive mechanism for necessary heat

production. We recently showed that severely burned adults exhibit acutely altered mitochondrial function compared to healthy non-burned adults[47]. The coupling of oxygen consumption to ATP production was diminished along with increased ratio of uncoupling respiration going towards thermogenesis[47]. Additionally, increased skeletal muscle thermogenesis was associated with hypermetabolism, suggesting that skeletal muscle mitochondrial contributes to hypermetabolism.

Skeletal muscle mitochondria in healthy humans are well coupled with ATP production (>80%), whereas in other tissues, particularly brown adipose tissue, where mitochondria predominantly function for non-shivering thermogenesis[174]. In severely burned patients with a diminished ability to thermoregulate [26] and subjected to prolonged immobilization, the increase in skeletal muscle mitochondrial thermogenesis may be advantageous.

As previously mentioned, hypermetabolism is known to persist up to two years after injury[6], and uncoupled skeletal muscle mitochondrial respiration contribute to hypermetabolism[47]. However, it is unknown if altered skeletal muscle mitochondrial function persists long-term beyond the acute hospitalization period. The purpose of this study was to determine skeletal muscle mitochondrial function in severely burned patients up to one year post-injury. We hypothesize that altered skeletal muscle mitochondrial function persists long-term after severe burn injury.

MATERIALS AND METHODS

Patients

Severely children encompassing more than 30% TBSA who were admitted to the Shriners Hospital for Children in Galveston for burn care were recruited for this study. Patients with burns wounds covering at least 40% TBSA were considered for consent. All patients received standard of burn care, including necessary drugs, early fluid resuscitation, and early wound debridement. Afterward, patients went through grafting procedures with autologous skin to cover open burn wounds. They were also provided nutritional support with a low-fat, high carbohydrate feeding formula (82% carbohydrate, 3% fat, 15% protein, Vivonex T.E.N.; Nestle Health Science, Minneapolis, MN) given enterally through a nasogastric feeding tube at 1,500 kcal/m2 body area + 1,500 kcal/m2 body area burned.

Burned patients were studied approximately two weeks post-injury, and at six months and 12 month follow-up visits. Following an overnight fast, skeletal muscle biopsies were collected from the m. vastus lateralis under ketamine sedation and local anesthesia with 1% lidocaine. Muscle biopsies were obtained utilizing a suction-adapted Bergstrom biopsy needle [158]. Resting energy expenditure (REE) were measured by indirect calorimetry (Sensor Medics Vmax 29, Yorba Linda, CA).

Sixteen young healthy non-burned men were also recruited from the Galveston, TX area to serve as a young healthy control group. Each participant reported to the Institute for Translational Research – Clinical Research Center (ITS-CRC) at the University of Texas Medical Branch. Following an overnight fast, skeletal muscle biopsies were collected from the *m. vastus lateralis* under local anesthesia, as described previously. Due to ethical constraints, we were not able to obtain quadriceps muscle biopsies from healthy non-burned children. We chose to use healthy adults to serve as controls to study the impact of burn injury to locomotive muscles. All human research

protocols were reviewed and approved by the Institution Review Board at the University of Texas Medical Branch. Written informed consent was obtained from all patients and/or their legal guardians and healthy participants before the start of the study.

Resting metabolic rate

Resting energy expenditure (REE) of burned patients was determined by indirect calorimetry (Sensor Medics Vmax 29, Yorba Linda, CA). REE was calculated from whole body oxygen consumption and carbon dioxide production rates using the Weir equation[175] by the equation: REE = (3.9 x VO_2) + (1.1 x VCO_2). This measured value was compared to the predicted REE which was calculated using the Harris-Benedict equation[176] (male: REE = 66.5 + (13.75 × weight [kg]) + (5.003 × height [cm]) – (6.755 × age [years]; female: REE = 655.1 + (9.563 × weight [kg]) + (1.850 × height [cm]) – (4.676 × age [years]). This is a standard method for estimating the degree of hypermetabolism in burned patients.

Muscle biopsy analysis

Approximately 10 – 20 mg of fresh skeletal muscle tissue was placed in an ice-cold (pH 7.1) preservation buffer (containing 10mM Ca-EGTA, 0.1 μM free Ca^{2+}, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM creatine phosphate) immediately upon collection. Muscle samples were then transferred to the laboratory where it was separated manually into approximately 1 mg myofiber bundles using sharp forceps. The sarcolemmal membranes of myofiber bundles were then permeabilized in a sucrose buffer containing 5 μM saponin for 30 minutes at 4°C. Afterwards, approximately 2 mg of tissue was blotted dry, weighed, and transferred to the chambers of an O2K respirometer (Oroboros Instruments,

Innsbruck, Austria) containing 2 ml of respiration buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 10 mM sucrose, and 1 mg/ml bovine serum albumin) for high-resolution respirometry measurements.

High-resolution respirometry

Mitochondrial substrates and inhibitors were added sequentially to the oxygraph chambers to determine each respiratory states. Initially, the leak respiratory state was recorded with the myofiber bundles alone. Afterward, octanoyl-L-carnitine (1.5 mM), pyruvate (5mM), malate (2mM) and glutamate (10 mM) were added to the oxygraph chamber to induce uncoupled state 2 respiration supported by complex I of the electron transport chain. Afterward, saturating levels of ADP (5mM) were added to the oxygraph chamber to reach state 3 maximal coupled respiration by complex I. Then 10 mM succinate was added to the oxygraph chamber to provide electrons via complex II, thereby activating the maximal couple state 3 respiration (maximal oxidative phosphorylation capacity [OXPHOS]). Finally, 5 μM oligomycin, an inhibitor of the F_O unit of the ATP synthase, was added to the oxygraph chamber to inhibit ATP synthase and induce maximal uncoupled respiration (state 4_O).

Our protocol for mitochondrial respiratory flux allows us to calculate respiratory control ratios which represents intrinsic mitochondrial functions. The respiratory control ratio (RCR) for ADP was calculated by state 3 divided by state 2, to determine mitochondrial ADP sensitivity. The substrate control ratio (SCR) for succinate was calculated by Oxphos divided by state 3, providing the index of succinate sensivity and functions of complex II (succinate dehydrogenase). Finally, the coupling control ratio (CCR) for oligomycin was calculated as state 4O divided by Oxphos. From this, the coupling control factor (CCF) was derived by substracting CCR from one (1 – CCR). CCR is indicative the portion of maximal respiration coupled to ATP production while

CCF represents the portion of maximal respiration linked to uncoupling and thermogenesis.

Statistical analysis

All data are presented as group means ± SE. Differences in group means were analyzed by unpaired t-tests. Because three groups were compared for muscle biochemical measurements, a Bonferroni correction was applied. Statistical significance was reached when $p < 0.05$. Statistical analyses were performed using Graphpad Prism version 7 (GraphPad Software, La Jolla, CA).

RESULTS

Subject demographics

Demographics for burned patients are presented in Table 2. Overall, 69 children were studied (8 ± 5 years; mean ± SD) with 77% males and 23% females. Patients had severe burns encompassing 44 ± 12% TBSA with 26 ± 20% 3rd-degree burns. Burned patients were hospitalized for 25 ± 17 days. Muscle biopsies were collected from 16 young healthy men (26 ± 4 years) to serve as controls. Demographics for healthy and burned patients studied at 2 weeks, 6 months, and 12 months post-burn are presented in Table 3.

Table 2. Burn patient demographics

	Mean ± SD
n	65
Sex (males/females)	50/15
Age (y)	8 ± 5
Height (cm)	123 ± 30
Weight (kg)	32 ± 21
Burn size (% TBSA)	44 ± 12
3rd degree burns (%)	26 ± 20
Length of stay (days)	25 ± 17

TBSA, Total body surface area

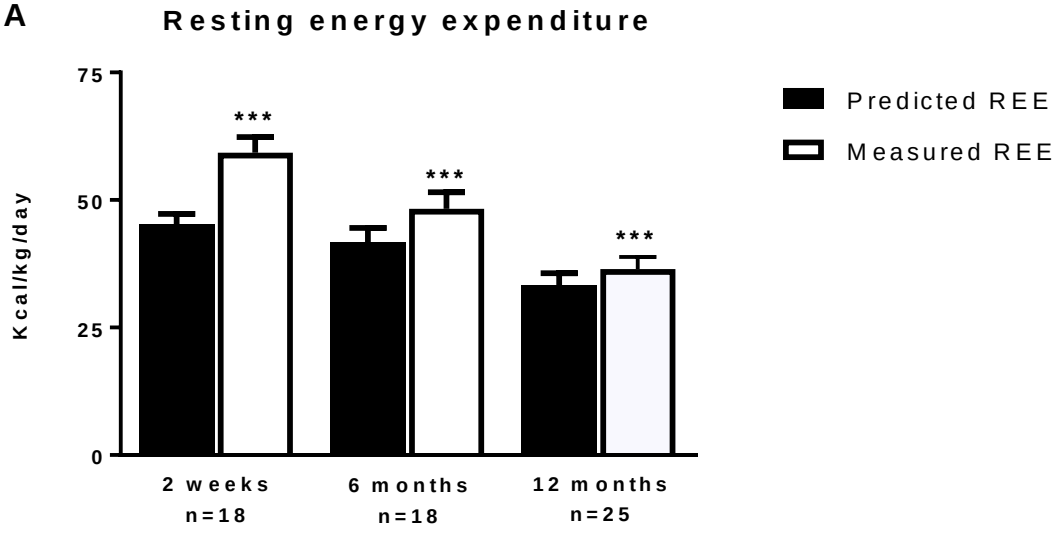
Table 3. Healthy control participants and burn patient cohort demographics

	Control (n=16)	**2 weeks (n=18)**	**6 months (n=25)**	**12 months (n=18)**
Age (y)	26 ± 4	5 ± 2	7 ± 5	11 ± 5
Height (cm)	179 ± 8	109 ± 2	122 ± 28	138 ± 28
Weight (kg)	82 ± 13	21 ± 8	31 ± 26	44 ± 27
Days post-burn	n/a	12 ± 7	191 ± 83	391 ± 103

Data are mean ± SD.

Burn hypermetabolism

REE for burned patients is presented in *Figure 5*. The degree of hypermetabolism was determined by comparing measured REE by indirect calorimetry to the predicted REE by the Harris-Benedict equation. The higher the discrepancy of measured REE to predicted REE indicates greater hypermetabolism. The greatest degree of metabolism was found at two weeks post-burn at 36% greater than predicted REE (46 ± 10 vs 62 ± kcal/kg/day, $p < 0.001$). Hypermetabolism persisted at 6 months (42 ± 10 vs 48 ± 11 kcal/kg/day, $p < 0.01$) and 12 months (36 ± 15 vs 39 ± 13 kcal/kg/day, $p < 0.01$). However, the degree of hypermetabolism decreased over time. When 2 weeks, the degree of hypermetabolism was significantly lower at 6 months (35 ± 33% vs 11 ± 29%, $p = 0.01$) and at 12 months (35 ± 33% vs 8 ± 2%, $p < 0.001$), suggesting that hypermetabolism may be resolved over time post-injury.



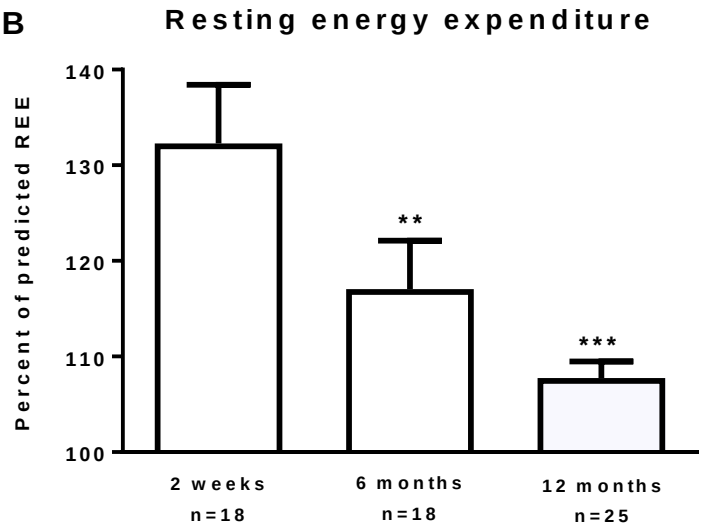


Figure 5. Predicted and measured REE.

REE predicted by the Harris-Benedict equation (Predicted REE) and measured from indirect calorimetry (Measured REE) is shown in (A) ***$p < 0.001$ vs predicted REE. Measured and predicted REE were compared by a paired t-test (B). **$p < 0.01$ and ***$p < 0.001$ vs 2 weeks post-burn. Values are presented as mean ± SE.

Skeletal muscle mitochondrial respiration

High-resolution respirometry data are presented in *Figure 6*. State 2 leak respiration with substrates of octanoyl-carnitine, pyruvate, malate, and glutamate was not different between groups. Compared to healthy control (40.3 ± 18.6 pmol/mg/sec), coupled state 3 respiration with the addition of ADP was significantly lower at 2 weeks (17.5 ± 5.7 pmol/mg/sec, $p < 0.001$), 6 months (24.1 ± 19.4 pmol/mg/sec, $p = 0.01$), and 12 months (22.3 ± 14.5 pmol/mg/sec, $p < 0.001$) post-burn (*Fig 6B*). Compared to 2 weeks, state 3 respiration was significantly greater at 6 months ($p = 0.017$).

Coupled Oxphos represents the maximal oxidative capacity of the electron transport chain after the addition of succinate shown in *Figure 6C*. Oxphos was significantly lower at 2 week (17.0 ± 8.3 pmol/mg/sec, $p < 0.001$), 6 months (41.8 ± 30.4

pmol/mg/sec, p = 0.03), and 12 months (35.8 ± 13.6 pmol/mg/sec, p = 0.001) when compared to healthy control (58.1 ± 12.7 pmol/mg/sec). Furthermore, oxphos was significantly greater at 6 months ($p < 0.01$) and 12 months ($p < 0.001$) when compared to 2 weeks post-burn.

Uncoupled state 4_O respiration was determined after the addition of oligomycin, the ATP synthase inhibitor (*Fig 6D*). State 4O respiration was significantly lower at 2 weeks (8.9 ± 4.3 vs 19.7 ± 7.4 pmol/mg/sec, $p < 0.001$), and 12 months (13.7 ± 4.4 vs 19.7 ± 7.4 pmol/mg/sec, p = 0.036), but not at 6 months (14.9 ± 2.6 vs 19.7 ± 7.4 pmol/mg/sec) when compared to healthy control.

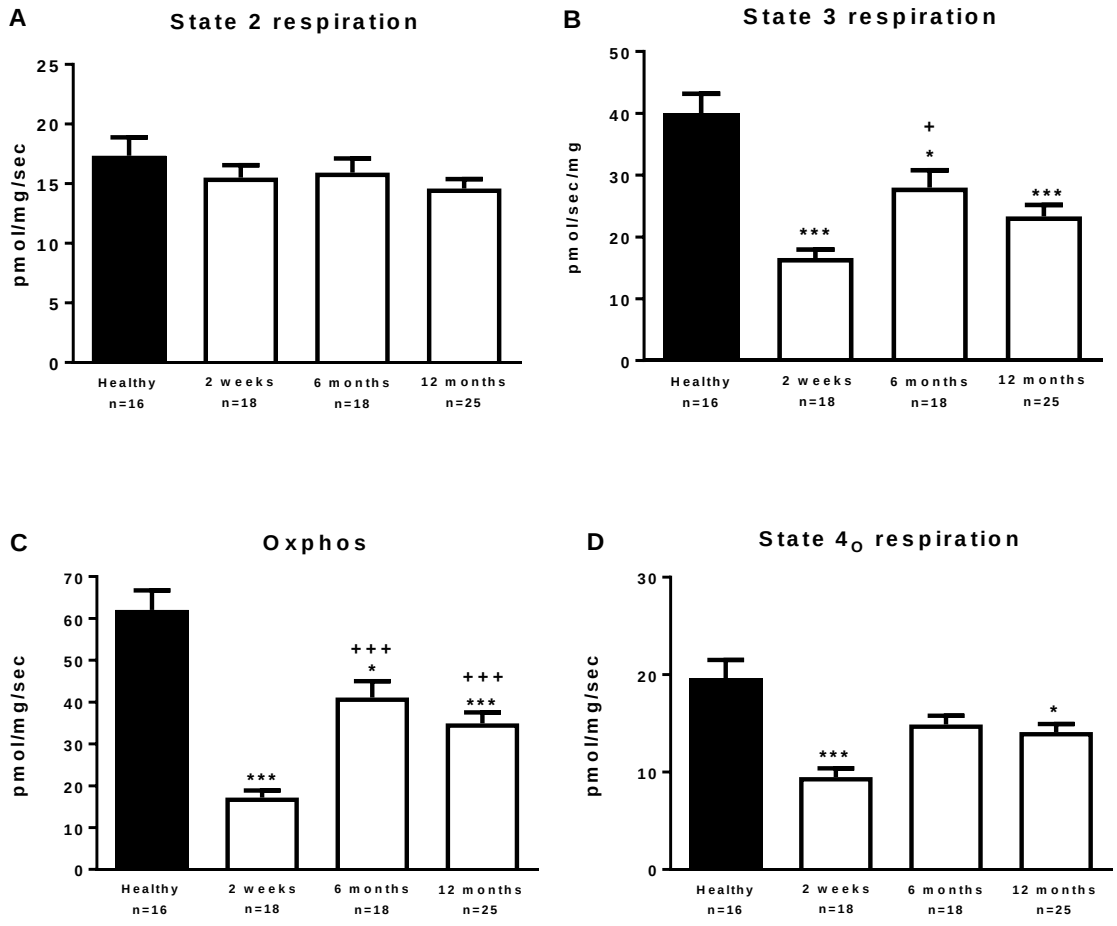


Figure 6. Mitochondrial respiration in healthy and burned muscles.

State 2 respiration in the leak state (+ octanoyl-carnitine [1.5mM], pyruvate [5mM], malate [2mM], and glutamate [10mM]) (A), state 3 coupled respiration with electron input through complex I of the electron transport chain (+ ADP [5mM]) (B), Oxphos coupled respiration with electron input through complexes I and II of the electron transport chain (+ succinate [10mM]) (C), and state 4_O leak respiration (+ oligomycin [5μM]) (D) where ATP synthase is inhibited by oligomycin . Data is modeled by analysis of variance to account for age, TBSA, and repeated measures. *$p < 0.05$ and ***$p < 0.001$ vs healthy control group. $^{+}p < 0.05$ and $^{+++}p < 0.01$ vs 2 weeks post burn. Values are presented by mean ± SE.

Skeletal muscle mitochondrial respiratory control

We calculated RCR to determine sensitivity to ADP, SCR for succinate, and CCR (and CCF) for oligomycin as measures for mitochondrial quality. RCR for ADP is shown in *Figure 7A*. RCR was significantly lower in 2 weeks (1.19 ± 0.06, $p < 0.001$), 6 months (1.68 ± 0.41, $p = 0.005$), and 12 months (1.61 ± 0.47, $p < 0.001$) when compared to healthy individuals (2.11 ± 0.81). This suggests that the ability of skeletal muscle mitochondria to make ATP is diminished after severe burn injury. Furthemore, RCR was significantly greater at 6 months ($p = 0.001$) and 12 months ($p = 0.005$) when compared to 2 weeks. This indicates improvement of mitochondrial capacity to produce ATP over time.

SCR for succinate is shown in *Figure 7B*. SCR was significantly lower in 2 weeks (1.19 ± 0.08 vs 1.42 ± 0.53, $p < 0.001$) when compared to healthy control suggesting that mitochondrial substrate sensitivity to succinate (complex II) is acutely reduced after severe burn injury. However, this was restored at 6 months post-burn. SCR was significantly greater at 6 months (1.44 ± 0.030 vs 1.19 ± 0.08, $p = 0.02$) and 12 months (1.54 ± 0.44 vs 1.19 ± 0.08, $p = 0.002$) when compared to 2 weeks post-burn.

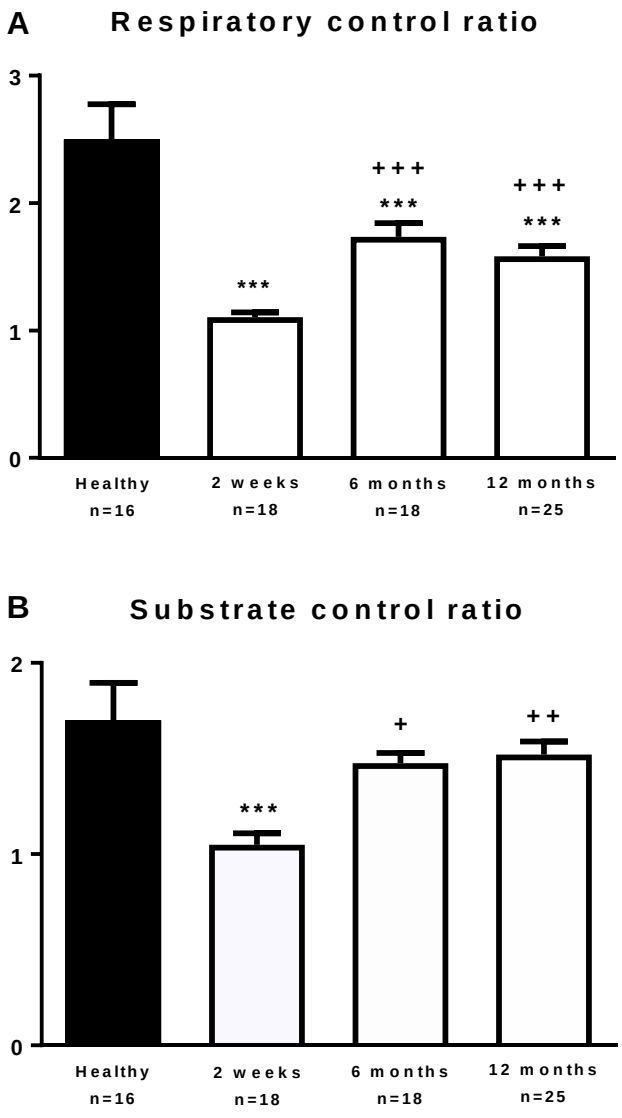


Figure 7. Mitochondrial respiratory ratio.

The RCR for ADP (A) and the SCR for succinate (B). Data was modeled by analysis of variance to account for age, TBSA, and repeated measures. ***$p < 0.001$ vs healthy control. $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$ vs 2 weeks post-burn. Values are presented as group mean with standard error. ADP, adenosine diphosphate; RCR, respiratory control ratio; SCR substrate control ratio; TBSA, total body surface area.

CCR for oligomycin, the ATP synthase inhibitor, is a ratio between zero and one. Therefore, CCF is determined by substracting CCR from 1 (1 – CCR). Thus, CCR represtions the fraction of respiration linked to phosphorylation, whereas CCF is linked to thermogenesis. These two fractions are presented as a percentage of maximal respiration in *Figure 8*. CCR was significantly higher in 2 weeks (0.53 ± 0.19 vs 0.32 ± 0.10, $p < 0.001$) and 12 months (0.38 ± 0.13 vs 0.32 ± 0.10, $p = 0.050$) in comparison to healthy control. CCR was numerically higher at 6 months post-burn, but that figure was not significant (0.32 ± 0.10 vs 0.35 ± 0.11, $p = 0.26$). Furthermore, when compared to 2 weeks post-burn, CCR was significantly lower at 6 months ($p = 0.02$), suggesting a marginal recovery of coupling control at this time post-injury.

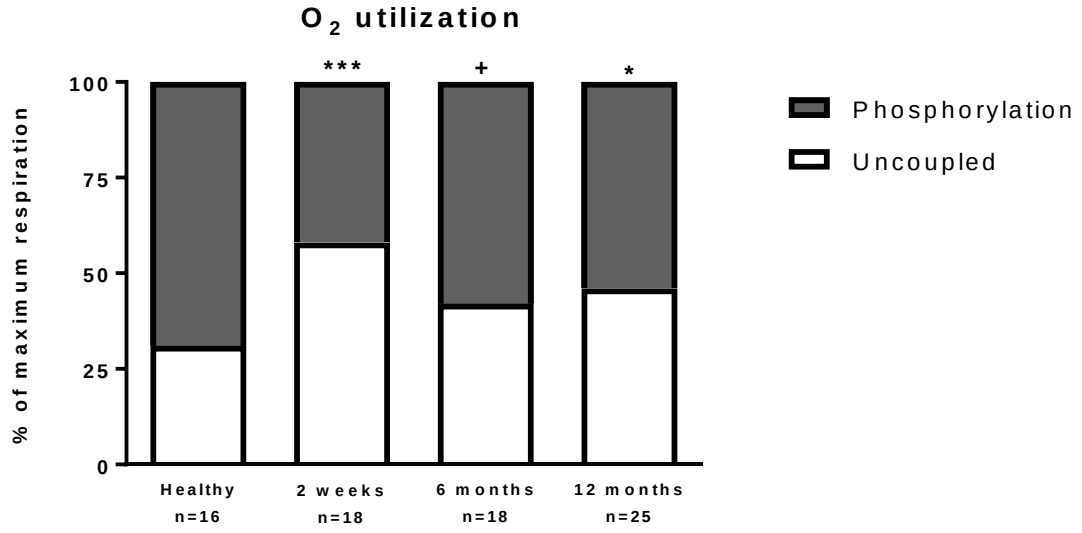


Figure 8. Coupling control ratio.

The CCR and CCF for oligomycin are shown. CCR shown as phosphorylation (grey), and CCF (white) shown as thermogenesis are represented as a percentage of maximal respiratory capacity. *$p < 0.05$ and ***$p < 0.001$ vs healthy control. $^{+}p < 0.05$ vs 2 weeks post-burn. CCR, coupling control ratio; CCF, coupling control factor.

Spearman correlation analysis was performed with REE and mitochondrial respiratory function and coupling controls. There were no significant correlations between state 2, state 3, or state 4_O (data not shown). However, there was a significant negative correlation between Oxphos and REE (R = -0.24, p = 0.03), suggesting a relationship between the degree of hypermetabolism and maximal oxidative capacity (*Figure 9*). Furthermore, RCR (R = -0.35, p = 0.001), SCR (R = -0.40, p < 0.001), and CCR (R = 0.28, p = 0.01) were all significantly correlated with REE, suggesting that diminished skeletal muscle mitochondrial control is associated with hypermetabolism in burned victims.

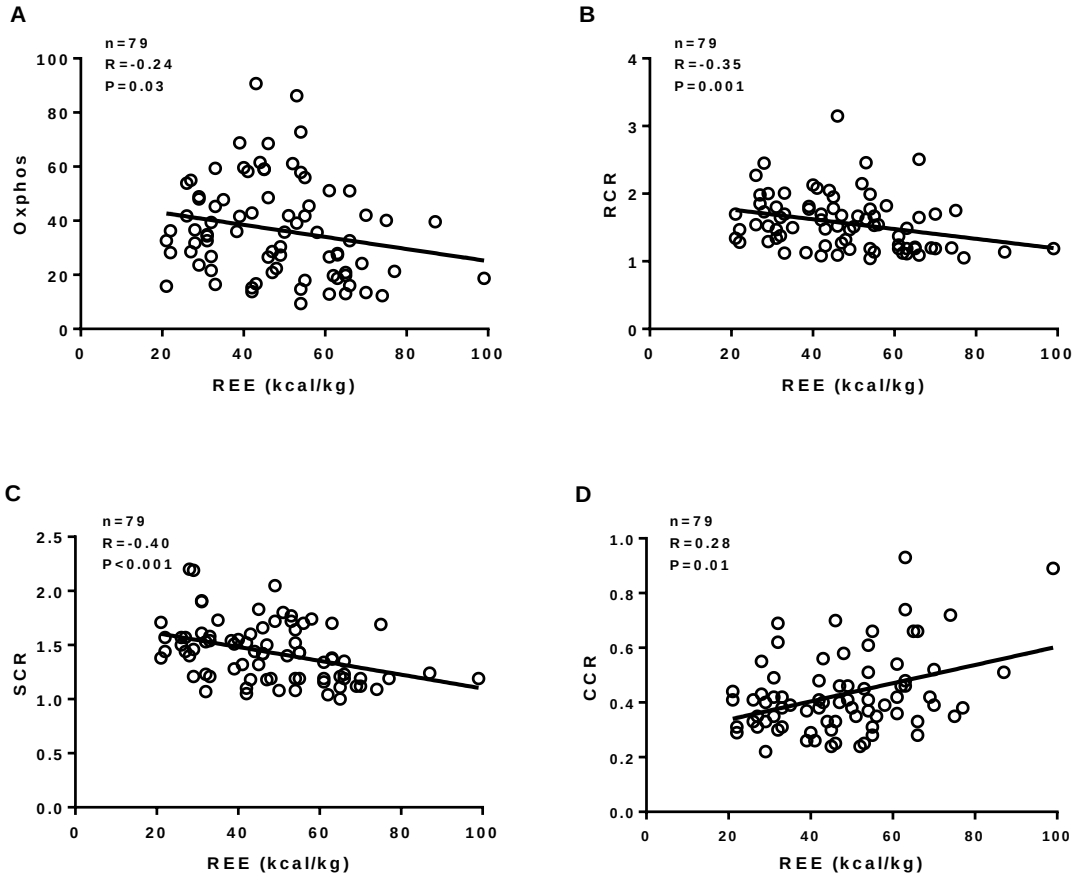


Figure 9. REE and respiration correlation.

Relationship between REE and Oxphos (A), RCR (B), SCR (C), and CCR (D) in burned children (n=79) ranging from 1 week to 2 years post-burn was determined by performing Spearman rank correlation analysis. There was a significant correlation between Oxphos, RCR, SCR, and CCR with REE. The Spearman correlation coefficient and p-value is reported on each graph. The straight line on each graph is the slope computed by linear regression. CCR, coupling control ratio; Oxphos, maximal oxidative phosphorylation; RCR, respiratory control ratio; REE, resting energy expenditure; SCR, substrate control ratio.

DISCUSSION

Severe burn injury results in a chronic pathophysiological stress response hallmarked by hypermetabolism, skeletal muscle wasting, morbidity and mortality[6,29,30,173]. Although several years of research have improved our understanding of burn-induced hypermetabolism, the complete biochemical understanding is lacking. In this study, we determined the skeletal muscle respiratory capacity and function in cohorts of severely burned children up to one year post-injury. We hypothesized that skeletal muscle mitochondrial thermogenesis contributes to burn-induced hypermetabolism. Our findings demonstrate the link between hypermetabolism and altered skeletal muscle mitochondrial function, which persists up to one year. In skeletal muscle mitochondria of burned victims, sensitivity to ADP and oligomycin was diminished for one year post-injury. Since this altered skeletal muscle mitochondrial coupling control is associated with increased whole-body REE, we suggest that altered skeletal muscle mitochondrial function contributes to hypermetabolism after severe burns.

Burn-induced hypermetabolism was previously shown to persist up to two years post-injury[6]. Acutely, this presents a problem with nutritional deficiency and the difficulty to provide adequate macronutrients[177,178], which may lead to increased loss of lean mass and prolong wound healing. Further, long-term effects of hypermetabolism may hinder rehabilitation and recovery, especially in severely burned children who show altered body composition and blunted growth compared to their age-matched non burned children[179]. Therefore, reducing hypermetabolism will likely improve recovery and outcomes in burn survivors.

Post-burn hypermetabolism is partially understood. Increased gluconeogenesis, lipolysis, substrate cycles, protein turnover have only explained about 60% of the hypermetabolic effect[12]. Additionally, Wilmore et al. demonstrated a relationship between burn severity, adrenergic stress, and degree of hypermetabolism. They also

showed a step-wise decrease in hypermetabolism with increasing ambient temperature[26]. Therefore, burn-damaged skin and increased adrenergic stress appear to mediate the hypermetabolic effect, which can partially be attributed to thermogenesis to maintain core body temperature in absence of a large surface area of skin. Supporting this supposition, our data show that uncoupled respiration linked to thermogenesis in burned victims is not different from healthy control. Although the maximal respiratory capacity in skeletal muscle mitochondria is reduced, uncoupled respiration is not diminished but rather maintained indicating that the ability to produce heat is not diminished. However, we do show diminished coupling effect of ADP indicating a qualitative shift in skeletal muscle respiratory function. This shift in coupling control is similar to our previous study with severely burned adults during their acute care[47]. Although our data shows that coupled respiration to ADP is improved at 6 and 12 months vs 2 weeks, the dimished response to ADP lasts up to one year after injury, suggesting a phenotypic change in skeletal muscle mitochondria away from ATP production and towards thermogenesis.

Our data shows diminished maximal respiratory response in skeletal muscle mitochondria. However, it is not likely that ATP availability will be limited in vivo. For example, muscle mitochondria only functions at maximal capacity or near maximal capacity during intense exercise. When a patient is at rest, or immobilized, ATP availability will likely be unaffected by a ~50% decrease in maximal ATP producing capacity due to lowered demands from inactivity. Studies in skeletal muscles of burned mice showed no significant differences in ATP concentration compared to non-burned control[41]. However, the long-term diminished coupling control for producing ATP may have profound impact in functional capacity of burned survivors. Studies showed skeletal muscle mitochondrial respiratory capacity correlates with whole-body VO_{2max} and insulin sensitivity in vivo[180-182]. Thus, the restoration of muscle respiratory capacity will have beneficial effects on metabolic functions in burned victims. Also, exercise training showed increase in whole-body O_2 consumption rates in burned victims[183]. This effect is

likely mediated, at least partially, by increased mitochondrial capacity in locomotive muscles.

Approximately 90% of whole body O_2 consumption occurs in mitochondria with about 80% coupled to ATP production[174]. A large portion of hypermetabolism in burned victims appears to be the exaggerated disparity of O_2 consumption rates over ATP production, suggesting a central role for mitochondrial coupling control. Skeletal muscle accounts for approximately 40% of body mass in healthy humans, and is responsible for about 20% of whole-body O_2 consumption at rest. Therefore, altered skeletal muscle mitochondrial coupling control would have a profound impact on whole-body O_2 consumption. Here, and in our previous study with severely burned adults[184], we show a loss of mitochondrial coupling control with diminished response to ADP and oligomycin. The reduced sensitivity to stimulation (ADP) or inhibition (oligomycin) indicates that skeletal muscle mitochondria becomes more uncoupled, or thermogenic, after severe burn injury.

While this cross-sectional study does not definitively prove that skeletal muscle mitochondrial thermogenesis contributes to hypermetabolism in burned victims, the results show a clear association. To further support this, we found that hypermetabolism decreases overtime with concomitant improvement in skeletal muscle mitochondrial coupling control. Additionally, correlation analysis showed a significant relationship with maximal coupled respiration and REE, where greater degree of hypermetabolism is associated with decreased ATP producing capacity. Additionally, the reduced response to ADP and oligomycin was also associated with hypermetabolism, indicating an association with skeletal muscle mitochondria and energy expenditure.

A full explanation elucidating skeletal muscle mitochondrial uncoupling after severe burn injury is lacking. Severe burn results in a more “leaky” inner mitochondrial membrane allowing protons to re-enter the matrix independent of ATP synthase. However, this “leakiness” is mediated by other specific mitochondrial proteins

such as adenine nucleotide translocase and uncoupling proteins (UCPs). Several studies showed the role of UCP1 in brown fat to generate heat by dissipating the electrochemical potential of mitochondrial membranes[20,25,38]. Others found an increase in UCP2 (a homologue of UCP1) expression in skeletal muscle of burned victims[185]. However, the physiological role of UCP2 remains unclear and requires further research.

A limitation to this study was that we could not get an age matched cohort to serve as healthy controls, but rather utilized muscle biopsies from healthy adults. Although it was possible to use abdominal muscle biopsies from children going through elective surgery, we felt that it was important to compare the same type of muscle rather than comparing locomotive to postural muscles which were shown to differ in mitochondrial function[186]. Although skeletal muscle mitochondrial function declines with aging[187], it may be largely due to decreased physical activity and fitness since young and older adults with similar fitness level have similar skeletal muscle mitochondrial function[188]. Therefore, we believe it is reasonable to suggest that skeletal muscle mitochondrial function is not different between active young adults and healthy children. Also, we found similar results in our previous study with severely burned adults indicating that these results are not altered within this age range.

CHAPTER 4

The impact of combined treatment with oxandrolone and propranolol on protein and lipid metabolism in severely burned children

INTRODUCTION

The pathophysiological response following severe burn injury results in a chronic hypermetabolic and hypercatabolic stress response that may persist up to 36 months [6,29,30,173]. This stress response is characterized by of a surge of catecholamines, increased peripheral lipolysis, elevated resting energy expenditure (REE), wasting of skeletal muscle, and altered mitochondrial function [40,41,43,107].

Skeletal muscle mass in healthy humans is maintained by a dynamic balance between muscle protein synthesis and breakdown, which over time are equal resulting in no net change in muscle mass [65]. However, severe burn injury results in a significant increase in skeletal muscle breakdown that cannot be matched by increased muscle protein synthesis [30]. In a cross-leg study utilizing stable isotope tracers of amino acids, Hart et al. found that children severely burned over 40% of their total body surface area had elevated amino acid breakdown at six and nine months. They also found these burned patients had elevated REE and loss of lean mass measured by dual x-ray absorptiometry (DEXA). This was further supported by our recent study utilizing bolus injections of amino acid isotope tracers in severely burned children. We studied these burned patients in the postprandial state from their acute care until one-year post injury and found that whole-body skeletal muscle protein breakdown was elevated at all time-points. Protein synthesis was also elevated but at a lower magnitude equating to a negative net protein balance (Chapter 1, Figures 3). Our results indicate that therapeutic targets to counter

skeletal muscle wasting should be focused on blunting protein breakdown following burn injury.

Skeletal muscle is abundant with mitochondria where its main role is to produce ATP necessary for locomotion and physical functions. With this in mind, we can consider skeletal muscle mitochondria function as a proxy of skeletal muscle quality. Previous studies indicate that severe thermal trauma induces mitochondrial dysfunction [40,41,43]. Cree et al. found that maximal coupled oxidative capacity was diminished in children who suffered burns covering over 40% of their TBSA. Indeed, our previous study in severely burned adults showed decreased maximal mitochondrial respiration in their acute care when compared healthy young adults[47]. In that study, we also found that the abundance of skeletal muscle mitochondria was significantly less than non-burned adults[184]. Furthermore, when we compared maximal oxidative phosphorylation normalized to mitochondrial content, we found that burned individuals had higher maximal oxidative capacity, which is possibly a compensatory mechanism from decreased mitochondrial abundance. What we found particularly interesting was the 40% decrease in coupling respiration, which was linked to a 40% increase in hypermetabolism. We mentioned here previously that severely burned children also showed diminished maximal oxidative capacity[189]. After severe burn, we found that altered skeletal muscle mitochondrial functions persist up to one year. Similar to burned adults, skeletal muscle mitochondria in severely burned children showed a switch in phenotype away from phosphorylation and more towards thermogenesis[189]. This indicated that the perturbation in mitochondrial function is not age dependent, but rather a hallmark of severe burns.

Lipolysis, the breakdown of peripheral fat, is accelerated after severe burns exceeding the rate of oxidation[190]. Because of this disparity, circulation of free fatty acid is increased resulting in ectopic fat deposition in tissues such as liver and muscle[107,191,192]. Additionally, when they're not oxidized, these free fatty acids get re-esterified back into

triglycerides[193]. This re-esterification of fatty acid has been called susbtrate recycling, which contributes to the burn-induced hypermetabolism[13]. Wolfe et al. found that this rate if triglyceride-fatty acid cycle increased by 450% in 18 burned children and adults[13]. In his review, Yu et al. found that the total fatty acid cycle contributes approximately 17% of the increase in energy expenditure after burn injury[12].

Propranolol is a non-selective β-adrenergic receptor blocker that has been extensively studied to mitigate the hypermetabolic stress response to severe burn injury. Its mechanism of action is to block the effect of the burn-induced surge in catecholamine. By blocking β_2-adrenergic receptors, propranolol reduces lipolysis seen in severely burned individuals, which was not found when the β1-adrenergic receptor blocker, metoprolol was used[109]. By reducing lipolysis, propranolol inhibits ectopic fat accretion in tissues and organs, such as the liver [107,108]. A large randomized controlled trial with severely burned pediatric patients found an 82% increase in skeletal muscle protein net balance with propranolol treatment versus a decrease of 27% in patients who did not receive propranolol [111]. The anti-catabolic effect of propranolol is thought to be mediated through the reduction of energy expenditure [104] by decreasing cardiac output, heart rate, and rate-pressure product [106].

Oxandrolone is synthetic testosterone that has been used clinically to treat muscle wasting in a number of diseases including severe burn[119,194,195]. Oxandrolone is viewed as a promising candidate to improve protein synthesis during hospitalization because it is less expensive than other anabolic agents such as rhGH, easily administered orally, with substantially less virilization potential than testosterone analogues[121]. By stimulating androgen receptors in skeletal muscles, oxandrolone stimulates protein synthesis and anabolism. Previous studies in severely burned children using stable isotope infusion show increased protein synthesis and greater amino acid utilization in skeletal muscle when treated with oxandrolone[119,120]. Other benefits of oxandrolone for burned patients

include improved body composition, maintained lean body mass, increased bone mineral density and content, shortened the hospital length of stay, and reduced mortality[123-126].

Individual treatment with either oxandrolone or propranolol confers metabolic benefits for severely burned patients. While oxandrolone works by stimulating protein synthesis and anabolism, the benefits of propranolol are mediated through the reduction of the hypermetabolic response through β-blockade. Recently, our colleagues studied 612 burned pediatric patients and found that one year of oxandrolone and propranolol administration increased growth rate over children who were given oxandrolone only, propranolol only, or standard of care only[196]. However, the effect of oxandrolone and propranolol on skeletal muscle metabolism, lipid metabolism, and mitochondrial function is still unknown. The purpose of this study is to investigate the combined use of oxandrolone and propranolol (Oxprop) on skeletal muscle protein turnover, lipolysis, and mitochondrial function. We hypothesize that burned children treated with Oxprop will demonstrate improved muscle and lipid metabolism.

METHODS AND MATERIALS

Patients

Severely burned children admitted to the Shriners Hospital for Children in Galveston, TX were recruited for this study. Children ages 2 - 17 with burns wounds covering at least 30% TBSA without any existing illness prior to their burn injury were considered. This study was conducted at the Shriners Hospitals for Children and approved by the Institutional Review Board at the University of Texas Medical Branch. Informed consent was obtained from each patient's parent or guardian prior to enrollment in the study.

Standard of care

As described in Chapter 2, each patient underwent the standard of care procedure within 48 hours of admission[121]. Total burn wound excision and grafting with autograft skin and allograft. After the autograft donor sites healed (usually 6 – 8 days from the previous operation), patients would return to the operating room for re-harvest. Sequential staged surgical procedures for repeat excision and grafting were undertaken until the wounds were healed. Patients were intubated for operations, and extubation was immediately conducted afterwards. Ventilator settings for those who remained intubated followed ARDS-NET recommendations[154]. Any occurrence of sepsis was recorded. Patients were at bed rest after excision and grafting procedures for five days. Afterwards, patients ambulated daily until the next excision and grafting procedure. Patients were treated identically regarding rehabilitation and mobilization.

As described in Chapter 2, each patient received enteral nutrition via a naso-duodenal tube with Vivonex TEN (Nestle Health Science, Minneapolis, MN). Vivonex is composed of 82% carbohydrate, 15% protein, and 3% fat. Daily caloric intake was

administered at a rate calculated to deliver 1,500 kcal/m^2 TBSA burned + 1,500 kcal/m^2 TBSA. This feeding regimen started at admission and continued at a constant rate until the wounds were healed. While in hospitalization, total caloric intake remained constant. Insulin was administered intravenously to keep serum glucose below 200 mg/dL, in accordance with standard accepted clinical practice.

Study Design

After informed ascent was received, subjects were randomized into the drug (standard of care with Oxprop) or control (standard of care only) group. Metabolic studies were conducted approximately three or four days after surgical procedures. Subjects were fasted overnight prior to the morning of the metabolic study. Study 1 was conducted approximately two weeks after burn injury and study 2 was performed approximately four weeks after injury.

Drug administration

After subjects were randomized to the drug (Oxprop) or control (placebo) groups, drug administration began no later than 96 hours post-admission. Oxandrolone was administered at 0.1 mg/kg (BTG Pharmaceuticals, Iselin, NJ) administered in a liquid form or capsule twice a day during the patients entire hospitalization. Propranolol was administered at a dose of 0.33 mg/kg of body weight every four hours (1.98 mg/kg/day). This dose was titrated up to a maximum dose of 4 mg/kg/day to achieve a 20% decrease in heart rate for each patient. When mean blood pressure fell below 65 mmHg, propranolol was withheld or decreased in dosage. Then the dose was increased incrementally to meet the study goal of a 20% decrease in heart rate from established

base-line levels as tolerated. Propranolol was given according to schedule during surgical procedures.

Experimental procedure

ISOTOPES

L-[ring-$^{13}C_6$] phenylalanine and L-[^{15}N] phenylalanine were utilized to determine protein kinetics. [1,1,2,3,3-$^{2}H_5$] glycerol was infused to assess whole body lipolysis. [U-$^{13}C_{16}$] palmitate to assess fatty acid kinetics. All stable isotope tracers were purchased from Cambridge Isotope Laboratories (Woburn, MA).

PROTOCOL

Protocol of the metabolic study is shown in *Figure 10*. Background blood samples were collected prior to the administration of stable isotope tracers. A bolus injection of primed (18 μmol/kg) was followed immediately by a 2-hr constant infusion (0.12 μmol/kg/min) of [$^{2}H_5$] glycerol and [U-$^{13}C_{16}$] palmitate (0.02 μmol/kg/min). L-[ring-$^{13}C_6$]Phe (5.56 mg/kg) and L-[^{15}N]Phe (5.40 mg/kg) in 3ml of 0.45% saline were injected at time 0 and 30 minutes, respectively. Frequent blood samples (~1 ml each) were taken at minutes 5, 10, 15, 20, 29, 35, 40, 50, and 60 to measure the enrichment decay in blood for each phenylalanine tracer (as described in Chapter 2). Muscle samples were obtained from the *m. vastus lateralis* at minutes 10 and 60 (~70 mg) to measure enrichment decay in the muscle intracellular free pool. After collection, the muscle samples were washed in ice-cold saline to remove visible blood. Samples were immediately frozen in liquid nitrogen and stored at -80°C for later processing. Additional blood samples were taken at 100, 110, and 120 minutes to determine plasma glycerol and palmitate enrichment. Blood samples were immediately spun in a centrifuge at 3,000 rpm for 20 minutes. Plasma (~0.8ml) was aliquoted into cryotubes for later processing. A third muscle biopsy was

taken at the end of the study and was immediately placed in an ice-cold (pH 7.1) preservation buffer (containing 10mM Ca-EGTA, 0.1 μM free Ca^{2+}, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM creatine phosphate) to measure mitochondrial respiration within 24 hours.

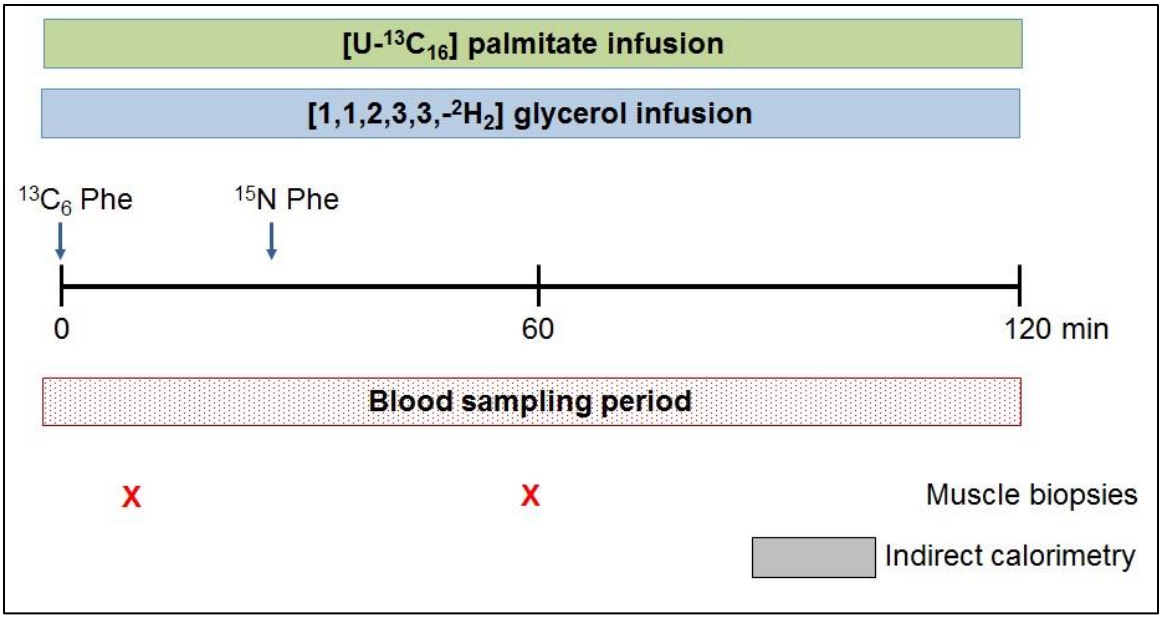


Figure 10. Timeline of stable isotope tracer protocol.

Timeline of the metabolic study. A primed (18 μmol/kg) $^2H_5$ glycerol (0.12 μmol/kg/min) was infused to measure whole-body lipolysis. U-$^{13}C_{16}$ palmitate (0.02 μmol/kg/min) was infused to assess FFA kinetics. Bolus injections of $^{13}C_6$ phe and ^{15}N phe were used to determine protein turnover. Blood samples were taken at designated times through the study. Skeletal muscle biopsies were taken from the *m. vastus lateralis* at 10 and 60 minutes into the study. REE was measured by indirect calorimetry 90 minutes into the study (Sensor Medics Vmax 29, Yorba Linda, CA). All stable isotope tracers were provided by Cambridge Isotope Laboratories (Woburn, MA). FFA, free fatty acid; Phe, phenylalanine; REE, resting energy expenditure.

SAMPLE ANALYSIS

To measure plasma enrichment, the 500 μL of plasma were pipette into a glass tube with 15% sulfosalicylic acid. These samples were centrifuged at 3,000 rpm for ten mins. Supernatants were loaded into amino acid column prepared for GCMS analysis. After draining, samples were placed into a speedvac overnight and allowed to dry. These samples were processed with t-butyldimethyl-silyl (TBDMS) derivatives of amino acids.[197] To measure free phenylalanine enrichment in muscle, a tissue internal standard solution containing 6 μmol/l of L-[ring-$^{2}H_5$] phe was added to ~30 mg of muscle (1 μl/mg of muscle). The muscle sample would be homogenized in 10% perchloric acid. The pooled supernatant was processed for the TMDMS derivatives.[197] The protein precipitates were thoroughly washed to remove free amino acids and fat and were dried overnight in an oven at 80°C to obtain dry protein pellets.[198] The dry protein pellets were hydrolyzed and then processed for the N-acetyl, n-propyl ester (NAP) derivatives of amino acids.

Measurement of plasma FFA and glycerol enrichment were described in detail previously[199]. Briefly, 250 μL of plasma were pipette into 13 x 100 mm screw top tubes with equal amount of heptadecanoic acid (C17:0) internal standard (0.23 μmol/mL in heptane) and water were added. After samples were shaken on a vortex, 3 mL of ice-cold acetone were added to precipitate plasma proteins, shaken again, then placed in -20C for 15 minutes. Samples were then centrifuged at 2500 rpm at 4C for 20 minutes. Supernatant was poured into 16 x 125 mm screw-top tubes. Then, 3 mL of hexane and 3 mL of ddH_2O were added before being taken to a horizontal shaker for 15 minutes. Samples were centrifuged once more (2500 rpm at 4C for 15 minute) to separate the solvent and aqueous phase. The upper (hexane) phase was transferred to a corresponding 13 x 100 mm screw top tube to measure palmitate, while the lower aqueous solvent was used to process glycerol. All samples were dried overnight in a Speed-Vac (40 – 55C).

Once dried, 250 μL TBA-phosphate buffer (0.2 M dibasic potassium phosphate and 0.05 M tetrabuylammonium hydrogen sulfate, pH adusted to 9.0 with tribasic potassium phosphate) and 250 μL iodemethane-dichloromethane (1:10 vol) were added and shaken for 15 minutes. Samples were then sonicated for 15 minutes. Afterwards, 3 mL hexane and shaken for 15 minutes. Samples were centrifuged as previous for 15 minutes. The upper later was transferred to another screw top tube and set to dry for 45 minutes in a speedvac. Once dry, 150 mL heptane were added to the samples vortexed, then transferred to vials for gas chromatography mass spectrometry (GCMS).

Isotopic enrichments in blood and muscle supernatants were measured on an Agilent 6890 GCMS. Ions were selectively monitored at mass-to-charge (m/z) ratios of 336, 337, 342, and 346 for Phe enrichment, 253,254, and 257 for glycerol enrichment, and 270, 286 for palmitate enrichment . Isotopic enrichments were expressed as a tracer-to-tracee ratio for all samples.

Calculations

Similar to Chapter 2, muscle fractional synthesis rate (FSR) was calculated by Eq. 1 and fractional breakdown rate (FBR) was calculated by Eq 2,[161] where the free to bound ratio of phenylalanine is represented by Q_M/T. The amount of free phenylalanine in the obtained muscle sample was measured and normalized to micromoles of free phenylalanine per gram of muscle. It was previously shown that one gram of dry muscle protein contains 150 μmol of phenylalanine.[200] The content of protein-bound phe in one gram of muscle was calculated by [(250 μmol/g) x (%dry protein in muscle)].

WHOLE BODY LIPOLYSIS AND FATTY ACID KINETICS

An isotopic steady state was achieved in the last 15 minutes of the infusion protocol. The glycerol rate of appearance (Ra gly) was calculated by dividing the glycerol infusion rate by the tracer-to-tracee ratio[24]. Plasma FFA rate of appearance (Ra FFA) was calculated by dividing Ra by the fractional contribution of palmitate to the total FFA concentration, determined by gas chromatography mass spectrometry. Intracellular recycling was calculated as: 3*Ra gly – Ra FFA.

HIGH-RESOLUTION RESPIROMETRY

Mitochondrial substrates and inhibitors were added sequentially to the oxygraph O2K chambers (Oroboros Instruments, Innsbruck, Austria) to determine each respiratory state. All experiments were performed in a rigourously stirred buffer (750 rpm) at 37°C. The respiration buffer used has the following composition (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 10 mM sucrose, and 1 mg/ml bovine serum albumin). O_2 concentration was determined at 2 sec intervals on O_2 flux per mg of tissue computes (Datlab, Oroboros Instruments, Innsbruck, Austria). Initially, the leak respiratory state was recorded with the myofiber bundles alone. Afterward, octanoyl-L-carnitine (1.5 mM), pyruvate (5mM), malate (2mM) and glutamate (10 mM) were added to the oxygraph chamber to induce uncoupled state 2 respiration supported by complex I of the electron transport chain. Afterward, saturating levels of ADP (5mM) were added to the oxygraph chamber to reach state 3 maximal coupled respiration by complex I. Then 10 mM succinate was added to the oxygraph chamber to provide electrons via complex II, thereby activating the maximal couple state 3 respiration (maximal oxidative phosphorylation capacity [OXPHOS]). Finally, 5 μM oligomycin, an inhibitor of the F_O unit of the ATP synthase, was added to the oxygraph chamber to inhibit ATP synthase and induce maximal uncoupled respiration (state 4_O).

RESTING METABOLIC RATE

Resting energy expenditure (REE) of burned patients was determined by indirect calorimetry (Sensor Medics Vmax 29, Yorba Linda, CA). REE was calculated from whole body oxygen consumption and carbon dioxide production rates using the Weir equation [175], which is REE = (3.9 x VO_2) + (1.1 x VCO_2). This measured value was compared to the predicted REE which was calculated using the Harris-Benedict equation [176] (male: REE = 66.5 + (13.75 × weight [kg]) + (5.003 × height [cm]) – (6.755 × age [years]; female: REE = 655.1 + (9.563 × weight [kg]) + (1.850 × height [cm]) – (4.676 × age [years]). This is a standard method for estimating the degree of hypermetabolism in burned patients.

BODY COMPOSITION

Lean body mass was measured by dual x-ray absorptiometry (DEXA) with QDR 4500 software (Hologics, Waltham, MA). The estimated precision of DEXA for human body composition is 2 – 3%[201]. Whole-body scans were performed according to the manufacturer's instructions.

Statistical analysis

All data are presented as group means ± SE. Differences between group means were analyzed by unpaired t-tests and differences within group means were analyzed by paired t-tests. Statistical significance was reached when $p < 0.05$. Statistical analyses were performed using Graphpad Prism version 7 (GraphPad Software, La Jolla, CA).

RESULTS

We were able to study 50 severely burned pediatric patients. There were 28 subjects in the control group (22 male, 6 female) and 22 in the oxprop group (15 male, 7 female). Subjects in both groups had similar age, severity of burn, days from burn injury to admission, and in-hospital length of stay. Demographics for the subjects are shown in Table 4.

Table 4. Subject characteristics

	Control	**Oxprop**
n	28	22
Male / Female	22 / 6	15 / 7
Age (y)	8 ± 5	8 ± 5
Burn size (%TBSA)	50 ± 16	46 ± 10
3rd degree (%)	36 ± 19	29 ± 15
Days to admit	3 ± 3	3 ± 3
Length of stay (days)	35 ± 5	31 ± 17

Values are mean ± SD.

Body composition

DEXA measurements were taken near the time of the second study. We compared the lean mass normalized to their height (kg/m^2) and found that the oxprop had significantly more mass than the control group (12.56 ± 0.26 vs 13.74 ± 0.53 kg/m^2, $p = 0.04$). Full DEXA measurements are listed in Table 5.

Table 5. DEXA analysis

	Control (n=28)	**Oxprop (n=20)**
Total body mass (kg)	32.83 ± 2.55	38.80 ± 4.00
Bone mineral content (kg)	1.06 ± 0.10	1.19 ± 0.13
Lean mass (kg)	22.29 ± 1.90	26.54 ± 2.69
Fat mass (kg)	9.48 ± 0.78	11.07 ± 1.33
% Lean mass	67.2 ± 1.1	68.6 ± 1.1
% Fat mass	29.6 ± 1.2	28.3 ± 1.1
BMI	17.89 ± 0.41	19.46 ± 0.94
LMI	11.96 ± 0.23	13.30 ± 0.60*

Values are mean ± SE. *p < 0.05. BMI, body mass index; LMI, lean mass index.

Resting energy expenditure

REE was significantly lower in the oxprop group than the control group at the first study (145 ± 4.2% vs 110 ± 4.6%, p < 0.001) and the second study (143 ± 5.9% vs 115 ± 4.6%, p < 0.001) (*Fig. 11*). No differences were seen from the first to the second study within the control group (p = 0.49) or the oxprop group (p = 0.64).

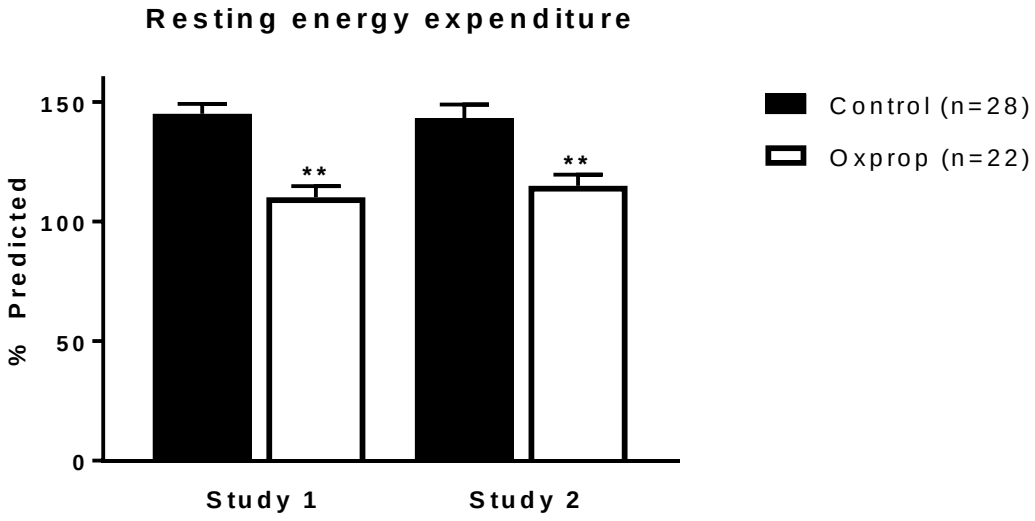


Figure 11. Resting energy expenditure in control and oxprop.

Resting energy expenditure was measured by indirect calorimetry and compared to the predicted energy expenditure by the Harris-Benedict equation. Units are in % of the predicted resting energy expenditure. **$p < 0.001$ vs. control.

Whole-body lipolysis

Whole-body lipolysis was significantly lower in the oxprop than control in the first study (10.91 ± 1.29 vs 7.49 ± 0.56 μmol/kg/min, $p < 0.05$). However, we found that lipolysis was similar between control and oxprop in the second study (10.64 ± 1.01 vs 9.8 ± 1.10 μmol/kg/min, $p = 0.83$). Lipolysis did not change in the control group between the first and second study (10.91 ± 1.29 vs 10.64 ± 1.01 μmol/kg/min, $p = 0.46$). We did find a significant increase in the rate of lipolysis in the oxprop group between the first and second study (7.49 ± 0.56 vs 9.8 ± 1.10 μmol/kg/min, $p < 0.05$) shown in *Figure 12*.

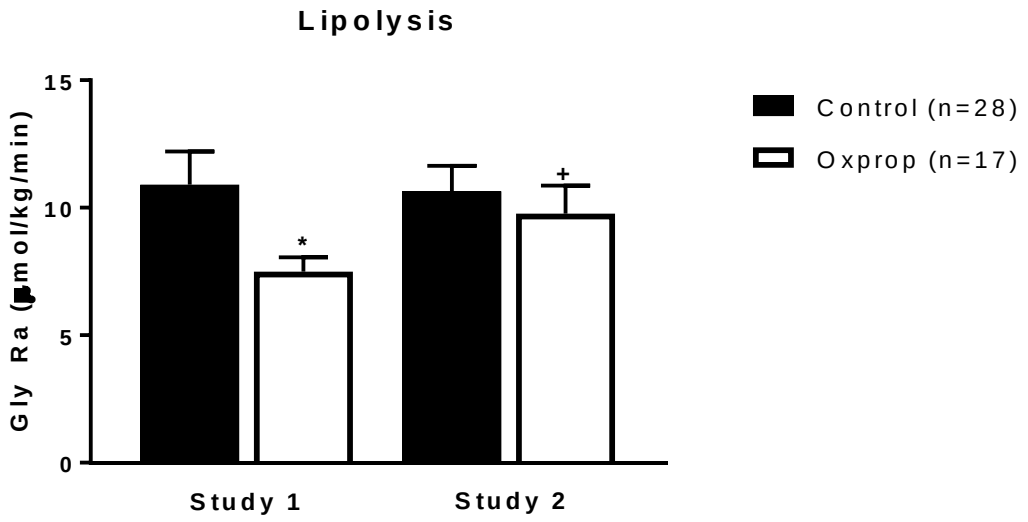


Figure 12. Rate of lipolysis in control and oxprop.

Rate of whole-body lipolysis was calculated using $^{2}H_5$ glycerol (0.12 μmol/kg/min) infusion in control and oxprop treated patients. Whole-body lipolysis was determined by the glycerol rate of appearance (Ra) measured in μmol/kg/min. *p < 0.05 vs control. +p < 0.05 vs study 1.

No differences were found in circulating free fatty acid between control and Oxprop at study 1 (17.72 ± 1.33 vs 18.72 ± 1.85 μmol/kg/min, p = 0.66) or at study 2 (21.06 ± 2.29 vs 17.69 ± 1.9 μmol/kg/min, p = 0.27) (*Fig 13*). No differences were found from study 1 to study 2 in control (p = 0.14) or Oxprop (p = 0.69).

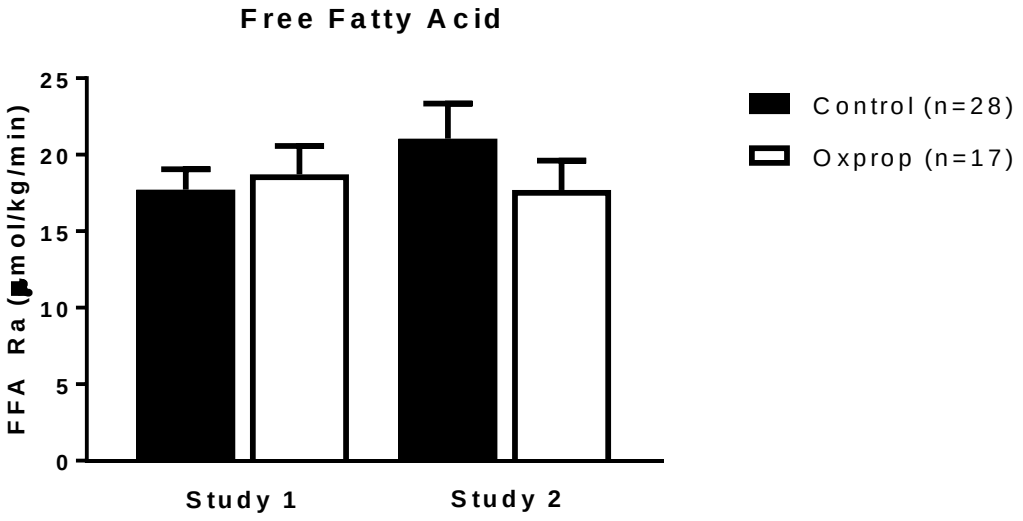


Figure 13. Free fatty acid circulation in control and oxprop.

The amount of circulating free fatty acid was determined by U-$^{13}C_{16}$ palmitate (0.02 μmol/kg/min) infusion in control and Oxprop treatment. The rate of lipolysis was determined by the palmitate rate of appearance (Ra) measured in μmol/kg/min.

We determined triglyceride-fatty acid intracellular recycling under notion that three fatty acids are released for every one glycerol back bone. Fewer fatty acid release from adipocyte would indicate that the fatty acid was re-esterified back into triglyceride[202]. We found that in study 1, FA recycling was significantly less in Oxprop than control (15.48 ± 4.39 vs 3.44 ± 1.68 μmol FA/kg/min, $p = 0.01$), but not in study 2 (9.54 ± 3.14 vs 11.06 ± 2.12 μmol FA/kg/min, $p = 0.69$), shown in *Figure 14*. No significant change was found from study 1 to study 2 in control ($p = 0.2$). However, we found a significant increase in FA recycling from study 1 to study 2 in Oxprop ($p < 0.01$).

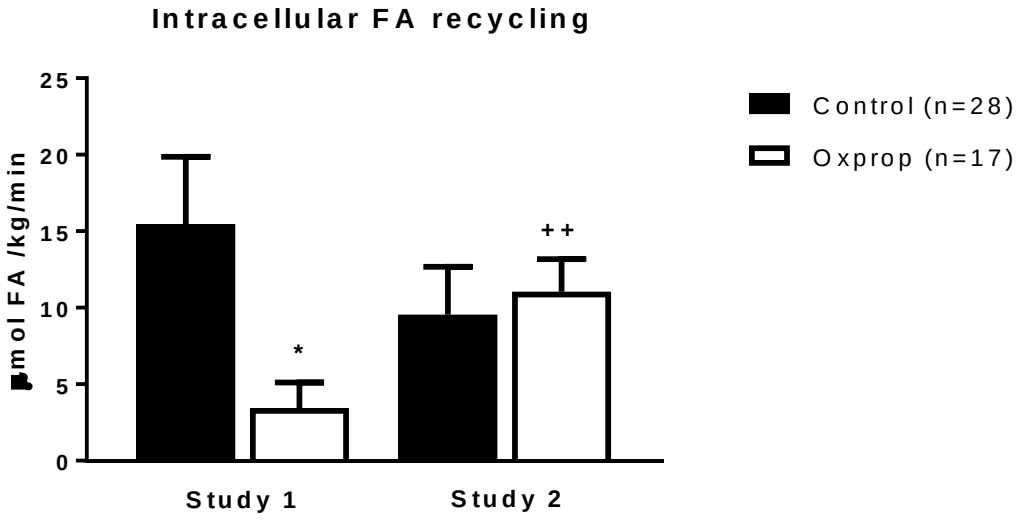


Figure 14. Rate of lipolysis in control and oxprop.

Recycling of fatty acid was determined in control and Oxprop treated patients. Recycling was determined by using both glycerol RA and FFA Ra. Calculation of intracellular recycling was determined by: intracellular recycling = 3*Ra Gly – Ra FFA. Intracellular recycling was measured in μmol of FA/kg/min. *$p < 0.05$ vs control. $^{++}p < 0.01$ vs study 1. FA, fatty acid; FFA, free fatty acid.

Muscle protein turnover

FSR was not different in the first study between control and oxprop (0.10 ± 0.02 vs 0.07 ± 0.01 %/h, $p = 0.26$) or the second study (0.15 ± 0.02 vs 0.12 ± 0.02 %/h, $p = 0.45$). No difference was found within the control from the first to the second study (0.10 ± 0.02 vs 0.15 ± 0.02 %/h, $p = 0.15$). However, we found a significant increase in FSR in the oxprop group from the first to the second study (0.07 ± 0.01 vs 0.12 ± 0.02 %/h, $p < 0.01$). There were no differences in the change in FSR between control and oxprop ($p = 0.89$) (*Fig. 15A*). FBR was not different in the first study between control and oxprop (0.27 ± 0.04 vs 0.28 ± 0.04 %/h, $p = 0.78$). In the second study, FBR was significantly

lower in the oxprop group compared to control (0.35 ± 0.04 vs 0.21 ± 0.03 %/h, $p < 0.01$) (*Fig. 15C*). No difference was found in the control group between the first and second study (0.27 ± 0.04 vs (0.35 ± 0.04, $p = 0.25$). There was a significant reduction in FBR in the oxprop group from the first to the second study (0.28 ± 0.04 vs 0.21 ± 0.03 %/h, $p = 0.02$). Net protein balance was derived by subtracting FBR from FSR. Net balance was not different between control and oxprop in the first study (-0.16 ± 0.03 vs -0.21 ± 0.04 %/h, $p = 0.39$). In the second study, net balance was significantly lower in the oxprop group when compared to control (-0.19 ± 0.04 vs -0.09 ± 0.03 %/h, $p = 0.04$) (*Fig. 15E*). No difference was found between the first and second study in the control group (-0.16 ± 0.03 vs -0.19 ± 0.04 %/h, $p = 0.97$). We found that net protein balance significantly improved in the oxprop group from the first to the second study (-0.21 ± 0.04 %/h vs -0.09 ± 0.03 %/h, $p < 0.01$). We calculated the change in FSR, FBR, and net balance by subtracting study 1 from study 2. Shown in *Figure 15B*, FSR showed similar trends in both control and oxprop and they were not significantly different (0.05 ± 0.03 vs 0.05 ± 0.02 %/h, $p = 0.89$). FBR showed an increasing trend in the control group in study 1 to study 2 (0.06 ± 0.05 %/h), while FBR decreased in the oxprop group from study 1 to study 2 (-0.08 ± 0.03 %/h) which was significantly different from control ($p = 0.02$) (*Fig 15D*). Because there was a greater increase in FBR over FSR in the control group, it resulted in a slightly greater negative protein net balance from study 1 to study 2 (-0.001 ± 0.04 %/h). The oxprop group showed significant improvement in net balance from study 1 to study 2 (0.13 ± 0.04, $p = 0.03$ vs control) due to the positive increase in FSR compounded by a reduction in FBR (*Fig. 15F*).

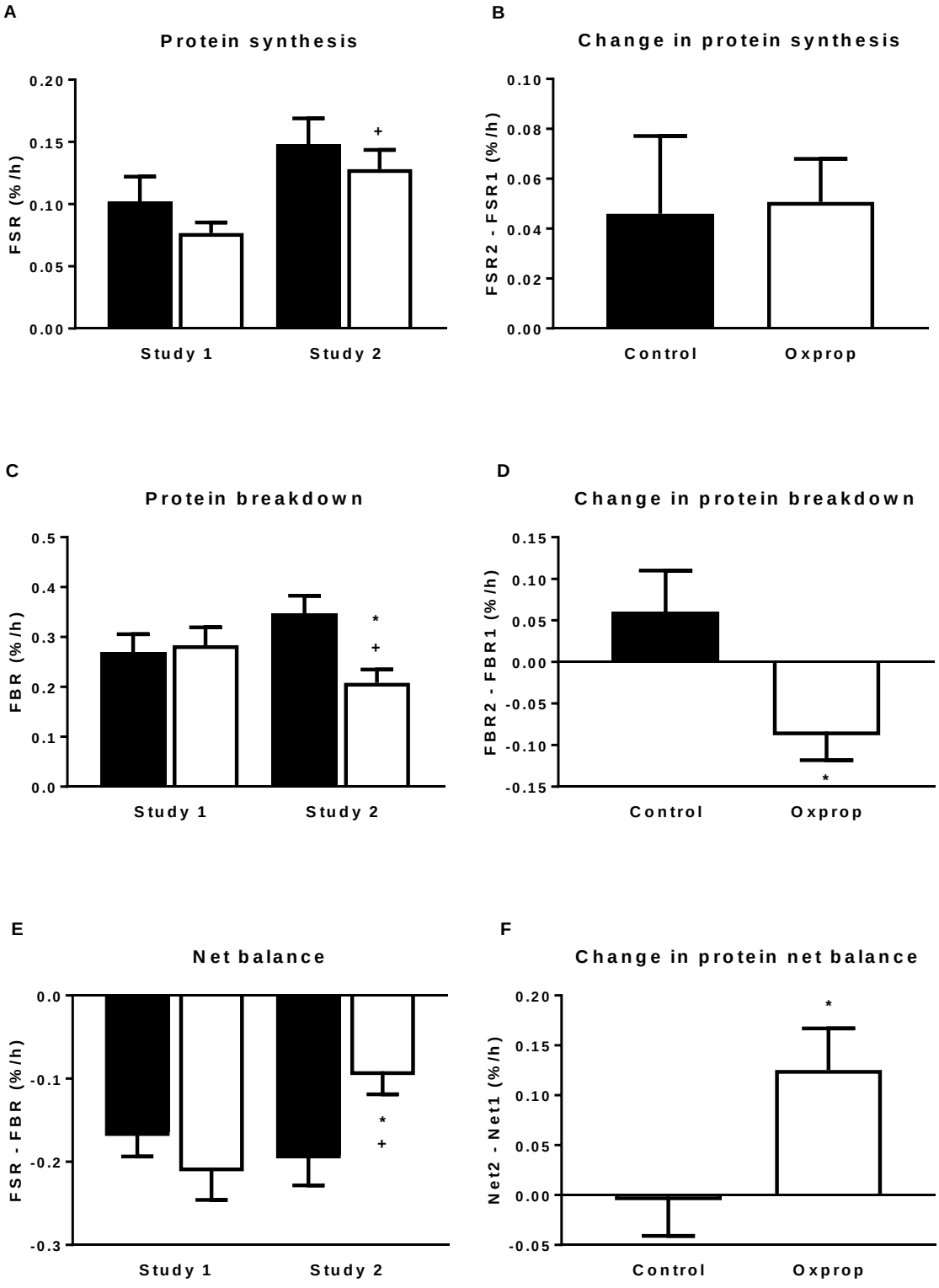

A
Protein synthesis
FSR (%/h)
0.20
0.15
0.10
0.05
0.00
+
Study 1
Study 2
B
Change in protein synthesis
FSR2 - FSR1 (%/h)
0.10
0.08
0.06
0.04
0.02
0.00
Control
Oxprop
C
Protein breakdown
FBR (%/h)
0.5
0.4
0.3
0.2
0.1
0.0
*
+
Study 1
Study 2
D
Change in protein breakdown
FBR2 - FBR1 (%/h)
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
*
Control
Oxprop
E
Net balance
FSR - FBR (%/h)
0.0
-0.1
-0.2
-0.3
*
+
Study 1
Study 2
F
Change in protein net balance
Net2 - Net1 (%/h)
0.20
0.15
0.10
0.05
0.00
-0.05
*
Control
Oxprop

Figure 15. Protein turnover in control and oxprop.

Protein turnover was measured in controls (n=28, black) and oxprop (n=22, white) Protein synthesis (A), breakdown (C), and net balance (E) were measured utilizing stable isotope tracers of phenylalanine. The change in protein synthesis (B), breakdown (D), and net balance (F) were measured in control and oxprop from study 1 to study 2. FSR, fractional synthesis rate; FBR, fractional breakdown rate. +$p < 0.05$ vs study 1, *$p < 0.05$ vs control.

Skeletal muscle mitochondrial respiration

High-resolution respirometry data are presented in *Figure 16***.** State 2 leak respiration was not different between control and oxprop in study 1 (17.03 ± 1.0 vs 18.55 ± 3.04 pmol/mg/s, p = 0.64) and in study 2 (13.55 ± 0.9 vs 15.33 ± 1.5 pmol/mg/s, p = 0.33). Coupled state 3 respiration was not different between control and oxprop groups in both study 1 (35.83 ± 2.4 vs 35.77 ± 4.9 pmol/mg/s, p = 0.99) and study 2 (31.13 ± 2.6 vs 29.43 ± 3.2 pmol/mg/s, p = 0.69). Maximal coupled respiration (OXPHOS) was not different in control vs oxprop group in study 1 (49.09 ± 2.69 vs 48.25 ± 5.27 pmol/mg/s, p = 0.89) and in study 2 (41.34 ± 3.36 vs 39.76 ± 4.08 pmol/mg/s, p = 0.77). State 4_O oligomycin-insensitive leak respiration was not significantly different in control compared with the oxprop group in study 1 (18.03 ± 1.23 vs 17.48 ± 1.608, p = 0.79) and study 2 (14.91 ± 0.78 vs 13.51 ± 1.4 pmol/mg/s, p = 0.39).

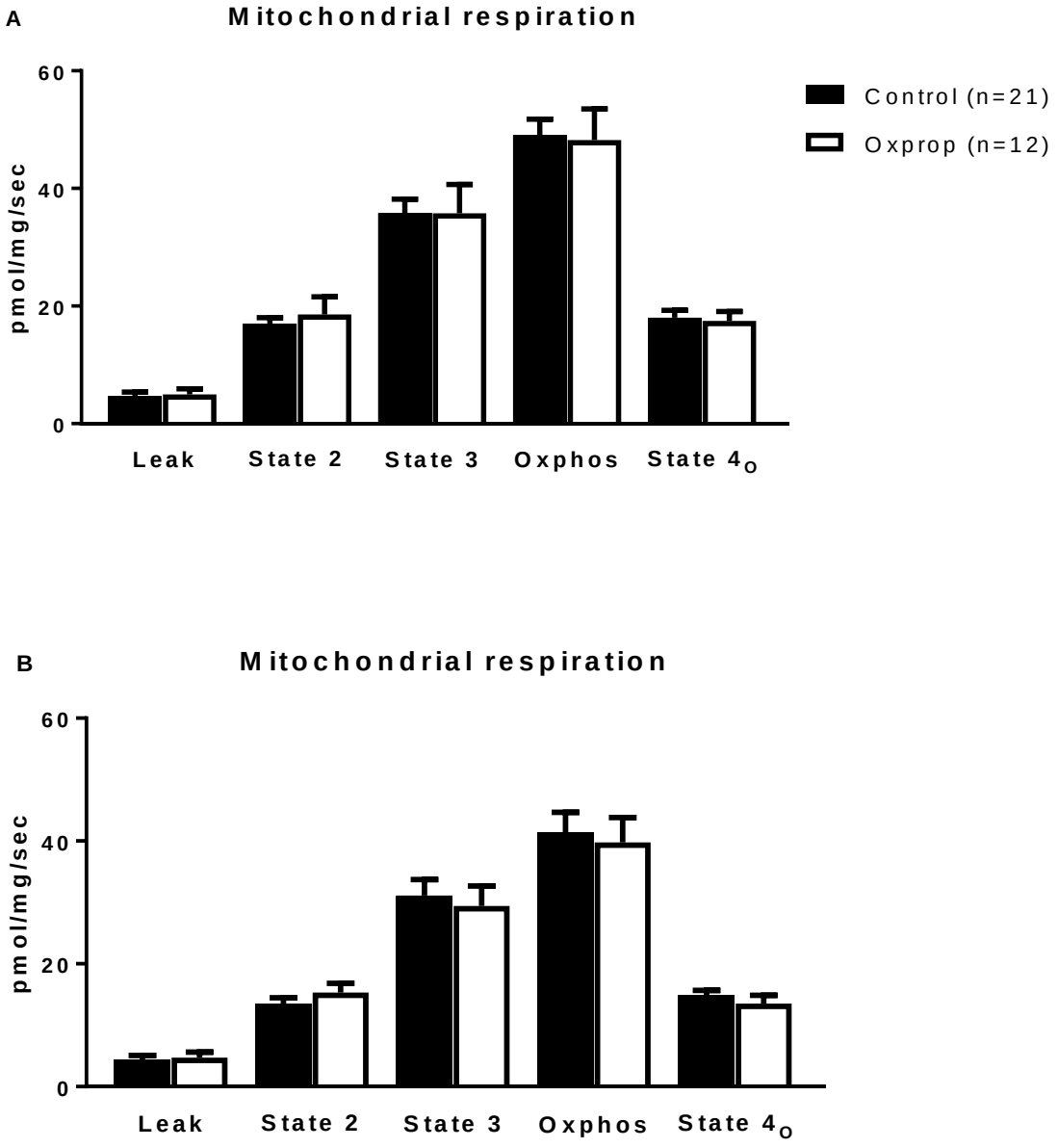


Figure 16. Mitochondrial respiration in control and oxprop.

Mitochondrial respiration in permeabilized myofiber from control and oxprop in the study 1(A) and study 2(B). Units are in pmol/mg/sec. Leak (sample and respiration buffer), state 2 (+ octanoyl-carnitine [1.5mM], pyruvate [5mM], malate [2mM], and glutamate [10mM]), state 3 (+ ADP [5mM]), oxphos (+ succinate [10mM]), and state 4_O (+ oligomycin [5μM]) respiration were determined sequentially in the sample tissue preparation.

Skeletal muscle mitochondrial respiratory control

The respiratory control ratio for ADP was not different in skeletal muscle mitochondria from control vs oxprop in study 1 (2.23 ± 0.21 vs 2.01 ± 0.18, p = 0.45) and study 2 (2.23 ± 0.21 vs 1.94 ± 0.16, p = 0.10). The substrate control ratio for succinate was not different between control and oxprop in study 1 (1.40 ± 0.04 vs 1.42 ± 0.08, p = 0.86) and study 2 (1.37 ± 0.05 vs 1.42 ± 0.09, p = 0.49). The flux control ratio for the ATP synthase inhibitor oligomycin was not different between control and oxprop in both study 1 (0.39 ± 0.04 vs 0.40 ± 0.05, p = 0.83) and study 2 (0.41 ± 0.04 vs 0.38 ± 0.04, p = 0.56). Flux control ratio data for oligomycin were used to calculate the proportion of maximal OXPHOS respiration that was linked to phosphorylation (oligomycin-sensitive) and thermogenesis (oligomycin-insensitive).

DISCUSSION

Severe burn injury results in a prolonged hypermetabolic and hypercatabolic stress response[6,173]. Skeletal muscle wasting, severe lipolysis, and increased energy expenditure are all hallmarks of this stress response[29,30]. We recently showed that severe burn also induces skeletal muscle mitochondrial dysfunction[40,47]. Treatment with propranolol or oxandrolone has individually shown beneficial effects on severe burn trauma[104,111,119,121]. Through their different modes of action, we hypothesized that the combined treatment of oxandrolone and propranolol would ameliorate the hypermetabolic and hypercatabolic effect of severe burn trauma. To test this hypothesis, we conducted metabolic studies on severely burned pediatric patients during their hospitalization. Indeed, REE was consistently significantly lower in the oxprop than the control group which can be attributed to the β-adrenergic blockade of propranolol which has been supported by several studies and reviews[104,114]. Our data shows that combining oxandrolone with propranolol still significantly reduces REE.

The rate of lipolysis was significantly reduced in the oxprop vs control group in the first study. However, lipolysis increased in the oxprop group from the first to the second study abolishing any difference between the two groups. Previous studies showing a reduction in lipolysis with propranolol was limited to just one week of treatment[107,109,191]. In a study utilizing stable isotopes and epinephrine infusion in severely burned children, Wolfe et al. suggested the possibility of burned patients becoming desensitized to the lipolytic effect of catecholamins[107] because prolonged epinephrine up-regulation causes a negative feedback loop to down-regulate its receptors[203,204]. Thus, it presents a possibility that lipolysis may have a tachyphylaxis effect to propranolol. Further investigations with the long term use of propranolol on lipolysis are warranted. However, our data are in agreement with previous results

showing that antagonism of β2 receptors in adipose tissue blunts peripheral lipolysis in patients with severe burns.

The rate of fatty acid recycling was significantly reduced in Oxprop vs control in the first study. Similar to lipolysis, recycling was significantly increased to at the second study. Intracellular fatty acid cycling is ~ 17-fold elevated in burned patients compared to healthy people[202]. The diminished recycling we found in Oxprop was expected due to the reduced rate of lipolysis by β-blockade. Intracellular fatty acid cycling is ATP dependent and therefore contributes to energy expenditure[13], therefore contributing to hypermetabolism. It was suggested that this reaction contributes approximately 17% to the hypermetabolic effect[12]. The reduction in fatty acid recycling rate by Oxprop treatment in the current study may explain part of the reduction in hypermetabolism. However, we did not find an increase in hypermetabolism when both lipolysis and fatty acid recycling was increased at the second study which was approximately four weeks post-injury. It is possible that other physiological functions continue to with drugs improve over, such as decreased protein turnover, that hypermetabolism (REE) continue to improve despite the resurgence of lipolysis and fatty acid recycling. The literature on the link between fatty acid recycling and post-burn hypermetabolism is limited. Future research to help us understand this tachyphylaxis phenomenom with lipolysis and fatty acid recycling may bring exciting new insight to potential drug therapies.

Although a modest, but not significant, increase was seen in FSR of our control group, we found a significant increase in FSR of our oxprop group. This is consistent with previous studies with treatment of either oxandrolone or propranolol alone which showed a significant increase in protein synthesis efficiency[111,119]. However, when we compared the change in protein synthesis between the first and second study, the two groups were not significantly different. We recently showed that skeletal muscle FSR is up-regulated above normal levels in burned children[205], suggesting that protein synthesis

may be approaching its upper limit, and the focus should be towards reducing protein breakdown.

We were unable to find any differences in protein breakdown in the first study between the oxprop and control groups. However, oxprop treatment significantly reduced FBR, where FBR was also significantly lower in the oxprop group compared to the control group. We show here for the first time that oxprop attenuates skeletal muscle protein breakdown in severely burned children. Previously, Hart and colleagues showed that oxandrolone improved protein net balance mediated by a significant increase in protein synthesis. However, there was no effect on protein breakdown[119]. Similarly, Herndon and colleagues showed that a significant improvement in protein net balance was achieved through protein synthesis, but no effect was seen in muscle protein breakdown of phenylalanine between their control and propranolol group (0.184 ± 0.030 vs 0.287 ± 0.048 μmol/min/100 ml of leg volume, respectively, $p = 0.20$). Thus, our data suggest that the anti-catabolic effect of combined oxandrolone and propranolol therapy is greater than its individual effect of either drug. By combining the significant increase in FSR and the significant reduction in FBR, we found a significant improvement in protein net balance in the second study. Because we did not find any differences between control and oxprop in the first study, suggests that there is a time-dependent effect of oxprop which is more evident after ~ 4 weeks of treatment. Our data for the anti-catabolic effect of oxprop is further supported with DEXA scans near the time of discharge showing that our oxprop treated subjects has significantly more lean mass when normalized to their height than subjects who did not receive oxprop.

Our previous work on skeletal muscle mitochondria in severely burned adults showed a link in increased mitochondrial uncoupling with increased REE[47]. Despite a significant reduction in whole body REE with oxprop treatment, no changes in mitochondria respiratory function were seen between the control and oxprop group, suggesting that Oxprop treatment doesn't have measurable effect on maximal

mitochondrial respiratory fluxes or coupling control ration. However, these measurements were made in vitro, and we cannot rule out the potential that Oxprop modulates mitochondrial function more subtly in vivo. Further research is warranted to elucidate the mechanism responsible for altered skeletal mitochondrial function in burn victims.

Here, we show the beneficial impact of oxprop therapy on severe burn trauma. However, there are limitations to our study. First, our study on protein turnover provides a snapshot data point while the patient is fasted. In reality, with the aggressive nutritional support, it is rare that a severely burned patient is in a fasted state. It may be worthwhile to the scientific community to investigate the effects of protein turnover in a fed state in patients treated with Oxprop. This may provide insight to the sensitivity of the rates of protein synthesis and breakdown to nutrients when they are treated with Oxprop. However, improvements in skeletal muscle amino acid turnover were accompanied by an more favorable LMI in Oxprop patients, suggesting that blunting muscle catabolism acutely may have meaningful implications for body composition

Despite these limitations, our study provides substantial data on the beneficial metabolic effects of the combination of oxandrolone and propranol treatment on skeletal muscle protein turnover, lipolysis, and energy expenditure. For the first time, we show the impact of the anti-catabolic effect of oxprop in reducing protein breakdown in severely burned children. Indeed, our study suggests that combination of β-blockade with synthetic testosterone is more beneficial than its individual treatment. Lastly, while mitochondrial function data were not different between Oxprop and control groups, reductions in ATP consuming processes such as protein and lipid substrate cycling my explain the reduction in REE observed in Oxprop patients, The data provided here should pave the way for multi-center randomized trial to further improve the treatment of burn care.

CHAPTER 5

Impact of rehabilitative exercise training with and without oxandrolone and propranolol treatment on muscle mass and function in severely burned children

INTRODUCTION

Severe burns encompassing over 30% of the total body surface area (TBSA) leaves victims in critically in conditions with profound metabolic dysregulation[30,48,173]. Increased catabolic conditions compounded by prolonged bed rest results in cachexia and loss of muscle function[29,131,206]. This loss in function and strength last up to several year after their burn injury[130]. Additionally, difficulty walking, running, feeling of weakness and fatigue were reported even after 17 years beyond burn injury[207], indicating the importance of finding a therapeutic method to restore muscle mass and function.

A 12-week exercise program has been shown to be a safe and effective approach to rehabilitate burn-injured patients without further exacerbating hypermetabolism[139] . Resistive and aerobic exercises improved skeletal muscle strength, increased lean body mass, and improved joint range of motion[132,208]. Exercise also improved pulmonary function[134] and cardiorespiratory performance[140].

Oxandrolone is a testosterone analog that has been shown to safely improve lean body mass and shorten the hospitalization period in severely burned individuals[117,119,121]. When combined with exercise, patients showed increased muscle strength and cardiopulmonary capacity[136]. Additionally, body weight significantly increased in patients taking oxandrolone with exercise compared to patients who did not take oxandrolone during exercise, and patients who exercised but did not take oxandrolone[136].

Propranolol is a β-adrenergic receptor black that has been used to reduce hypermetabolism in severe burn trauma[11,106,111,114]. It also increased lean mass by improving protein synthesis efficiency resulting in an improved protein net balance[104,111]. Patients were able to safely tolerate rehabilitative exercise while taking propranolol[135]. Patients who were treated with propranolol during their rehabilitation showed greater improvement in cardiorespiratory fitness than those who did not take propranolol[135].

Treatment with either oxandrolone or propranolol during out-patient rehabilitative exercise has been shown beneficial effects above exercise training alone. However, the impact of the combined use of oxandrolone and propranolol with a hospital-based out-patient rehabilitative exercise program on muscle mass and function in severely burned children remains unknown. The purpose of this study is to determine the effects of oxandrolone and propranolol (Oxprop) during a 6-week resistive and aerobic exercise rehabilitation program in severely burned children.

METHODS AND MATERIALS

Patients

This study was approved by the Institutional Review Board at the University of Texas Medical Branch. Severely burned children ages 7 – 17 years old with severe burns over 30% TBSA who were admitted to the Shriners Hospitals for Children in Galveston were considered for this study. In our hospital, rehabilitative exercise training has been a standard if care in the out-patient rehabilitation of children over the age of 7. All patients received standard acute burn care as described in Chapters 2 and 3, which included nutritional support, early excision and grafting, antibiotics, or anesthetics. Patients who were consented to this study were randomized into a control group, receiving the standard of care only, or the Oxprop group where they received the standard of care and oxandrolone and propranolol. Administration of Oxprop drugs began within 96 hours after admission and continued through the course of their hospitalization period. After discharge, all patients were enrolled into a 6-week in-hospital rehabilitation program. The control group participated in the standard exercise rehabilitation program, while the oxprop group participated in the exercise rehabilitation program and continued administration of oxandrolone and propranolol. Baseline exercise tests and metabolic studies were conducted at discharge (DC) from hospital and prior to beginning the exercise training program began, and again at the end of the 6-weeks exercise program (post exercise; PE).

DRUG ADMINISTRATION

Dosage for oxandrolone was given at 0.1 mg/kg (BTG Pharmaceuticals, Iselin, NJ) administered in a liquid form or capsule twice a day during their entire stay in hospital and the exercise training program. Propranolol was given at a dose of 0.33 mg/kg

of body weight every four hours (1.98 mg/kg/day). This dose was adjusted to achieve a 20% decrease in heart rate for each patient. Propranolol administration began within 96 hours post-admission and continued through the acute hospitalization period and out-patient exercise program.

REHABILITATIVE EXERCISE TRAINING

All subjects received similar training program in terms of frequency, intensity, duration, and mode of exercise. The training program included both resistive and aerobic components. All exercise sessions will be supervised by an exercise specialist and follow the standards and guidelines set forth by the American College of Sports Medicine (ACSM) and the American Academy of Pediatrics[209-212].

Resistive exercise

Eight different resistive exercises were used: bench press, squats, shoulder press, leg press, biceps curl, leg curl, triceps extension, and calf raises. Training apparatus were modified to accommodate the injury characteristics of each patient when appropriate. All exercises were performed on resistance machines or utilized free-weights. Subjects performed three sets of upper and lower body exercises with a 2-minute rest interval between each set. Resistive exercise sessions were done on Mondays, Wednesdays, and Fridays. No other strength training types of activities were permitted outside of the supervised session. However, there were no limitations on their normal daily activities. Subjects were familiarized with the exercise equipment and taught proper techniques during the first week. The intensity for the first week was set at 50 – 60% of their three-repetitions maximum (3RM) with a target number of repetitions of 15 – 20 per set. The second week increased their intensity to 80 – 85% of their 3RM to target 8 – 12

repetitions, and continued for the remaining sessions. Adjustments in the load were made as applicable when the subject was able to consistently perform three sets of 14 – 15 repetitions.

Aerobic exercise

Aerobic exercise was performed on a treadmill or cycle ergometer for approximately 25 - 45 minutes for three days each week. The intensity was set at 60 – 75% of their VO_{2peak}, which was determined during a modified Bruce treadmill test (see below). Each session started with a five-minute warm-up and subjects were asked for their rated perceived exertion (RPE) at 10, 15, 20, and 25 minutes into the exercise session. Heart rate and O_2 saturation were monitored during the session using a Radical Signal Extraction pulse oximeter (Masimo Corp, Irvine, CA) or an Imara HRM wrist monitor (Nike, Beaverton, OR). The exercise intensity was adjusted according to the exercise heart rate and RPE.

BODY COMPOSITION

Lean body mass was measured by Dual Emission X-Ray Absorptiometry (DEXA) and the images were analyzed with QDR 4500A software (Hologics, Waltham, MA). This method has a 2 – 3% margin of error for human body composition[201]. Whole-body scans were performed according to the manufacturer's instructions.

ASSESSMENT OF MUSCLE FUNCTION

Muscle strength test were performed using a Biodex dynanamometer (Shirley, NY). The isokinetic test was performed on the dominant leg and tested at an angular

velocity of 150° per second. Patients were seated and stabilized with straps across the mid-thigh, pelvis, and trunk following the guidelines of the Biodex Multi-Joint System 3 Testing and Rehabilitation System User's Guide. The test administrator first demonstrated the test, followed by an explanation to the subject, followed by one practice set where the subject can be familiar with the movement without any load. The subject was asked to perform 10 maximal voluntary muscle contractions at full knee flexion and extension without rest between each repetition. A 2-minute rest period was given and the same test was performed for a second time. Peak torque (Newton meter, Nm) and average power (Watts, W) was calculated using the Biodex software system. The highest value of the two trials was recorded.

ASSESSMENT OF CARDIOVASCULAR FUNCTION

Cardiovascular function test was performed utilizing a treadmill test (modified Bruce Protocol). The rate of O_2 uptake (VO_2), the rate of CO_2 production (VCO_2), and ventilator equivalence (V_E) were measured using the Medgraphic CardiO2 Combined O2/ECG Exercise System (St. Paul, Mn)[132]. Inspired and expired gas, flow, and volume were measured continuously through a hose attached to a face mask. The treadmill speed was 1.7 mph at the start of the test with a 0% grade elevation. Afterwards, the speed and elevation increased every three minutes. Subjects were constantly encouraged to give their maximal effort. The test ended once volitional fatigue was achieved, or the subject has an unwillingness to exercise further. The VO_{2peak} was based on an exercise heart rate above 195 bpm, respiratory exchange ratio greater than 1.05, unsteady gait, or a VO_2 plateau.

ASSESSMENT OF MUSCLE PROTEIN TURNOVER

As described in Chapter 2 and 3, Skeletal muscle protein synthesis and breakdown were measured utilizing stable isotope tracers of L-[ring-$^{13}C_6$] phenylalanine (L-[ring-$^{13}C_6$]Phe; 99% enriched) and L-[15N] phenylalanine (99% enriched) (Cambridge Isotopes, Cambridge, MA) [161,205]. Briefly, bolus injections of stable isotope tracers were injected at time 0 and 30 minutes of the protocol. Blood draws were taken periodically for one hour. Skeletal muscle biopsies were taken from the *m. vastus lateralis* at 10 and 60 minutes into the protocol. Muscle biopsies samples were snap frozen with liquid nitrogen and stored for future analysis. Blood samples were centrifuged and stored in -80°C freezers for future analysis. Isotope enrichments were determined by gas chromatography mass spectrometry (GCMS). Skeletal muscle fractional synethesis (FSR) and breakdown (FBR) rates were calculated by the precursor-product method[161].

ASSESSMENT OF RESTING ENERGY EXPENDITURE

Resting energy expenditure (REE) of burned patients was determined by indirect calorimetry (Sensor Medics Vmax 29, Yorba Linda, CA). REE was calculated from whole body oxygen consumption and carbon dioxide production rates using the Weir equation [175] as described in Chapter 3. This measured value was compared to the predicted REE which was calculated using the Harris-Benedict equation [176]. This is a standard method for estimating the degree of hypermetabolism in burned patients.

STATISTICAL ANALYSIS

All data are presented as group means ± SE unless stated otherwise. Differences between group means were analyzed by unpaired t-tests and differences within group

means were analyzed by paired t-tests. Statistical significance was reached when $p < 0.05$. Statistical analyses were performed using Graphpad Prism version 7 (GraphPad Software, La Jolla, CA).

RESULTS

We were able to study 36 severely burned pediatric patients with 19 in control and 17 in Oxprop group. The subject demographics are shown in Table 6. Control and Oxprop groups were similar in age, severity of burn, length of hospital stay, and total number of days they performed exercises (Table 6). They were also similar in height, weight, and BMI at the time of discharge (Table 6).

Table 6. Subject characteristics

	Control	**Oxprop**
n	19	17
Male / Female	15 / 4	12 / 5
Age (y)	12 ± 3	12 ± 4
Burn size (%TBSA)	47 ± 12	44 ± 9
3rd degree (%)	34 ± 18	27 ± 15
Length of stay (days)	31 ± 15	28 ± 9
# of days exercised	25 ± 6	27 ± 3

Values are group means ± SD.

Body composition

Total body mass increased from DC to PE in control (40.4 ± 2.3 vs 42.6 ± 2.6 kg, $p < 0.05$) and Oxprop (42.4 ± 3.4 vs 45 ± 3.8, $p < 0.05$). Lean mass also increased from DC to PE in control (28.6 ± 1.9 vs 30.8 ± 2.0 kg, $p < 0.05$) and Oxprop (29.6 ± 2.2 vs 30.2 ± 2.3, $p < 0.05$). BMI increased from DC to PE in control (17.8 ± 0.5 vs 18.9 ± 0.5, $p < 0.05$) and Oxprop (19.7 ± 0.8 vs 20.7 ± 1, $p < 0.050$. We determined Lean Mass Index (LMI) by calculating the total amount of lean mass (kg) divided by height (m^2). LMI significantly improved from DC to PE in control (12.5 ± 0.3 vs 13.3 ± 0.3, $p < 0.001$) and in Oxprop (13.7 ± 0.5 vs 14.0 ± 0.5, $p < 0.01$) treated children. Additionally, LMI in Oxprop was significantly greater than control at DC ($p < 0.05$), but no difference was found between groups at PE ($p = 0.26$). Difference in percent lean mass was not

found from DC to PE in either group. The magnitude of change in total body mass, lean mass, BMI, or LMI were not different between control and Oxprop, suggesting that the improvement in these measures were due to exercise training with no effects from drugs. All measures from DEXA analysis is shown in Table 7.

Table 7. DEXA analysis

	Control (n=19)		**Oxprop (n=17)**	
	Discharge	Post Exercise	Discharge	Post Exercise
Total body mass (kg)	40.4 ± 2.3	42.6 ± 2.6*	42.4 ± 3.4	45 ± 3.8*
Lean mass (kg)	28.6 ± 1.9	30.8 ± 2.0*	29.6 ± 2.2	30.2 ± 2.3*
% Lean mass	71 ± 2	71 ± 1	69 ± 2	69 ± 1
BMI	17.8 ± 0.5	18.9 ± 0.5*	19.7 ± 0.8	20.7 ± 1*
LMI	12.5 ± 0.3	13.3 ± 0.3*	13.7 ± 0.5^{+}	14.0 ± 0.5*

Values are mean ± SE. *p<0.05 vs discharge, +p<0.05 vs control.

Resting energy expenditure

We measured REE as a percentage of the predicted REE by the Harris-Benedict equation and found significant reduction in REE from DC to PE in control (148 ± 6% vs 122 ± 7%, $p < 0.01$) and in Oxprop treated patients (121 ± 6% vs 102 ± 3%, $p < 0.03$). Furthermore, Oxprop treated patients had a significantly lower REE than the control group at both DC ($p < 0.01$) and PE ($p = 0.01$) (*Fig. 17*).

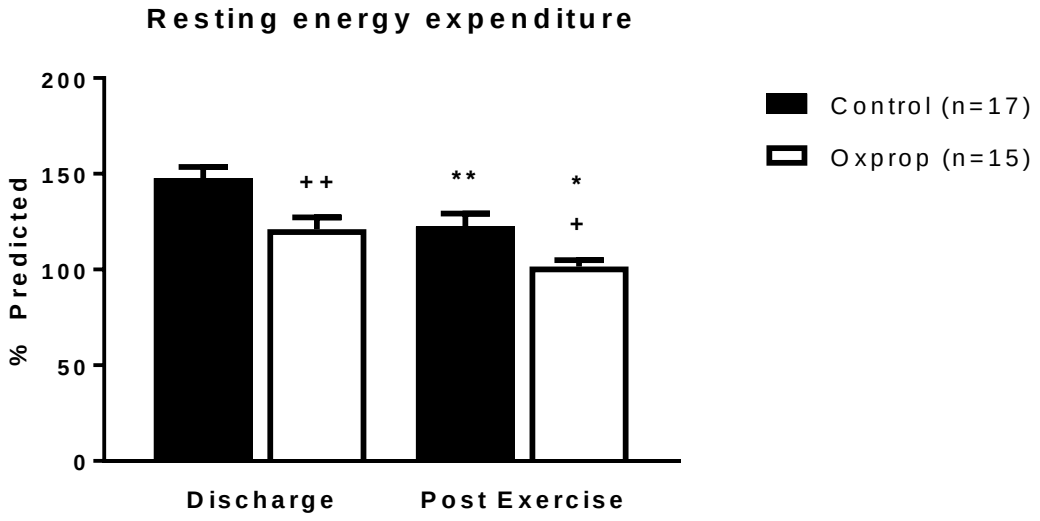


Figure 17. Resting energy expenditure in control and oxprop.

Resting energy expenditure in control and oxprop treated children with severe burns before an after a 6-week out-patient exercise training program. Resting energy expenditure (REE) was measured by indirect calorimetry and compared to the predicted value by Harris-Benedict equation. The measure of %predicted was derived by dividing the measured REE by predicted REE. *$p < 0.05$ vs discharge, **$p < 0.01$ vs discharge, *$p < 0.05$ vs control, ++$p < 0.01$ vs control.

Cardiac workload

Resting heart rate (RHR) improved from DC to PE in control (119 ± 3 vs 111 ± 3 bpm, $p = 0.03$) and oxprop (104 ± 4 vs ± 91 ± 4, $p = 0.02$). Additionally, RHR in oxprop was significantly lower than control at DC ($p < 0.01$) and at PE ($p < 0.001$) (*Fig. 18*). However, the magnitude of change from DC to PE were not significantly different between groups ($p = 0.3$).

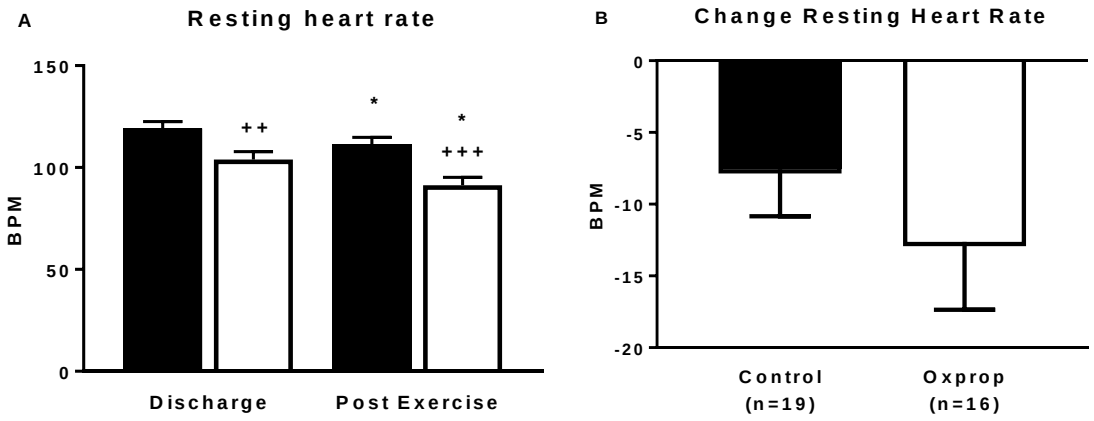


Figure 18. Resting heart rate in children with Oxprop or placebo.

Resting heart rate in children with severe burns randomized to long term oxprop or placebo (control) treatment before (discharge) and after (post exercise) an out-patient exercise training program. Resting heart rate was measured in control and oxprop (A). The change in resting heart rate was determined by the difference seen PE minus DC . Bpm, beats per minute. *p < 0.05 vs Discharge; ++p < 0.01 vs control; +++p < 0.001 vs control.

Cardiorespiratory fitness

VO_{2peak} significantly improved from DC to PE in control (22.84 ± 1.63 vs 29.06 ± 1.51 ml/kg/min, p < 0.001) and oxprop (23.53 ± 2.06 vs 30.45 ± 2.01 ml/kg/min, p < 0.001) (*Fig. 19*). No significant differences were found in the change of $VO2_{peak}$ (p = 0.2), and no differences were found between groups at DC and PE.

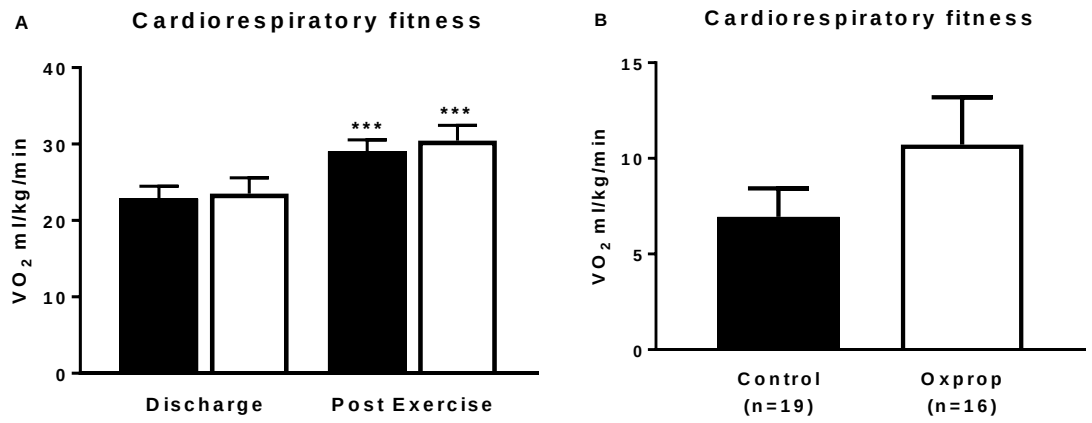


Figure 19. Cardiorespiratory fitness in control and oxprop.

Cardiorespiratory fitness in control and oxprop treated children with severe burns before and after a 6-week exercise training program (A).Cardiorespiratory fitness was measured by a treadmill test. The change in cardiorespiratory fitness was determined by subtracting inintial value from post exercise value (B). Peak VO2 (ml/kg/min) was measured. ***p < 0.001 vs Discharge.

Muscle strength

Strength significantly improved from DC to PE in the control group (40.5 ± 4.3 vs 55.2 ± 5.0 Nm, $p < 0.001$) and the oxprop group (41.9 ± 6.0 vs 65.4 ± 7.7 Nm, $p < 0.001$). The change from DC to PE was significantly greater in the oxprop group vs control (14.7 ± 1.8 vs 22.2 ± 2.2 Nm, $p = 0.01$). However, no significant differences in strength were seen between control and oxprop at DC or PE. Similar results were found when we normalized their peak torque relative to their body weight. Relative to their body weight, muscle strength improved from DC to PE in control (1.0 ± 0.1 vs 1.3 ± 0.1 Nm/kg, $p < 0.001$) and oxprop (0.9 ± 0.1 vs 1.4 ± 0.1 Nm/kg, $p < 0.001$). No differences were found between groups at DC or PE. The change in improvement was significantly higher in oxprop group when to the control group (0.27 ± 0.04 vs 0.41 ± 0.04 Nm/kg, $p = 0.03$) (*Fig. 20*).

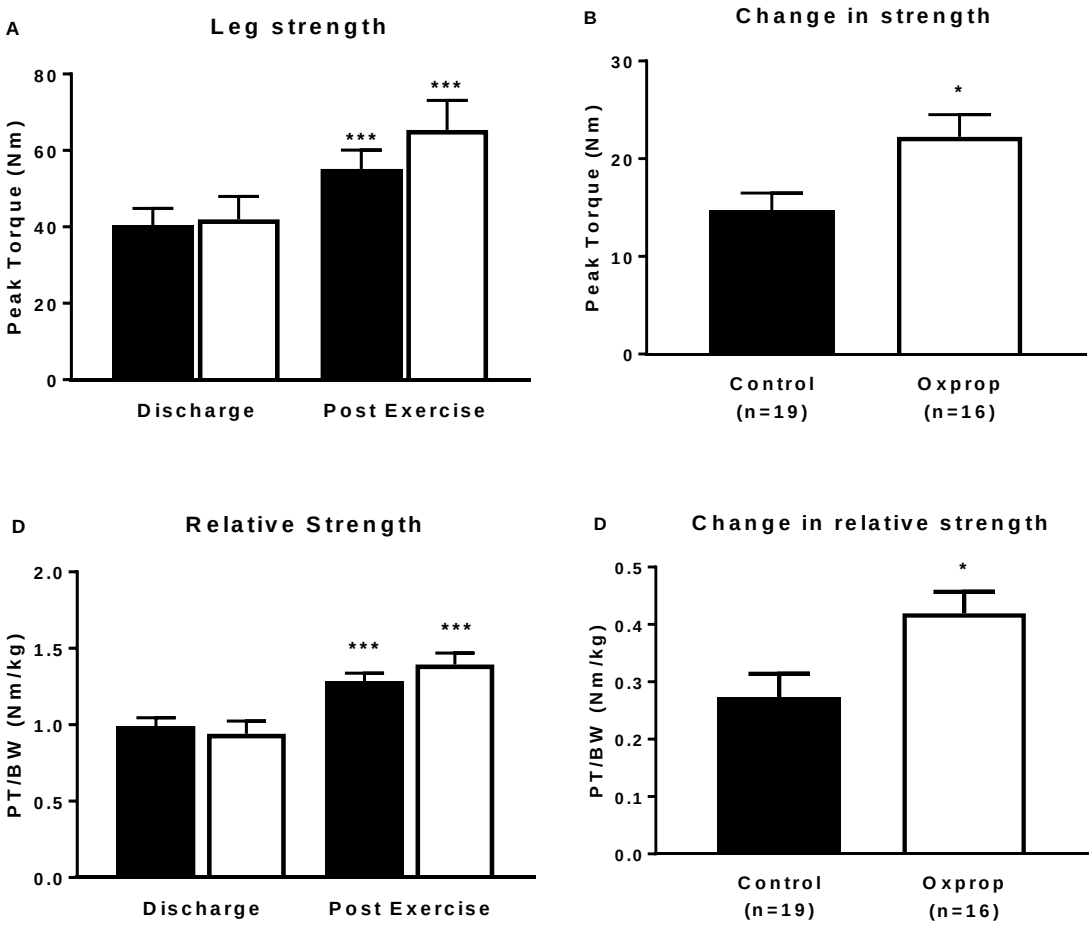


Figure 20. Peak torque in control and oxprop treated patients.

Peak torque in control and oxprop treated patients before and after a 6-week exercise training program. Peak torque was measured in Newton meters (Nm) by biodex Pre- and post-exercise (A). Change in strength (B). ***$p < 0.001$ vs Discharge; *$p < 0.05$ vs control.

Muscle power

Average power significantly improved from DC to PE in the control group (52.3 ± 6.8 vs 69.7 ± 6.9 W, $p < 0.001$) and oxprop group (54.9 ± 8.7 vs 86.9 ± 11.4 W, $p < 0.001$). The magnitude of change was significantly greater in the oxprop group vs control (17.4 ± 2.7 vs 30.5 ± 3.0 W, $p < 0.01$) (*Fig. 21*). No significant differences in average power were found between groups at DC or PE.

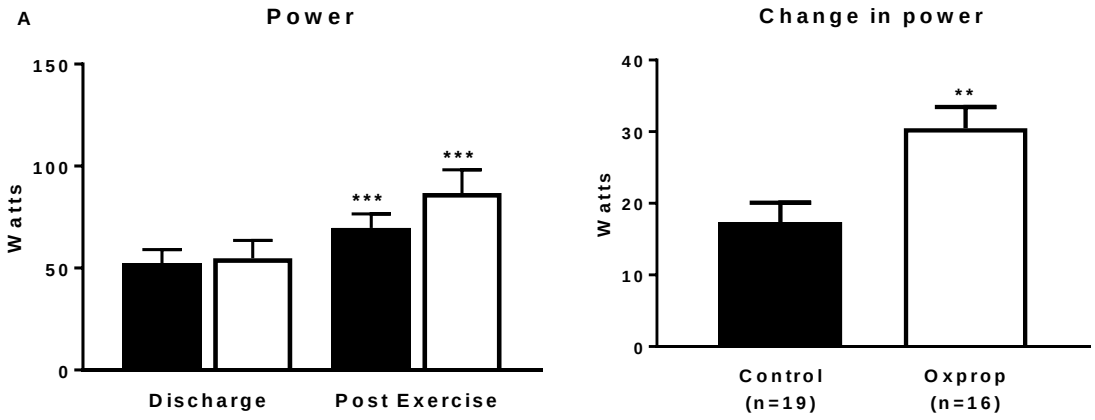


Figure 21. Muscle power in control and oxprop.

Muscle power in control and oxprop treated children with severe burns before and after a 6-week exercise training program. Tests were performed by biodex measured by Watts (W) at discharge and post-exercise (A). Change in strength (B). ***$p < 0.001$ vs Discharge; **$p < 0.01$ vs control.

Muscle protein turnover

FSR did not significantly change from DC to PE in control (0.17 ± 0.03 vs 0.07 vs 0.01 %/h, $p = 0.11$) or oxprop treated patients (0.09 ± 0.01 vs 0.11 ± 0.09 %/h, $p = 0.81$). FSR was not significantly different in control vs oxprop at DC ($p = 0.14$). However, FSR was significantly higher in the oxprop vs. control group at PE ($p < 0.01$). FBR tended to improve from DC to PE in control treated patients (0.35 ± 0.06 vs 0.19 ± 0.04 %/h, $p = 0.053$), but this was not statistically significant. FBR was not significantly changed in the oxprop group from DC to PE (0.14 ± 0.01 vs 0.11 ± 0.02, $p = 0.18$). However, FBR was significantly lower at DC in oxprop vs control ($p < 0.01$). FBR tended to be lower in oxprop vs control at PE ($p = 0.06$), but it was not statistically significant. Protein net balance was determined by subtracting FBR from FSR. No improvement was found from DC to PE in control (-0.18 ± 0.06 vs -0.12 ± 0.04, $p = 0.84$) or Oxprop (-0.05 ± 0.02 vs 0.01 ± 0.03, $p = 0.30$). Protein net balance was less negative in Oxprop vs control at DC ($p < 0.05$) and at PE ($p < 0.05$) (*Fig 22*).

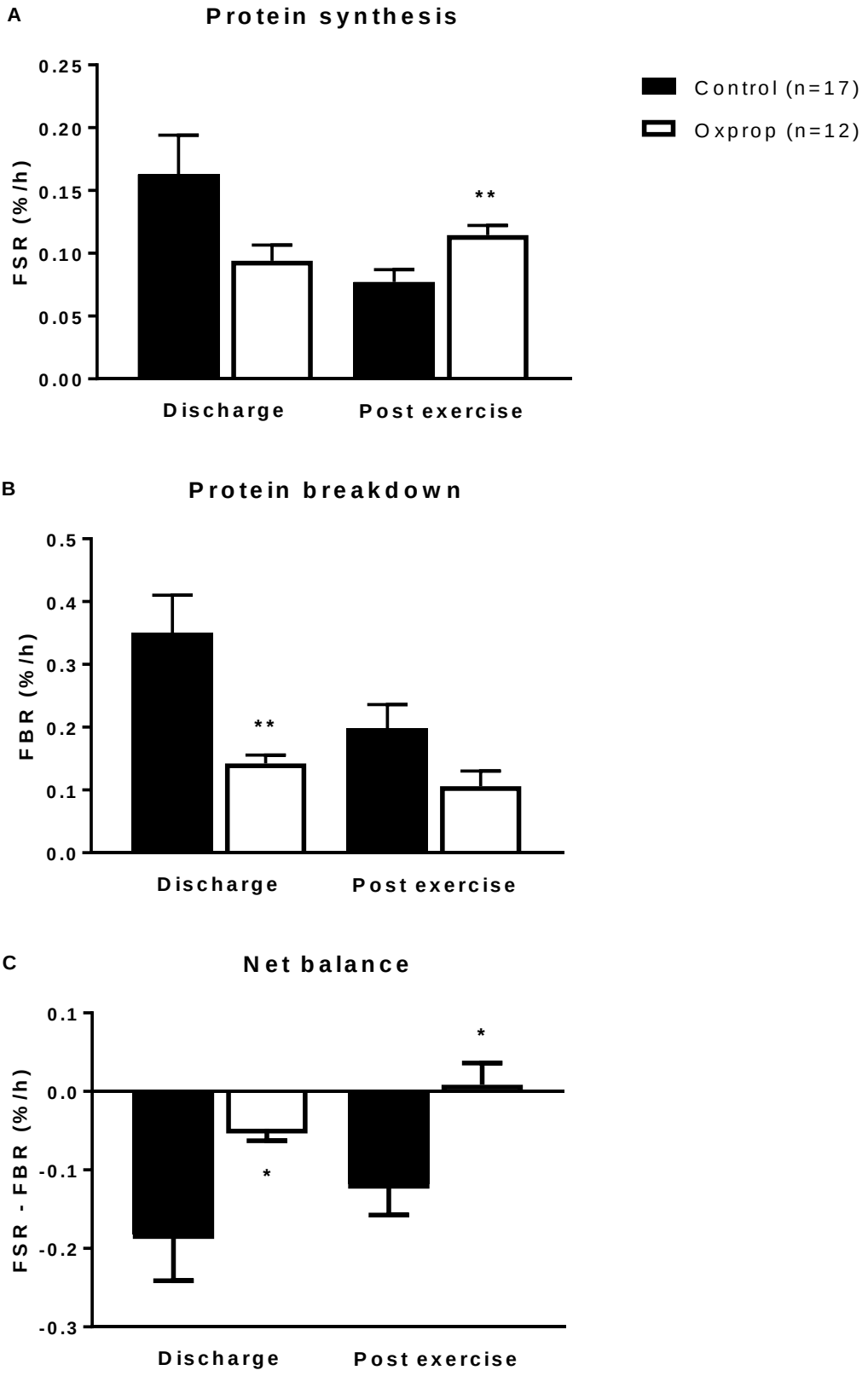

A
Protein synthesis
FSR (%/h)
0.25
0.20
0.15
0.10
0.05
0.00
**
Discharge
Post exercise
Control (n=17)
Oxprop (n=12)
B
Protein breakdown
FBR (%/h)
0.5
0.4
0.3
0.2
0.1
0.0
**
Discharge
Post exercise
C
Net balance
FSR - FBR (%/h)
0.1
0.0
-0.1
-0.2
-0.3
*
*
Discharge
Post exercise

Figure 22. Skeletal muscle protein turnover in control and oxprop.

Skeletal muscle protein turnover in control and oxprop treated children with severe burns before and after a 6-week outpatient exercise training program. Protein synthesis (A), breakdown (B), and net balance (C) were measured utilizing stable isotope tracers of phenylalanine. FSR, fractional synthesis rate; FBR, fractional breakdown rate. *$p < 0.05$ vs control, **$p < 0.01$ vs control.

DISCUSSION

Severe burn injury results in an extreme pathophysiological response characterized by increased REE, elevated HR, and extreme loss of skeletal muscle mass and function[6,29,30,131,173]. Severe loss of muscle mass increases morbidity and mortality[31,65]. Individuals with severe burns showed limited functional capacity even one year after their injury, long after their wounds have healed[130]. Therefore, it is imperative to develop a standard rehabilitation strategy to restore skeletal muscle mass and function. Indeed, a 12-week supervised hospital based rehabilitation exercise program performed at 6-9 months post injury showed improved muscle strength, power, and lean body mass of severely burned children compared to those who followed an outpatient rehabilitation program without exercise[132,140]. Our study shows for the first time, that pharmacological treatment with the combination of oxandrolone and propranolol augment the beneficial outcomes of an out-patient exercise rehabilitation program.

In the current study there was a significant improvement in absolute muscle strength, muscle strength relative to body weight, and average power in both the control and Oxprop group. This is consistent another study by Porro and colleagues showed that patients who participated in exercise (EX) and patients who took propranolol while participating in exercise (PROPEX) improved in muscular leg strength measured in peak torque[135]. However, the magnitude of change was not different indicating that propranolol did not offer additional benefits to exercise for leg strength. Similarly, Przkora and colleagues found improvements in muscular strength in burn-injured children who participated in exercise (EX) and burn-injured children who took oxandrolone and exercised (OXEX)[136]. Again, no differences were found in the magnitude of change between those groups. In our study, not only did we find that both oxprop and control improved in leg muscle strength, but the magnitude of change in oxprop was significantly greater than the change in control group. This suggests that

combined treatment with oxandrolone and propranolol is able to augment the improvement in strength brought about by exercise training, but either agent alone does not have this effect. A significantly greater improvement was also seen in oxprop over control when we normalized muscle strength change according to their body weight.

For the first time, we provide evidence that muscle power is increased in in burned children following 6-weeks of rehabilitative exercise. Additionally, the increase in muscle power in Oxprop treated patients was significantly greater than the increase seen in control placebo treated patients, indicating that the combined treatment of propranolol and oxandrolone further improved neuromuscular function with exercise. Muscle strength and endurance are important indicators for muscle quality. However, utilizing power as an indicator of muscle control may be equally important[213]. This is worth considering in severely burned patients where neuromuscular control can provide insight to their ability or limitation in movement velocity. For activities of daily living, it is worth noting that improved strength may help them function independently, but also the capability of how quickly they can move and react may also contribute to their functional independence.

We showed improvement in cardiorespiratory fitness in both groups. However, we were not able to find any differences between the changes in VO_{2peak} in Oxprop vs control. Oxandrolone by itself did not add any additional benefits to VO_{2peak}, but administration of propranolol alone did show greater improvement in VO_{2peak} than just exercise[135]. It was speculated that this effect may be attributed to the reduced capillary blood flow allowing greater gas exchange in exercising muscles[135]. However, the literature on exercise and propranolol on cardiorespiratory fitness in severely burned individuals is limited. To determine if treatment with oxandrolone impedes this effect by propranolol requires further investigation.

An extremely important finding in our study is the reduction of hypermetabolism following a 6-week exercise training supplemented with O xprop therapy. Both groups showed a reduction in REE and RHR. Additionally, REE and RHR in Oxprop was

significantly lower at DC and PE than control. After exercise rehabilitation, we found the Oxprop subjects had a mean REE at 102% by the Harris-Benedict equation and RHR at 91 bpm. This shows a restoration of physiological functioning towards normal values as the measured REE converges to the Harris-Benedict predicted calculation and a RHR of 91 bpm is within normal range between 60 and 100 according to the American Heart Association[214]. This suggests that there is an effect of Oxprop drugs on HR and REE. The reduction in REE may be attributed to the reduction in protein turnover.

We found improvements in lean body mass in both groups after exercise. However, the change in lean mass was not significantly different between each group. LMI was significantly higher in Oxprop than control, suggesting that oxandrolone and propranolol therapy significantly improve the maintenance of lean mass during their acute hospitalization period. The study utilizing oxandrolone alone found that lean body mass increased significantly greater than exercise without oxandrolone[136]. However, their exercise program persisted for 12-weeks and was performed at 6-9 months post injury. Since this protocol was double the length of this exercise training program employed in the current protocol, and was also performed at a different time frame post injury, may offer an explanation for this discrepancy. It is reasonable to suggest that longer training periods with oxandrolone will likely produce greater outcome than shorter training periods.

We did not find significant changes in FSR changes over time. Control subjects tended to show a reduction in FSR after exercise, which is in agreement with the previous findings of Hardee et al. where FSR significantly decreased following a 12-week exercise training[140]. Here, we found that FBR in control tended to decrease from DC to PE, although again not significant. The trend in reduction of both FSR and FBR may indicate the reduction in overall protein turnover over time and as a result of exercise training. As we explained previously, the rate of protein synthesis in severely burned children may be attributed to the elevated rate of breakdown by continuously providing amino acid into

the intracellular free pool[205]. We show that FBR was significantly lower in the Oxprop group than the control group at DC and PE, and also FSR is significantly greater in Oxprop than control at PE. This is consistent with our previous work showing that Oxprop treatment has a strong anti-catabolic effect by reducing protein breakdown acutely post burn (Chapter 3). Additionally, exercise therapy combined with oxandrolone further stimulates the anabolic drive. Protein net balance was significantly more positive in Oxprop than control at both DC and PE time-points. Similar to what we found with REE and RHR, Oxprop therapy following exercise training seem to bring muscle protein turnover back to normal physiological range.

Collectively, the whole body function and skeletal muscle metabolism data presented here supports a role for Oxprop treatment in augmenting exercise induced improvements in body composition and function, particularly in regard to muscle strength and performance. Interestingly, these results were more favorable that those reported in studies testing the effect of either oxandrolone or propranolol alone in augmenting exercise induced improvements in body composition and exercise performance in burned children, providing the first evidence that combined treatment of both agents may be synergist, which is in line with recent data showing that Oxprop treatment blunts growth arrest in burned children more than treatment with either propranolol or oxandrolone alone[196].

There are some limitations to our study. First, our protein turnover analysis can only provide a snapshot of the rates of protein synthesis and breakdown while they were in a fasted state. Secondly, nutrition and physical activity were not directly monitored during the intervention. The change in weight can be highly influenced by diet. Although all burned patients received nutritional counseling, they have the free will to choose the amount or kind of food to consume. However, this may provide insight in the effectiveness of treatment in free-living patients. Also, because these subjects are young children, they tend to be active and engage in daily play activities. Their extra daily

activities can contribute to their progression during the exercise training program. Furthermore, the literature on drug and exercise therapy is limited. We were only able to compare our study utilizing oxandrolone and propranolol in a 6-week exercise program beginning at the time of discharge. Previous studies on oxandrolone or propranolol and exercise incorporated a 12-week exercise that began 6 months after burn injury. Perhaps if we doubled the length of our exercise program, we could have showed a greater effect in our Oxprop group. Lastly, these exercise tests are largely limited to the motivation of the subject. Pain and discomfort can largely limit their performance during these tests rather than their actual maximal effort.

Despite these limitations, our data shows that the combined treatment of oxandrolone and propranolol can augment the beneficial effect of a 6-week exercise rehabilitation program on muscle strength, power, lean body mass, and improving protein turnover. Additionally, utilizing Oxprop and exercise may have the potential to abrogate the hypermetabolic and hypercatabolic effect of severe burn injury. We show here that utilizing Oxprop can decrease REE and heart rate after exercise training, thereby returning it back within normal range. Further research on levels of cytokines, catecholamines, genes and proteins that may be responsible for these effects we show in this study may help us further understand mechanisms of post-burn hypermetabolism. Our data shows promising results in improving burn care rehabilitation through a multi-model approach by combining testosterone and β-blockade in additional to exercise rehabilitation.

CHAPTER 6

General Discussion and Conclusions

Severe burn injury affects millions of people around the world and has a huge impact to the cost of healthcare[1,4,5]. Severe burns covering at least 30% of the total body surface area result in significant and long-lasting metabolic abnormalities[6]. Elevated heart rate, resting energy expenditure, extreme muscle wasting, and loss of muscle function are all hallmarks of this stress response, which can last up to three years after the injury[6,30,131]. Over the past few decades, advancements in surgical, nutritional, pharmacological, and environmental intervention have greatly improved the outcomes of burn-injured patients[3,50]. However, there are several unanswered questions on the etiology of burn sequela and in particular, how to best treat the acute stress response to burns and promote the long term rehabilitation of burn survivors. Thus, we sought to investigate a novel pharmacological method and employ new methods to test our hypothesis.

In chapter 2 and 3, our aim was to measure and document skeletal muscle protein turnover and mitochondrial function in severely burned children, both acutely and over the long term. As previously mentioned, severe muscle wasting occurs as a result of severe burn. Therefore, it has clinical importance to be able to measure the rate of protein turnover and how it responds to treatment and healing. We utilized a new bolus injection method of stable isotope tracers of the amino acid, phenylalanine. This new bolus method was beneficial to the patients because the protocol is shorter and less invasive than the previous cross-leg method which may reduce discomfort for the subject. Additionally, we utilized a high-resolution respirometry to measure mitochondrial function in skeletal muscle *ex vivo*. This method allowed us to determine the efficiency of energy production

by the electron transport chain. We found that skeletal muscle protein turnover was up-regulated above normal levels in burned children, where the increase of muscle protein breakdown was particularly remarkable. Additionally, we found the capacity of muscle mitochondria for ATP production was diminished in burned victims, which persisted up to one year after bur injury. Thus, not only are burned victims losing muscles at an alarming rate, but the ability to produce energy is also reduced, suggesting both muscle mass and muscle quality are lost after severe burn trauma. Therefore, we concluded that treatments that focus on reducing acute skeletal muscle protein breakdown and restore muscle function in the long term would likely have the best impact on burn patients in terms of hastening recovery and improving quality of life.

Several pharmacological agents have been investigated to improve outcomes in severe burn trauma. Oxandrolone, a testosterone analog, has been extensively studied to alter muscle protein metabolism and improve lean body mass in burn survivors. Propranolol, a non-selective β-adrenergic receptor blocker, has also been studied extensively with an aim of reducing HR and metabolic rate. Both of these drugs showed promising results with few side effects. Since these drugs act through difference mechanisms, we hypothesized that the combined use of these drugs will yield beneficial outcomes in terms of blunting muscle catabolism, and muscle mass and function in severely burned children. In chapter 4, we utilized the bolus injection method with labeled Phe, infusions of labeled glycerol and palmitate, and high-resolution respirometry to determine the impact of Oxprop administration on protein turnover, lipid kinetics, and skeletal muscle mitochondrial function. Indeed, we found that treatment with Oxprop improved protein turnover, predominantly by reducing protein breakdown. These results were further supported when we found that the drug treated group had significantly more lean mass when normalized to their height than the control group near the time of discharge. Additionally, we found that hypermetabolism and cardiac work were also reduced with Oxprop treatment relative to a placebo group. While mitochondrial function

was not different in skeletal muscle of Oxprop and control treated patients, reduced protein and lipid turnover as well as a significantly lower heart rate like contribute to the reduction in REE seen in Oxprop treated burned children.

The next step in progression was to follow these same patients to their outpatient rehabilitation treatment. In Chapter 5, we hypothesized that Oxprop treatment along with a 6-week exercise rehabilitation program will elicit more beneficial response in muscle mass and strength, cardiorespiratory fitness, and cardiac work than just exercise alone. Our results indicated that drug treatments and exercise had significantly greater muscle strength and power gains over just exercise alone. Additionally, resting energy expenditure and heart rate was significantly reduced in our drug and exercise group versus exercise alone. Indeed, these results showed that there was a drug effect on muscle function and cardiac work. This study further supports the notion of employing an exercise program as a standard part of rehabilitative care, with additional benefits with oxandrolone and propranolol administration.

In summary, the studies presented within this thesis provide evidence for the benefits of oxandrolone and propranolol treatment for severe burn injury. Specifically, we showed that the combined use of these drugs blunts muscle wasting by directly reducing skeletal muscle protein breakdown, resulting in an improved protein net balance. Oxprop lowered REE, probably by reducing lipid and protein turnover, and decreasing HR, thereby decreasing ATP turnover. With exercise therapy, Oxprop augments exercise related improvements in muscle function by mediating increases in strength and power. Therefore, b-blockade and androgen replacement should be considered as an adjunct therapy for severely burned children. However, additional multi-center trials, particularly in adults and both sexes are still needed.

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Vita

Tony grew up in Dallas, Texas where he enlisted in the United States Marine Corps right after graduating high school in May, 2000. After serving four years of active duty and receiving his honorable discharge, Tony moved to Houston where he pursued a B.S. in exercise science at the University of Houston. Afterwards, he wanted to specialize in clinical rehabilitation so he went for his M.S. in clinical exercise physiology at Texas A&M University in College Station. While at Texas A&M, he was encouraged by faculty to pursue a Ph.D. In the fall of 2012, Tony was accepted into the Ph.D. in Rehabilitation Science program at UTMB and joined the metabolism unit at the Shriners Hospital for Children. As a UTMB student, he was awarded several scholarships and recognitions for his academic excellence and contributions to UTMB by serving in student leadership roles such as the Student Government Association and Students Together for Service. His research in pediatric burn trauma also earned him recognition and awards at national conferences, such as the Shock Society – Department of Defense Battlefield Health and Trauma Fellowship Award. He will continue his scientific endeavors as a postdoctoral fellow at the United States Army Institute of Surgical Research in San Antonio, Texas.

EDUCATION

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PUBLICATIONS

1. Ogunbileje J, Porter C, Herndon D, **Chao T**, Abdelrahman D, Papadimitriou A, Chondronikola M, Zimmers T, Reidy P, Rasmussen B, Sidossis L. Hypermetabolism and

hypercatabolism of skeletal muscle accompany mitochondrial stress following severe burn trauma. *Am J Physiol Endocrinol Metab.* (In press).

2. Porter C, Herndon DN, Chondronikola M, **Chao T**, Annamalai P, Bhattarai N, Saraf MK, Capek K, Reidy PT, Daquinag AC, Kolonin MG, Rasmussen BB, Borsheim E, Toliver-Kinsky T, Sidossis LS. Human and mouse brown adipose tissue mitochondria have comparable UCP1 function. *Cell Metabolism*. (In press).

3. Chondronikola M, Volpi E, Borsheim E, Porter C, Saraf MK, Annamalai P, Yfanti C, **Chao T**, Wong D, Shinoda K, Labbe SM, Hurren NM, Cesani F, Kajimura S, Sidossis LS. Brown Adipose Tissue Activation Is Linked to Distinct Systemic Effects on Lipid Metabolism in Humans. *Cell Metabolism*. 2016 Jun;23(6):1200-1206.

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11. Porter C, Herndon DN, Børsheim E, **Chao T**, Reidy PT, Borack MS, Rasmussen BB, Chondronikola M, Saraf MK, Sidossis LS. Uncoupled skeletal muscle mitochondria

contribute to hypermetabolism in severely burned adults. *Am J Physiol Endocrinol Metab.* 2014 Sep;307(5):E462-7.

ABSTRACTS

1. **Chao T**, Porter C, Suman OE, Ali A, Sidossis LS, Finnerty CC, Herndon DN. Metformin treatment improves skeletal muscle protein turnover in severely burned children. Thirty-Ninth Annual Conference on Shock. Austin, TX. June 14, 2016.

2. **Chao T**, Herndon DN, Porter C, Suman OE, Sidossis. Combined oxandrolone and propranolol treatment improves skeletal muscle nitrogen balance in severely burned children by blunting proteolysis. American Burn Association Annual Meeting. May 3, 2016. Las Vegas, NV.

3. **Chao T**, Herndon DN, Porter C, Reda Abdelrahman D, Suman, OE, Sidossis, LS. Combined oxandrolone and propranolol therapy attenuate protein breakdown in severely burned pediatric patients. Experimental Biology. Apr 5, 2016. San Diego, CA.

4. **Chao T**, Herndon DN, Porter C, Bohanon FJ, Chaidemenou N, Sidossis LS. Prolonged elevated skeletal muscle protein turnover following severe burn injury. American Burn Association Annual Meeting. Apr 24, 2015. Chicago, IL.

5. **Chao T**, Porter C, Bhattarai N, Herndon DN, Toliver-Kinskey T, Sidossis LS. Severe burn injury induces thermogenically functional mitochondria within inguinal white adipose tissue of mice. Keystone Symposia. Beige and Brown Fat: Basic Biology and Novel Therapeutics. Apr 17, 2015. Snowbird, UT.

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7. **Chao T**, Herndon DN, Bhattarai, Reidy P, Borack M, Rasmussen B, Sidossis LS, Porter C. Skeletal muscle mitochondrial dysfunction following severe thermal trauma. Experimental Biology. Apr 26, 2014. San Diego, CA.

PROFESSIONAL PRESENTATIONS

1. **Chao T**. Metformin treatment improves skeletal muscle protein turnover in severely burned children. *Thirty-Ninth Annual Conference on Shock*. Austin, TX. June 14, 2016.

2. **Chao T**. Combined oxandrolone and propranolol treatment improves skeletal muscle nitrogen balance in severely burned children by blunting proteolysis*. American Burn Association Annual Meeting*. May 3, 2016. Las Vegas, NV.

3. **Chao T.** Utilizing the Bolus Method to Measure Protein Turnover in Pediatric Burn Patients. *8th Annual Isotope Tracers in Metabolic Research*. Cleveland, OH. Nov 18, 2015.

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