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Original Article

Beneficial Effects of Insulin on Ischemia Reperfusion Injury in Human Skeletal Muscle

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Purpose: Exaggerated leucocyte activity is a crucial step in the pathophysiology of skeletal muscle ischemia-reperfusion injury (IRI). We tested the hypothesis that insulin, via its' anti-leukocyte activity, attenuates skeletal muscle IRI in humans.

Materials and Methods: This randomized, blinded, placebo-controlled trial was conducted in patients with skeletal muscle ischemia who required revascularization. Treatment protocols were similar among them except for the insulin group, which received an infusion of insulin at 2.5 U/h. The degree of endothelial adhesiveness; leukocyte activity and pro-inflammatory status via P-selectin, tumor necrosis factor (TNF)-alpha, and myeloperoxidase (MPO) levels in the venous effluent; and clinical outcomes were measured.

Results: Twenty-four consenting patients were randomized to the insulin or control group. There were no significant differences between the two groups except for the median serum insulin level, which was higher in the insulin group (P<0.01). No serious intervention-related adverse events were observed. P-selectin (55.04-99.86 pg/mL; P<0.001), MPO (110.8-160.6 pg/mL; P<0.001), and TNF-alpha (12.16-36.01 pg/mL; P<0.001) levels demonstrated a significant increase post-reperfusion in the 'control' group, reaching a peak value at 2 hours post-reperfusion. The increase in all three markers from baseline was significantly diminished in the insulin group at the two-hour (P-selectin, P=0.001; MPO, P=0.001; TNF-alpha, P=0.005) and four-hour (P-selectin, P=0.003; MPO, P=0.002; TNF-alpha, P=0.01) intervals. The differences in clinical outcomes between the insulin and control groups were not statistically significant.

Conclusion: In clinical practice, insulin has the potential to attenuate the severity of skeletal muscle IRI inhibiting P-selectin, MPO, and TNF-alpha levels.

Key Words: Skeletal muscle, Ischemia, Reperfusion injury, Insulin

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INTRODUCTION

Ischemia is a fundamental cause of tissue injury and organ dysfunction. Paradoxically, reperfusion exacerbates existing ischemic injury and contributes to poorer outcomes than expected owing to ischemia-reperfusion injury (IRI). The search for interventions to overcome IRI is ongoing. Understanding the mechanism of IRI is crucial in this respect.

Leukocyte-endothelial interactions are a crucial com-

ponent in the pathogenesis of IRI. Leukocyte activation involves multiple processes, including rolling, adhesion, and transmigration. It is mediated by complex interactions of a multitude of cellular adhesion molecules [1]. Following activation, highly toxic reactive oxygen species (ROS) are released from neutrophils, resulting in cellular damage [2].

Studies using animal models have demonstrated that soluble P-selectin, tumor necrosis factor alpha (TNF-α), and myeloperoxidase (MPO) are highly sensitive surrogate markers of IRI and integral to the pro-inflammatory state and exaggerated leukocyte-endothelial activity.

P-selectin is a cellular adhesion molecule derived from endothelial cells and platelets. It is overexpressed during reperfusion and regulates leukocyte rolling. Its role in IRI has been demonstrated by Singbartl et al. [3] who reported the attenuation of acute renal failure following reperfusion in P-selectin-deficient mice and by Chen et al. [4] who demonstrated attenuation of hepatic insufficiency in mice treated with the P-selectin blocking agent tetramethylpyrazine.

Adhered leukocytes transmigrate through the endothelium and subsequently become activated [5,6]. Upon activation, they release MPO, that plays a crucial role in the formation of reactive oxygen species (such as hypochlorite) during IRI, resulting in tissue damage [7]. The role of MPO in IRI was demonstrated by Li et al. who established the protective effects of an MPO inhibitor, phloroglucinol, during ischemia-reperfusion of the rat myocardium [8].

TNF-α, a proinflammatory cytokine, is released by a multitude of cells under stress conditions. Its role was first demonstrated by Colletti et al. [9] in 1990 in a murine model subjected to hepatic ischemia-reperfusion. Trials using animal models have demonstrated that TNF-α is released during IRI, leading to the expression of various cell adhesion molecules and cytokines. Seekamp et al. [10] used a soluble TNF receptor construct and an anti–TNF-α antibody to abrogate TNF-α activity in skeletal muscle, resulting in decreased muscle capillary permeability and edema.

P-selectin, MPO, and TNF-α were therefore selected to represent the key elements of IRI, denoting endothelial-leukocyte interaction, polymorphonuclear leukocyte activity, and pro-inflammatory status, respectively. Clinical outcomes are affected by many variables in real-life clinical settings and are therefore prone to heterogeneity; however, ultimately, the expectation is that the results of the study would lead to a beneficial clinical outcome. Therefore, clinical endpoints were also measured in both groups along with serum molecular markers.

Insulin, which is primarily used for its hypoglycemic effect, has recently been recognized for its anti-inflammatory and immunomodulatory effects. The cytotoxic functions of neutrophil and generation of ROS are attenuated during reperfusion [11,12].

In a rabbit model, Ji et al. [13] demonstrated that glucose-insulin-potassium (GIK) infusion attenuated myocardial injury with a significant decrease in the coronary endothelial expression of P-selectin and intracellular adhesions molecule-1; this inhibited the adherence of polymorphonuclear leukocytes to the coronary endothelium. In similar animal models, insulin was shown to abolish the increase in TNF-α and MPO activity [14].

Sodi-Pallares et al. [15] and Opie [16] suggested that a GIK mixture may protect ischemic cardiomyocytes after revascularization. A more recent work has shown that insulin exerts powerful anti-inflammatory effects on endothelial cells in vitro and on circulating mononuclear cells in vivo when infused at a dose of 2.5 U/h in non-diabetic subjects [17,18].

However, the effect of insulin on P-selectin, MPO, and TNF-α in reperfused ischemic skeletal muscles in humans remains unknown. We hypothesized that in patients with acute lower extremity skeletal muscle ischemia, an infusion of insulin at 2.5 U/h attenuates reperfusion injury, as seen by the diminution of these molecular markers and improved clinical outcomes.

MATERIALS AND METHODS

1) The study

This was a randomized, single-blinded, placebo-controlled clinical trial. The authors designed the study, collected, and analyzed the data, and wrote the manuscript. The trial was performed in accordance with Sri Lankan law. The study protocol was approved by the University of Colombo Sri Lanka Ethics Committee (ref no. EC-11-166), and the trial was registered in the Sri Lanka Clinical Trials Registry (no. SLCTR/2014/005). All the participants provided written informed consent.

2) Study population

Patients aged >18 years who presented with acute lower limb ischemia were eligible for enrollment. These included patients who presented with acute arterial occlusion due to thrombosis or embolism.

The decision to surgically revascularize or amputate in patients with thromboembolic acute limb ischemia was made by an independent surgeon. The exclusion criteria included the following: patients for whom amputation rather than revascularization was indicated, patients with obesity (body mass index >30 kg/m^2), patients with insulin-treated

diabetes mellitus, and patients on glucocorticoid treatment.

3) Experimental protocol

Those who met the inclusion criterion were subsequently block-randomized into a control or insulin group. The participants and clinicians involved in the surgery, except for the anesthetists, were blinded to the code's identity.

All patients received unfractionated heparin (Heparin Leo injection; Leo Pharma Inc., Hurley, Berkshire, UK). A bolus of 80 units/kg (approximately 5,000 IU) heparin was administered at the point of diagnosis, with the aim of preventing further propagation. Subsequently, an infusion of unfractionated heparin at 18 units/kg was commenced until definitive surgery and continued in the early postoperative period. Heparin infusion was titrated to maintain an activated partial thromboplastin time ratio of 2.0-2.5. Participants in the 'Insulin group' were administered an infusion of GIK solution (40 mmol KCl, 10% dextrose at 60 mL/h, and soluble insulin [Novo Nordisk Pharmaceuticals, Bagsvaerd, Denmark] at 2.5 U/h, separately). The infusion was commenced at the point of diagnosis and continued for 12 hours post-reperfusion. The dextrose/potassium infusion was titrated to maintain glucose levels between 80 and 200 mg/dL and potassium levels between 4 and 5 mmol/L. The range of glucose and potassium levels and infusions mentioned above were based on the protocol described by Chaudhuri et al. [19].

Patients in the control group were infused with normal saline instead of a GIK solution. Owing to the absence of a standard therapy for preventing reperfusion injury, normal saline was used as the placebo for comparison.

4) Sampling

A challenge faced in human studies is the development of a strategy for identifying surrogate markers with high sensitivity. Sampling of the locoregional venous effluent with an indwelling venous catheter has been demonstrated to be more specific and effective than sampling the systemic circulation via a peripheral cannula. A femoral vein catheter was used by Rowlands and Homer-Vanniasinkam [20] to study the presence of lower limb ischemia during aortic aneurysmal repair, and a jugular venous catheter was used by Wijeyaratne et al. [21] to study cerebral ischemia during carotid endarterectomy.

Ethical approval was obtained for the insertion of an ipsilateral indwelling femoral venous catheter and for it to be left in situ for 12 hours post reperfusion to demonstrate the temporal evolution of reperfusion markers.

5) Outcome measures

① Biochemical markers

The biochemical markers measured were plasma P-selectin, TNF-α, and MPO levels in the venous effluent in the control and insulin groups. Loco-regional sampling was performed at 0, +2, +4, +6, and +12 hours post reperfusion to establish the temporal evolution of P-selectin, TNF, and MPO in the venous effluent.

ELISA was used to measure plasma MPO (ng/mL; R&D Systems Inc., Minneapolis, MN, USA), soluble P-selectin (ng/mL; R&D Systems Inc.), and TNF-α (pg/mL; Cayman Chemical Company, Ann Arbor, MI, USA) levels.

② Clinical outcomes

The clinical outcomes measured were the local and systemic clinical effects of IRI IRI in both groups. Systemic clinical effects included non-cardiogenic pulmonary edema (assessed by the presence of dyspnea and bi-basal lung fine crepitation and bedside echocardiography), acute renal failure (assessed by the presence of oliguric renal failure despite adequate hydration and elevated serum creatinine/urea levels), myocardial reperfusion injury (determined by electrocardiographic evidence of standard term elevation myocardial infarction or non-ST elevation myocardial infarction and cardiac arrhythmia), and death. Local clinical sequelae included compartment syndrome (assessed via compartment pressure manometry when clinically determined) and major lower limb amputation (defined as amputation proximal to the ankle).

6) Statistical analysis

Sample size was calculated using equations, which are used for clinical trials that have a quantitative outcome or sample sizes that are likely to be small. Due to the absence of previous clinical data on the efficacy of insulin in skeletal muscle IRI, the present study was designed as a pilot clinical trial with randomized and controlled parallel groups. Variables were guided by previous clinical trials of insulin use in myocardial ischemia and of the use of highly sensitive surrogate serological markers of reperfusion injury. Twenty-four patients were recruited, with 12 in each group.

Continuous numerical data are presented as medians and interquartile ranges and were compared using the non-parametric Mann–Whitney U-test. Categorical variables were compared using Fischer's exact test.

Univariate analysis was performed to assess the impact of the independent variables on the primary endpoints. Linear regression analysis was used to test the association between serum insulin levels and the markers of reperfu-

sion injury. Variables that were found to be significantly associated with the dependent variable were inserted into the regression model as independent variables. To overcome the heterogeneity of baseline serum levels of reperfusion markers, the increment in the respective serum markers at different time points (when compared to the initial baseline value) was calculated and used as the outcome variable. Regression models with linear prediction were constructed using a stepwise approach that involved including significant correlations into the model to test the association between insulin levels and reperfusion markers. Any violation of the linear regression model was excluded via residual analysis prior to data analysis.

Table 1. Baseline characteristics of the study population

Characteristic	Control (n=12)	Insulin (n=12)	P-value
Sex, male	12	12	-
Age (yr)	61.8	64.6	0.332
Hypertension	9	5	0.090
Diabetes	2	2	-
Dyslipidemia	6	7	0.410
History of ischemic heart disease	5	4	0.654
Smoking	8	8	-
Active smoker	5	3	0.380
Ex-smokers	3	5	0.450
Level of arterial occlusion			
Aortic	6	7	0.640
Femoral	5	4	0.570
Popliteal	1	1	-

RESULTS

1) Characteristics of the study population

Twenty-four consenting patients were block-randomized into either a 'control' (n=12) or 'insulin' group (n=12).

Univariate analysis showed that the insulin and control groups were similar in age, comorbidities, duration of smoking, pack-years of smoking, nature of surgery, and level of ischemia (Table 1). The disease-related characteristics of the participants in the two groups are listed in Table 2 and 3. The baseline values of the markers of reperfusion injury were statistically similar between the control and insulin groups.

The median age of the study participants was 63.2 years, and all were male. Hypertension, ischemic heart disease, arrhythmia, dyslipidemia, and a history of smoking were common comorbidities and risk factors. All procedures involved open vascular intervention.

The onset of ischemia was defined as the time at which ischemic pain was first experienced. Median duration of ischemia between control and insulin groups was not statistically significant (174.5 vs. 190.3 min; P=0.323).

2) Insulin levels

Serum insulin levels were measured before insulin infusion commenced, during the perioperative period at 6 and 12 hours, and post-operatively. The median serum insulin level was significantly greater in the insulin group at 6 hours (31.8 vs. 21.9 mu/mL, P<0.01) and 12 hours (32.1 vs. 22.1 mu/mL, P<0.01). Insulin levels pre-infusion were similar in both groups (22 vs. 22.2 mu/mL, P>0.05).

Table 2. Disease-related characteristics of the control group population (n=12)

No.	Level of occlusion	Ischaemic status	Duration of ischemia (min)	Intervention
1	Femoral	Rutherford IIb	240	Femoral thromboembolectomy
2	Femoral	Rutherford IIb	90	Femoral thromboembolectomy
3	Femoral	Rutherford IIa	150	Femoral thromboembolectomy
4	Aorta	Rutherford IIa	105	Saddle thrombectomy
5	Aorta	Rutherford IIa	200	Bilateral femoral thromboembolectomy
6	Femoral	Rutherford IIb	65	Femoral thromboembolectomy
7	Aorta	Rutherford IIb	140	Saddle thrombectomy
8	Aorta	Rutherford IIa	315	Saddle thromboembolectomy
9	Aorta	Rutherford IIb	395	Saddle embolectomy
10	Popliteal	Rutherford IIb	155	Emergency bypass of thrombosed popliteal aneurysm
11	Femoral	Rutherford IIb	135	Femoral thromboembolectomy
12	Aorta	Rutherford IIb	105	Axillo-bifemoral bypass

Table 3. Disease-related characteristics of the insulin group population (n=12)

No	Level of occlusion	Ischaemic status	Duration of ischemia (min)	Intervention
1	Femoral	Rutherford IIb	315	Femoral thromboembolectomy
2	Aorta	Rutherford IIb	230	Saddle thrombectomy
3	Aorta	Rutherford IIa	140	Axillo-bifemoral bypass
4	Femoral	Rutherford IIa	80	Femoral thrombectomy
5	Aorta	Rutherford IIb	130	Saddle thrombectomy
6	Aorta	Rutherford IIb	144	Saddle thrombectomy
7	Femoral	Rutherford IIb	375	Femoral thromboembolectomy
8	Aorta	Rutherford IIb	240	Axillo-femoral and femoro-femoral bypass
9	Aorta	Rutherford IIa	135	Saddle thromboembolectomy
10	Aorta	Rutherford IIa	115	Saddle thromboembolectomy
11	Popliteal	Rutherford IIb	130	Popliteal thromboembolectomy
12	Femoral	Rutherford IIb	250	Femoral thromboembolectomy

Table 4. Baseline and 2- and 4-hours concentrations of reperfusion injury markers of the control (n=12) and insulin (n=12) groups

Reperfusion injury marker (pg/mL)	Baseline		2-hour level		4-hour level	
	Control group	Insulin group	Control group	Insulin group	Control group	Insulin group
P-selectin	52.02±4.9	63.93±7.5	101.12±5.5	78.22±9.4*	95.25±7.3	73.12±7.1#
Myeloperoxidase	115.92±3.8	139.74±8.5	172.22±8.3	150.72±7.3*	148.91±7.1	148.62±7.2#
TNF-alpha	12.89±1.8	14.89±2.5	38.12±5.3	21.73±5.3#	26.56±4.6	16.77±1.8#

All values are presented as mean±standard error of the mean; linear regression analysis was applied for statistical analysis.
TNF, tumor necrosis factor.
*P-value<0.05 and #P-value<0.001 when the increment of markers of reperfusion injury at the 2-hour and 4-hour intervals and baseline was compared in the two groups.

3) Temporal evolution of reperfusion markers

P-selectin, TNF-α, and MPO levels in the reperfusate were measured via the indwelling femoral vein catheter at 0, +2, +4, +6, and +12 hours after reperfusion. Peak concentrations were observed 2 hours post-reperfusion for all three markers. The baseline and peak values in the control and insulin groups are shown in Table 4.

The increase in P-selectin, MPO, and TNF-α levels was compared between the control and insulin groups using a linear regression model at the aforementioned time intervals. We found that all three markers were significantly attenuated in the 'insulin group' at the 2- and 4-hour intervals (P-selectin levels at 2 [P=0.001] and 4 [P=0.003] hours after reperfusion; MPO levels at 2 [P=0.001] and 4 [P=0.002] hours after reperfusion; and TNF-α levels at 2 [P=0.005] and 4 [P=0.01] hours after reperfusion).

4) Clinical outcomes

No clinically significant hypoglycemic events were observed during the study period. Capillary blood glucose levels and serum potassium concentrations were not significantly affected by the use of insulin alone or combined with glucose-potassium solution.

Clinically apparent local and systemic sequelae of IRI, that is, the secondary end points, were monitored in the control and insulin groups.

The mortality rates of the control and insulin groups were 1/12 (8.3%) and 0/12 (0%), respectively. A 70-year-old male who underwent thromboembolectomy for an aortic saddle embolus developed myocardial infarction and arrhythmias. No patient progressed to proximal amputation due to the development of compartment syndrome following reperfusion in either group. The unit employs a low-threshold policy for prophylactic fasciotomy in patients with neurological deficits. All patients underwent intraoperative fasciotomy.

In the control group, two patients developed acute kidney injury, three developed myocardial infarction, and three developed cardiac arrhythmias. Three clinical events were recorded in the 'insulin group', including one acute kidney

injury and one cardiac arrhythmia. However, there seems to be a trend towards lower morbidity with insulin.

DISCUSSION

Studies on the pathogenesis of skeletal muscle reperfusion injury, as well as trials attempting to ameliorate IRI, have been primarily based on in vitro and animal models. Despite the complex pathophysiology and lack of clear understanding of the intricacies involved in IRI, the pivotal role of leukocyte-endothelial interaction has been established in animal models by Springer et al. [22] and Carden et al. [23]. Our study is the first to involve human skeletal muscle in a clinical setting to demonstrate a significant increase in the levels of P-selectin, MPO, and TNF-α in the reperfusate, corresponding to an exaggeration of endothelial adhesiveness, leukocyte activation, and inflammatory status, respectively.

None of the many therapeutic agents targeting the pathophysiology of IRI have transcended the clinical barrier. However, insulin has demonstrated a potential to modulate the leukocyte-endothelial interaction during IRI in in vitro and pre-clinical studies [14,24]. Insulin is also the most used therapeutic agent in clinical trials on IRI. Its role in myocardial reperfusion has been extensively studied. The ECLA [25], CREATE-ECLA [25], REVIVAL [26], and IMMEADATE trials [26] are some of the main randomized clinical trials that examined the potential of insulin in IRI. Although the most recent meta-analysis did not demonstrate an overall mortality benefit, these RCTs on insulin use in myocardial reperfusion injury have demonstrated a clear reduction in infarct size and leukocyte-endothelial interaction [27,28]. These promising results prompted us to study the role of insulin in the amelioration of skeletal muscle IRI.

Insulin administration was based on recommendations by previous studies on myocardial IRI. Insulin has previously been administrated as part of a GIK regimen, and Dandona et al. [18], with an infusion of 2.5 U/h, achieved supra-physiological levels of insulin. The same dose, route of administration, and dosage demonstrated both safety and efficacy in studies conducted by Chaudhuri et al. [19]. Therefore, we adopted a similar method of insulin administration in the present study.

Ji et al. [13] suggested that hyperinsulinemia, rather than changes in glucose levels, is key to the therapeutic effect of GIK. Although potassium and dextrose were not routinely administered to patients in the control group, there was no significant difference in glucose or potassium levels between the control and insulin groups, with the only difference being the statistically significant increase in insulin levels.

The primary endpoints of the study were based on the levels of surrogate markers of IRI in the venous effluent. This study was powered by the presence of these highly sensitive markers.

The control group demonstrated a characteristic elevation in soluble P-selectin, MPO, and TNF-α levels in the venous effluent of a reperfused skeletal muscle. Peak elevation was observed between 2 and 4 hours of reperfusion. The increase in P-selectin levels indicates that there is an exaggeration of both endothelial 'stickiness' and the ensuing tissue leukocyte infiltration during IRI in skeletal muscle. The increase in MPO levels indicates enhanced neutrophil activation with the release of deleterious ROS. TNF-α is a crucial 'player' in the acute inflammatory pathogenetic process, and its exaggeration confirms that reperfusion is associated with a significant pro-inflammatory burst.

All three reperfusion markers were significantly attenuated in the venous effluent of the 'insulin group' compared to that of the 'control group' at both 2 and 4 hours post-reperfusion. This is the first study to demonstrate that insulin, at higher-than-normal serum concentrations, can attenuate the increase in P-selectin, MPO, and TNF-α levels. Our findings support the argument that supraphysiological insulin levels exert an inhibitory effect on endothelial-leukocyte activity in skeletal muscle reperfusion injury.

The study was powered based on the primary endpoints, that is, levels of highly sensitive surrogate markers, rather than clinical outcomes. Many confounding variables are known to affect selected clinical endpoints of morbidity and mortality. The present study was not powered to confidently exclude the impact of these confounding variables on the clinical endpoints. Therefore, although improved clinical outcomes were noted in the insulin group, these must be viewed in light of the effects of such variables.

1) Limitations

Our study has several limitations. A larger sample size would have improved the validity of the data. Acute lower limb ischemia is an emergency. Patients are often quite ill and in pain and have many comorbidities. Although a significant difference in baseline characteristics was not observed between the two groups in this study, the authors agree that many non-quantifiable confounding factors are likely to have been present. The lack of previous clinical data on thromboembolic acute limb ischemia meant that this study had to be conducted as a pilot clinical trial.

Although it would have been preferable to study more patients who presented with established acute limb ischaemia, this was not possible from a single institution within the context of the study. Furthermore, obtaining consent

from patients who presented acutely with significant pain and discomfort proved exceedingly difficult.

Clinicians who were blinded to the study protocol assessed the clinical (secondary) endpoints. All patients were placed in high-dependency or intensive care units, and trained intensivists performed around-the-clock monitoring. They interpreted data at their discretion and clinical judgment; therefore, the effect of interpersonal variation was unavoidable. While this would affect the sensitivity of the secondary endpoints, it may also mean that the clinical endpoints of our study reflect true, real-life situations that are more clinically relevant.

2) Future direction

Although not statistically significant, improved clinical outcomes were observed in the insulin group. The power of our study was based on the primary endpoints, that is, levels of highly sensitive surrogate markers of reperfusion injury, rather than clinical outcomes. Based on our results, we estimated that approximately 200 to 250 patients per arm would be required to study clinical outcomes. It would also be useful to study whether higher doses of insulin would improve outcomes without deleterious side effects.

Future studies to further elucidate the pathogenesis of IRI would aid the understanding of the mechanism by which insulin attenuates the neutrophil inflammatory response. Pre-clinical studies and clinical trials on skeletal muscle IRI have been based on pre-treatment with a potential therapeutic agent. While such studies are important to further understand the complex pathogenesis of IRI, pre-treatment is not a therapeutic option in acute lower limb ischemia, where the clinical presentation is often sudden and unpredictable. In our study, insulin was administered to patients with established acute limb ischemia. Our study results demonstrate that insulin has the potential of being used as a therapeutic intervention in the clinical setting of acute limb ischemia.

CONCLUSION

The present study demonstrated that skeletal muscle IRI in humans is associated with an exaggeration of P-selectin, TNF-α, and MPO levels in the venous effluent, which are surrogate markers of endothelial-leukocyte activity. The use of soluble insulin at a dose of 2.5 U/h significantly reduced the levels of these markers in the venous effluent.

The ability of insulin to inhibit the increase of these highly sensitive markers supports the argument that insulin, at a higher-than-normal serum concentration, may be a potential candidate for therapeutic manipulation in skeletal muscle reperfusion injury in a clinical setting.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHOR CONTRIBUTIONS

Concept and design: all authors. Analysis and interpretation: all authors. Data collection: TDG. Writing the article: all authors. Critical revision of the article: all authors. Final approval of the article: all authors. Statistical analysis: TDG, SMW. Overall responsibility: all authors.

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