Published in final edited form as:

Pharm Res. 2013 May ; 30(5): 1338–1348. doi:10.1007/s11095-013-0973-z.

Brain extracellular γ -hydroxybutyrate concentrations are decreased by L-lactate in rats: Role in the treatment of overdoses

Samuel A. Roiko, Nisha Vijay, Melanie A. Felmlee, and Marilyn E. Morris

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Buffalo, New York 14214-8033, USA

Abstract

Purpose—L-lactate represents a potential treatment for GHB overdose by inhibiting GHB renal reabsorption mediated by monocarboxylate transporters. Our objective was to assess the dose-dependence of L-lactate treatment, with and without D-mannitol, on GHB toxicokinetics/toxicodynamics (TK/TD).

Methods—Rats were administered GHB 600 mg/kg i.v. with L-lactate (low and high doses), D-mannitol, or L-lactate (low dose) with D-mannitol. GHB-induced sleep time and GHB plasma, urine and brain extracellular fluid (ECF) concentrations (by LC/MS/MS) were determined. The effect of L-lactate and D-mannitol on the uptake and efflux of GHB was assessed in rat brain endothelial RBE4 cells.

Results—L-lactate treatment increased GHB renal clearance from 1.4 ± 0.1 ml/min/kg (control) to 2.4 ± 0.2 and 4.7 ± 0.5 ml/min/kg after low and high doses, respectively, and reduced brain ECF AUC values to 65 and 25% of control. Sleep time was decreased from 137 ± 12 minutes (control) to 91 ± 16 and 55 ± 5 minutes (low and high L-lactate, respectively). D-mannitol did not alter GHB TK/TD and did not alter L-lactate's effects on GHB TK/TD. L-lactate, but not D-mannitol, inhibited GHB uptake, and increased GHB efflux from RBE4 cells.

Conclusions—L-lactate decreases plasma and brain ECF concentrations of GHB, decreasing sedative/hypnotic effects.

Keywords

γ -hydroxybutyrate; monocarboxylate transporter; microdialysis; lactate; overdose

Introduction

γ -hydroxybutyrate (GHB) is an endogenous metabolite of γ -aminobutyric acid (GABA) and a putative neurotransmitter or neuromodulator with its own receptor (1, 2). GHB is used therapeutically, currently marketed under the trade name Xyrem® (Jazz Pharmaceuticals, Palo Alto, CA) for narcolepsy with cataplexy and is also available as a treatment for alcohol

Reprint requests should be submitted to: Marilyn E. Morris, Department of Pharmaceutical Sciences University at Buffalo, State University of New York, 352 Kapoor Hall, Buffalo, NY 14214-8033, memorris@buffalo.edu.

Authorship Contributions

Participated in research design: Morris, Roiko, Vijay, and Felmlee

Conducted experiments: Roiko, Vijay, and Felmlee

Contributed new reagents or analytic tools: Morris, Roiko, Vijay, and Felmlee

Performed data analysis: Roiko

Wrote or contributed to the writing of the manuscript: Morris, Roiko, Vijay, and Felmlee

withdrawal in Europe (3). However, the illicit use of GHB overshadows its therapeutic potential.

The illicit use and abuse of GHB and its precursors 1,4 butanediol and γ -butyrolactone (which are rapidly converted into GHB in the body) has been widely documented. Body builders use these compounds for their anabolic steroid-like effects (4). GHB is also used as a club drug due to its euphoric effects, and in drug-facilitated sexual assault because of its sedative/hypnotic and amnesic effects (5, 6). As the use and abuse of GHB has increased, so have cases of GHB overdose and death (7, 8). GHB toxicity is characterized by dizziness, respiratory depression, vomiting, coma, and death (9). There are currently no approved treatments for GHB toxicity other than supportive care.

GHB is a substrate for the proton-coupled monocarboxylate transporter 1 (MCT1), and also the isoforms MCT2 and MCT4 (10, 11). MCT1 and MCT2 are found throughout the body, including the kidney in the renal proximal tubules and the vascular endothelium at the blood-brain barrier (BBB) and can transport substrates in a bidirectional manner depending on proton concentration gradients (12, 13). Endogenous substrates for MCTs include lactate, pyruvate, and ketone bodies such as β -hydroxybutyrate and acetoacetate (14). Recent findings indicate that L-lactate inhibits MCT1-mediated reabsorption of GHB in the renal proximal tubule, increasing the renal clearance and reducing the sedative/hypnotic effects of GHB (15), suggesting that L-lactate may be a potential treatment for GHB overdose.

The sedative/hypnotic effects of GHB seen during toxicity and overdose are due to its effects in the brain, mediated (at least in part) by the GABA_B receptor (16, 17). Recent studies in our laboratory used brain microdialysis to measure free GHB concentrations in rat frontal cortex, indicating that GHB exhibits MCT-mediated uptake into the frontal cortex, which would be saturated at high GHB concentrations (18). In addition, GHB concentrations at the *offset* of its sedative/hypnotic effect (return of righting reflex, RRR) are equivalent across doses in frontal cortex ECF, plasma, and whole brain (19), indicating a relationship between GHB concentrations at these sites and sedative/hypnotic effects. The actual GHB concentrations in plasma, brain frontal cortex or frontal cortex ECF differed from one another, but the higher plasma concentrations were not due to plasma protein binding, since binding is negligible for GHB (20). Since MCT1 is present on brain microvessel endothelial cells at the BBB, we hypothesized that MCT1 inhibition with L-lactate would alter GHB brain distribution and serve as an additional site of action in treating GHB overdose.

The use of D-mannitol as an osmotic diuretic and its effects on the BBB have been well-documented (21). Previous work suggested that combining D-mannitol with Lactated Ringer's injection could increase the renal clearance and decrease the sleep time of GHB (1000 mg/kg), when Lactated Ringer's injection alone or D-mannitol alone had no effect on the TK/TD of GHB (15). The mechanism underlying this reported additive effect on GHB TK is unknown. We further explored this interaction by studying the effects of L-lactate and D-mannitol alone and in combination on GHB brain ECF concentrations *in vivo* using brain microdialysis, and on the TK/TD of GHB. In addition, the effects of these treatments on GHB transport at the BBB were further explored *in vitro* by characterizing the uptake and efflux of GHB from cultured rat brain endothelial cells.

Materials and Methods

Chemicals

γ -hydroxybutyrate (sodium salt) was obtained from NIDA (Rockville, MD). Ketamine, xylazine, buprenorphine, and carprofen were purchased from Butler Schein (Melville, NY). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston,

MA). Sodium L-lactate and alpha-cyano-4-hydroxycinnamate (CHC) were purchased from Sigma-Aldrich (St. Louis, MO), and D-mannitol was obtained from Fisher Scientific (Pittsburgh, PA). For LC/MS/MS analysis, formic acid was purchased from Sigma-Aldrich (St. Louis, MO), deuterated GHB (GHB-d6) was purchased from Cerilliant (Round Rock, TX), and HPLC-grade acetonitrile, methanol, water, and acetic acid were purchased from Honeywell Burdick and Jackson (Morristown, NJ).

Animals and surgery

All animal procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (280–320 g) were used in all studies and surgeries were performed as previously described (19). Briefly, rats were anesthetized with ketamine/xylazine (90/9 mg/kg), implanted with jugular and femoral vein cannulae, and then mounted in a stereotaxic frame (Harvard Apparatus, Holliston, MA). Microdialysis guide cannulae (CMA 11 guide cannulae, CMA Microdialysis, North Chelmsford, MA) containing dummy probes were implanted in the frontal cortex (AP + 3.2mm and ML $\pm$ 2.5 mm from Bregma, and DV –0.5mm from dura (22). The cannula was fixed in place and buprenorphine (0.05 mg/kg s.c.) and 0.9% saline (6 ml s.c.) were administered immediately post-surgery, and carprofen 5 mg/kg s.c. was administered daily for two days post-surgery. Rats were allowed at least 6 days for recovery prior to microdialysis probe implantation and conduct of the experiment.

Microdialysis probe implantation, retrodialysis, and sample collection

Microdialysis probes (CMA11, CMA Microdialysis, North Chelmsford, MA) were prepared as described by manufacturer's instructions, and implanted 24 hours prior to each experiment, which allows the blood-brain barrier to reform post-implantation (23). Experiments were conducted in metabolic cages using awake and freely moving rats with ad libitum access to water. The experimental procedure is shown in Figure 1. On the day of the experiment, microdialysis probes were perfused with aCSF at a flow rate of 2 μ l/min and allowed to stabilize for 2 hours. Microdialysate fractions were collected every 20 minutes. After stabilization, the probe was perfused with GHB 1 μ g/ml in aCSF for 80 minutes to calculate in vivo probe recovery of GHB for each rat using the established retrodialysis method (24). After retrodialysis, blank aCSF was perfused through the probe for 80 minutes to remove any residual GHB (washout). Although GHB is endogenous, no GHB was detected in the final 2 washout samples. Rats were then administered GHB (600 mg/kg i.v., same GHB dose in all studies) and corresponding treatments were administered as described below. Plasma and dialysate samples were collected for 6 hours post-dose, and times of loss of righting reflex and return to righting reflex (LRR and RRR) were recorded for each rat. Six hours after GHB administration, rats were sacrificed and the probe tracks were stained with dye. Brains were collected to confirm probe location and to ensure probe track condition. Blood samples were centrifuged at 1000 g for 15 min at 4°C, and plasma, urine, and dialysate samples were stored at –80°C until analysis. Rat exclusion criteria included highly inflamed probe tracks as determined by visual observation, poor retrodialysis recovery (< 5%), and GHB concentration-time profiles with concentrations that were greater than 3 standard deviations from group means. Following these criteria resulted in the exclusion of at least 2, but no more than 4, rats in each treatment group. Data presented are from rats that met the criteria.

Experimental study design

The effects of L-lactate on GHB brain ECF concentrations and sleep-time—To determine the dose-dependent effects of L-lactate administration on GHB brain ECF concentrations and sleep-time, rats (n = 3/group) were prepared as described above and were

administered GHB 600 mg/kg i.v. combined with L-lactate as an i.v. bolus followed by an i.v. infusion (low dose, bolus 66 mg/kg, infusion 302.5 mg/kg/hr; high dose, bolus 330 mg/kg, infusion 605 mg/kg/hr) 5 minutes after GHB administration. This lactate dose was calculated to elevate steady-state blood lactate concentrations from 1.5 to 2.5 mM for the low dose and above 4 mM for the high dose. Data from rats administered GHB 600 mg/kg alone was previously published (18) and used here for comparison purposes.

Effects of Mannitol on L-lactate administration and GHB TK/TD—The goal of this study was to determine if the administration of D-mannitol altered the effects of L-lactate on GHB TK/TD. Rats were prepared as above (n = 3/group) and administered either D-mannitol (500 mg/kg i.v.) alone or combined with L-lactate low dose (bolus 66 mg/kg, infusion 302.5 mg/kg/hr) 5 minutes after administration of GHB 600 mg/kg i.v.. Times for LRR and RRR were recorded and kinetic parameters determined; values were compared with rats treated with GHB alone and GHB + L-lactate low dose.

L-lactate pharmacokinetics in the presence and absence of GHB—In conjunction with the above studies, we characterized L-lactate plasma concentrations after a saline bolus, a saline bolus and infusion, and the administration of GHB 600 mg/kg, to determine if GHB administration or a saline infusion alters baseline L-lactate plasma concentrations. Rats were implanted with jugular and femoral cannulae and after 5 days of recovery, GHB or saline (vehicle) was administered and blood samples were collected over 6 hours. Plasma was obtained and stored at -80°C until L-lactate concentrations were measured. L-lactate was measured in plasma samples obtained from rats with microdialysis probes implanted that were treated with GHB alone, GHB + L-lactate (low and high doses), and GHB + L-lactate (low dose) + D-mannitol. Lactate concentrations were measured using the 1500 Sport Lactate Analyzer (YSI Inc, Yellow Springs, OH).

Plasma, microdialysate and urine sample preparation and LC/MS/MS analysis

Plasma, microdialysate and urine samples were all prepared as previously described (19, 25). Briefly, plasma (50 μl) was combined with 5 μl of GHB-d6 (1 mg/ml) and GHB stock solution (or double-distilled water for samples). Acetonitrile was added to plasma samples to precipitate proteins and the samples centrifuged. Double-distilled water was added to the supernatant and sample extraction was performed using Bond Elut SAX cartridges (Varian, Palo Alto, CA), prepared as described previously (19). Samples were reconstituted in 1.25 ml 0.1% formic acid in double-distilled water and 5% acetonitrile. For rats in the high dose lactate group and the low dose L-lactate + D-mannitol group, a separately validated modification of the sample preparation protocol was incorporated, omitting the solid-phase extraction step to simplify plasma sample preparation (detailed in (17)). Briefly, plasma proteins were precipitated using 0.1% formic acid in acetonitrile, samples were vortexed, and then centrifuged at 10,000g for 20 minutes. Supernatant was then evaporated under a stream of nitrogen gas and samples were reconstituted in aqueous mobile phase. Microdialysis samples were diluted with aCSF to bring concentrations within the range of the standard curve. GHB-d6 (5 μl of 5 $\mu\text{g}/\text{ml}$) was added to 35 μl of microdialysate sample or standard and injected directly onto the LC/MS/MS. Urine samples (50 μl) were prepared for analysis by adding GHB-d6 (10 μl of 200 $\mu\text{g}/\text{ml}$), double-distilled water and acetonitrile; samples were centrifuged and the supernatant was collected for LC/MS/MS analysis.

For all LC/MS/MS analyses, an Agilent 1100 series HPLC with an online degasser, binary pump and autosampler (Agilent Technologies, Palo Alto, CA) linked to a PE Sciex API triple-quadrupole tandem mass spectrometer with a turbo ion spray (Applied Biosystems, Foster City, MA) was used. HPLC conditions, mass spectrometer parameters, linear calibration ranges and accuracy/precision are detailed in (19).

GHB cell uptake studies

The immortalized rat brain capillary endothelial cell line (RBE4) was kindly provided by Professor P. Couraud (Institut Cochin, Paris). RBE4 cells (passages 39 – 44) were cultured as described previously (18). Briefly, cells were grown at 37°C with 5% CO₂ on Type-I collagen-coated flasks, and media was changed every 2–3 days. RBE4 culture media was 1:1 alpha-minimum essentials medium/Hams F10 nutrient mixture supplemented with L-glutamine (2.0 mM), geneticin (300 µg/ml), human recombinant fibroblast growth factor (1 ng/ml), gentamicin (50 µg/ml), and 10 % v/v qualified fetal bovine serum (FBS). Cells were passaged with 0.25% trypsin/EDTA and seeded on individual collagen-coated 35 × 10 mm wells for uptake and efflux studies, conducted 7 days post confluence (the time needed for formation of tight junctions)(26).

Uptake studies—For lactate inhibition studies in RBE4 cells, cells were washed and equilibrated for 30 minutes at 37°C with uptake buffer containing 138 mM NaCl, 1.8 mM CaCl₂, 5.4 mM KCl, 0.8 mM MgSO₄, 1.0 mM Na₂HPO₄, 5.5 mM D-glucose, and 20 mM HEPES (pH 7.4). Cells were then equilibrated to room temperature for five minutes and subsequently incubated for 15 seconds with 10 mM [³H]-GHB alone or with 1, 5, or 10 mM L-lactate or 10 or 100 mM D-mannitol. Previous work indicated that 15 seconds is within the time of linear uptake of GHB in RBE4 cells (18). A concentration of 10 mM GHB was used in these studies since it is similar to the high concentrations of GHB observed in rats after 600 mg/kg GHB i.v., and 1, 5, and 10 mM concentrations of L-lactate were used, as 1 mM approximates baseline L-lactate concentrations, 5 mM is a high L-lactate concentration reached after dosing (see Figure 3), and 10 mM represents a very high L-lactate plasma concentration. The D-mannitol concentrations were chosen since 10 mM represents a low D-mannitol concentration, and 100 mM is a maximal concentration calculated to be reached after infusion of 1 M D-mannitol (a clinical dosing regimen), assuming D-mannitol only distributes into extracellular fluid. After incubation, cells were washed 3 times with ice-cold buffer and lysed with 0.5 ml 1.0 N NaOH for 60 minutes at room temperature. After lysis, NaOH was neutralized with 0.5 ml 1.0 N HCl. [³H]-GHB accumulation was determined by liquid scintillation counting, and protein concentration was measured using bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. GHB uptake was normalized per mg of protein.

Efflux studies—The time course of GHB efflux and the effects of treatments on GHB efflux were determined. Confluent RBE4 cells on individual collagen-coated wells were incubated with 10 mM [³H]-GHB for 30 minutes at 37°C and subsequently allowed to equilibrate to room temperature (5 minutes). GHB-containing uptake buffer was removed and 1 ml uptake buffer was applied for 15 and 30 seconds and 1, 2, 5, and 10 minutes. At the designated time point, the uptake buffer was removed and the reaction was stopped with 3 washes of ice-cold uptake buffer, followed by cell lysis using 1.0 N NaOH. 1 N HCl was added to neutralize the NaOH and samples were aliquoted for liquid scintillation counting and analysis of protein expression using the bicinchoninic acid assay. The amount of remaining intracellular GHB was normalized per mg of protein. The effects of 100 mM D-mannitol, 10 mM L-lactate (alone and combined), 3 and 15 mM GHB with or without 10 mM L-lactate, and 2.5 mM α-cyano-4-hydroxycinnamate (CHC) (negative control) on the amount of GHB efflux were determined by applying these solutions (prepared in uptake buffer) for 0.5 and 2 minutes after removal of GHB-containing uptake buffer (onset of efflux). Three separate studies were performed with triplicate determinations in each study.

Data and statistical analysis

To calculate the relative *in vivo* recovery of GHB from the dialysis probe, retrodialysis recovery was used as described previously (19). The recovery of GHB from the probe was calculated using the equation below:

$$\text{Recovery}_{\text{Probe}} = \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{C_{\text{perfusate}}} \quad \text{Equation 1}$$

where $C_{\text{perfusate}}$ is the perfusate GHB concentration (inlet) and $C_{\text{dialysate}}$ is the dialysate GHB concentration (outlet). To determine GHB concentrations in frontal cortex ECF, dialysate GHB concentrations after i.v. administration of GHB were divided by the experimentally determined GHB probe recovery.

The area under the concentration-time curve (AUC) for GHB in plasma and brain ECF was calculated by the log-linear trapezoidal method using Phoenix WinNonlin 6.0 (Pharsight Corp., Mountain View, CA). Time-averaged renal clearance was calculated by dividing the total amount of GHB excreted in urine ($A_{e,inf}$) by the GHB plasma AUC. Overall unbound brain ECF/plasma partition coefficients for GHB ($K_{p,u,u}$) were calculated by dividing the brain GHB ECF AUC by the GHB plasma AUC. GHB is not bound to plasma proteins, so plasma concentrations represent unbound concentrations (20). Sleep time (minutes) was determined as the difference between RRR and LRR. GHB concentrations in ECF and plasma at RRR were calculated using noncompartmental methods. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Inc., San Diego, CA). Statistical differences between group means were assessed using one-way ANOVA with nonparametric analysis (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Results

The effects of L-lactate and D-mannitol administration on GHB TK/TD

Toxicokinetics—Plasma and frontal cortex extracellular GHB concentration-time profiles collected during *in vivo* microdialysis after administration of GHB 600 mg/kg alone or in the presence of two L-lactate dosing regimens (low dose, bolus 66 mg/kg, infusion 302.5 mg/kg/hr and high dose, bolus 330 mg/kg, infusion 605 mg/kg/hr) are shown in Figure 2. In addition, the osmotic diuretic D-mannitol was administered alone or combined with low dose L-lactate (Figure 2). When low dose L-lactate was administered, GHB plasma concentrations were substantially reduced beginning 90 minutes after GHB administration (Figure 2A). L-lactate also elicited a decline in GHB frontal cortex ECF concentrations 180 minutes post-GHB administration (Figure 2B). The administration of D-mannitol alone did not significantly alter GHB plasma or frontal cortex ECF concentration-time profiles, although a trend towards higher GHB ECF concentrations was observed. The combined administration of D-mannitol with L-lactate did not alter the effect of L-lactate alone on GHB plasma or ECF concentration-time profiles. Administration of the high L-lactate dose resulted in a substantial decrease in both plasma and ECF GHB concentrations. Plasma GHB concentrations in the presence of the high lactate dose were lower than other treatment groups 90 minutes post-GHB administration (Figure 2A), and average ECF GHB concentrations were lower immediately (midpoint of 10 minutes, during the first fraction collected (Figure 2B), although changes were not statistically different.

Noncompartmental analysis of the GHB plasma and brain ECF concentration-time profiles after the administration of L-lactate as low or high doses, D-mannitol alone, or combination of low-dose L-lactate and D-mannitol are shown in Table I. Administration of low-dose L-lactate increased GHB time-averaged renal clearance and reduced GHB plasma AUCs with

a trend towards lower ECF AUCs. This was reflected in similar brain:blood partition coefficients, as seen with GHB alone. The high L-lactate dose resulted in an even greater increase in GHB time-averaged renal clearance, as observed by the high fraction of the GHB dose excreted in urine, which reduced GHB plasma AUCs and significantly reduced GHB ECF AUCs and GHB brain:blood partitioning. D-mannitol alone did not significantly alter GHB renal clearance or GHB plasma and ECF AUCs.

Toxicodynamics—The low dose of L-lactate significantly reduced sleep-times after GHB administration from 137 minutes to 90 minutes (Table I, $p < 0.05$). Plasma and brain ECF concentrations of GHB at RRR were unchanged with the administration of this L-lactate dose. The high dose of L-lactate resulted in higher plasma concentrations of GHB at RRR ($p < 0.05$), but ECF GHB concentrations at RRR were unchanged. D-mannitol alone did not significantly alter GHB-induced sleep times compared to GHB alone. D-mannitol combined with L-lactate reduced sleep times from 137 minutes to 99 minutes, similar to the effect of L-lactate alone (Table I, $p < 0.05$). Brain plasma and ECF concentrations of GHB at RRR when D-mannitol was administered alone or with L-lactate were higher but not significantly different than those of GHB alone.

L-lactate pharmacokinetics after GHB and L-lactate administration

L-lactate concentrations were measured in rats administered a bolus of GHB or a bolus and infusion of saline to determine if GHB or a saline infusion altered L-lactate concentrations. GHB administration did not significantly alter baseline L-lactate concentrations (Data not shown). A saline bolus or a saline bolus plus infusion did not alter baseline L-lactate concentrations, and L-lactate plasma concentration-time data from the saline treatments and GHB 600 mg/kg i.v. were combined and compared to the effects of L-lactate administration.

The administration of the low dose of L-lactate (bolus 66 mg/kg, infusion 302.5 mg/kg/hr) resulted in a moderate increase in plasma L-lactate concentrations, from baseline concentrations of 1.5 mM to a value of 2.5 mM. Plasma L-lactate concentrations slightly declined to 2 mM during the final 4 hours of sampling. D-mannitol did not alter L-lactate plasma concentrations after the low dose, and the L-lactate plasma concentrations from both groups were combined. Administration of the high dose of L-lactate (bolus 330 mg/kg, infusion 605 mg/kg/hr) elicited a rapid increase in plasma lactate concentrations to 6.5 mM, and remained above 4 mM for 90 minutes, after which concentrations declined to about 3 mM for the remainder of the study.

Effects of L-lactate and D-mannitol on GHB uptake *in vitro*

Since MCT1 is highly expressed at the BBB (13), we examined the effects of L-lactate on the uptake of 10 mM GHB in RBE4 cells (which also expresses MCT1, (27)) as an *in vitro* model of the rat BBB. This concentration of GHB was used since it is near the K_m for uptake via MCT1 at pH 7.4 (Morse et al. 2012), and is similar to GHB plasma concentrations after the i.v. administration of 600 mg/kg GHB. When L-lactate was applied at a concentration of 5 or 10 mM, the uptake of 10 mM GHB was inhibited to 74% and 64% of control (Figure 3A, $p < 0.05$). When D-mannitol was added at concentrations of 10 and 100 mM acutely (15 seconds prior to GHB) or for a longer period (30 minutes preincubation at 37° C), the uptake of 10 mM GHB was not changed (Figure 3B).

GHB Efflux and effects of L-lactate, D-mannitol, and GHB *in vitro*

To further study the transport of GHB at the BBB, we conducted efflux studies in RBE4 cells to determine if D-mannitol or L-lactate altered GHB efflux. The time-course for GHB efflux from RBE4 cells is shown in Figure 4A. The time-points of 0.5 and 2 minutes after the onset of efflux were chosen to study the effects of treatments on GHB efflux from RBE4

cells. The addition of 100 mM D-mannitol increased the efflux of GHB at 0.5 minutes such that the remaining intracellular GHB was 78% of control ($p < 0.05$ compared to control); there was no effect at 2 minutes. The addition of 10 mM L-lactate resulted in a significant increase in GHB efflux (remaining intracellular GHB was 55% of control, $p < 0.05$), and combining D-mannitol with L-lactate also resulted in increased GHB efflux (intracellular GHB remaining was 38% of control, $p < 0.05$). To determine if GHB alone could increase its own efflux, we added unlabeled GHB at concentrations of 3 and 15 mM and observed increased GHB efflux (intracellular GHB remaining was 56% and 47% of control, $p < 0.05$), which also was further increased when L-lactate was added with GHB (all to less than 38% of control, $p < 0.05$). The MCT inhibitor CHC produced a modest but significant increase in GHB efflux at 0.5 minutes, but not at 2 minutes.

Discussion

L-lactate and GHB are both substrates for MCT1, a transporter present in the renal proximal tubule that is largely responsible for GHB renal reabsorption. Previous studies demonstrated that L-lactate increases GHB renal clearance and reduces GHB-induced sleep time (15, 19, 20). The work here extends the previous findings to study the dose-dependent effects of L-lactate administration on GHB TK/TD *in vivo* in rats, including effects on GHB frontal cortex ECF concentrations, and characterizes the mechanisms underlying GHB transport in an *in vitro* model of the rat BBB. Our results demonstrate that L-lactate administration dose-dependently increased GHB renal clearance with higher doses resulting in greater reductions in GHB plasma and frontal cortex ECF concentrations and reduced GHB sleep time.

Since MCT1 is present at the BBB (13) and is largely responsible for GHB brain uptake (18, 28), we hypothesized that L-lactate administration would inhibit GHB distribution into the brain. Our results indicated that a low dose of L-lactate (66 mg/kg bolus, 302.5 mg/kg/hr infusion) increased GHB renal clearance and reduced GHB exposure as indicated by plasma and brain ECF AUCs, but the overall brain:blood partition coefficient was not changed (Table I). This indicates no change in GHB transport at the BBB, and that GHB brain concentrations correlate to plasma concentrations, as demonstrated previously (18, 19). The plasma and brain ECF GHB concentrations at RRR were unchanged from GHB alone, consistent with previous findings that plasma and brain GHB concentrations correlate with RRR (18, 19). The high dose of L-lactate (330 mg/kg bolus, 605 mg/kg/hr infusion) resulted in a significant increase in GHB renal clearance and in the total fraction of the GHB dose excreted in urine (Table I). The GHB plasma AUC was not significantly reduced beyond that of the low L-lactate dose. However, the brain ECF AUC was significantly reduced, and GHB blood-brain partitioning was significantly lower as well, suggesting that GHB transport at the BBB was altered in the presence of the high L-lactate dose. This resulted in a significant reduction in GHB sleep time. The ECF GHB concentration at RRR was not changed; however, the plasma GHB concentration at RRR was significantly higher, consistent with the hypothesis that GHB concentrations in the frontal cortex ECF are more closely connected with GHB's effects in the brain.

The low dose of L-lactate administered in this study increased plasma L-lactate concentrations by 1 mM, which remained fairly stable throughout the 6 hour sampling period. The high dose of L-lactate produced plasma L-lactate concentrations of approximately 5 mM above baseline which declined to 1.5 mM above baseline by 120 minutes. Recent findings from our laboratory indicated that GHB itself may alter L-lactate plasma concentrations when GHB is administered at high doses (17); however, this was not observed at the dose level used in the present study. In the present study, we observed a greater effect of L-lactate on renal elimination, while effects on BBB transport were only observed with the high L-lactate dose. These findings can be attributed to the differences in

the pH gradients that are present in the renal tubules and at the BBB. MCT1 is dependent on the presence of a proton gradient and affinity of substrates for this transporter will vary, dependent on this gradient. The K_m for MCT1-mediated GHB transport shifts from 2.2 to 17 mM when the extracellular pH is raised from 6.5 to 7.4 (29). Similarly, while 2 mM L-lactate significantly inhibits GHB transport in rat kidney vesicles at pH 5.5 (30), the IC_{50} for L-lactate inhibition of GHB uptake is 19.1 mM in red blood cells at pH 7.4, with no inhibition observed when L-lactate concentrations are less than 5 mM (29). Therefore, L-lactate has a greater affinity towards inhibiting renal reabsorption in the renal proximal tubule lumen, where there is a pH gradient from lumen to blood. At the physiological pH of 7.4 present in blood perfusing the BBB, it would be expected that only very high concentrations of L-lactate would significantly inhibit MCT1-mediated GHB uptake into the brain, which is consistent with our current findings.

In vitro studies were conducted using RBE4 cells as an *in vitro* model of the rat BBB (31) to characterize the BBB transport of GHB. We previously characterized the time- and concentration-dependent uptake in RBE4 cells at pH 7.4 (18). Here, the uptake of 10 mM GHB was inhibited to 75% of control when L-lactate 5 mM was added, and inhibited to 64% of control with 10 mM L-lactate. When RBE4 cells were preincubated with GHB, both L-lactate and GHB increased GHB efflux from the cells. Since MCTs are bidirectional (32), these findings suggest that *trans* stimulation plays a role in increasing GHB efflux. The *in vivo* setting is likely complex, where multiple processes and transporters may be involved. It is probable that both the inhibition of GHB uptake and stimulation of GHB efflux play a role in the alteration of GHB BBB transport when high L-lactate concentrations are present.

The osmotic diuretic D-mannitol has been demonstrated to reduce intracranial pressure via increasing BBB permeability due to vasodilation and shrinkage of endothelial cells, thereby affecting BBB uptake (reviewed in (21)). Previous work from our laboratory had demonstrated that a bolus and infusion of 10% w/v D-mannitol reduced steady state plasma GHB concentrations and increased GHB renal and total clearances (20). Additionally, we previously reported that the co-administration of the osmotic diuretic D-mannitol, at the dose used in the present study (0.5 g/kg), enhanced the effects of Lactated Ringer's injection (containing 28 mM L-lactate) on GHB TK/TD *in vivo* by increasing the renal and total clearances of GHB following the administration of 1000 mg/kg GHB i.v. (15). However, it was not clear by what mechanism D-mannitol enhanced the effects of Lactated Ringer's injection. In the same study, D-mannitol 0.5 g/kg i.v. did not significantly affect GHB TK, beyond the effects of L-lactate alone (60 mg/kg/hr), when GHB was infused (208 mg/kg/hr) to steady state concentrations of 0.62 mg/ml. Here, experiments were performed using brain microdialysis *in vivo* and with rat brain endothelial RBE4 cells in order to gain mechanistic information regarding the effect of mannitol on GHB disposition and understand the controversial findings. D-mannitol itself had no significant effect on GHB TK/TD, and no effect on GHB TK/TD was observed after administration of D-mannitol with a low dose of L-lactate *in vivo*, as previously reported by Wang et al (15). The effects of Lactated Ringer's injection on GHB may be related to the higher dose of GHB administered in that study (1000 mg/kg compared with 600 mg/kg in the present study), or to the use of L-lactate in the form of Lactated Ringer's injection, or possibly due to the larger volume of fluid administered when L-lactate was administered as Lactated Ringer's injection (300 μ l/min). Therefore, further studies are needed to evaluate the effects of D-mannitol when administered in combination with Lactated Ringer's injection on GHB TK/TD. Our *in vitro* studies demonstrated that the uptake or efflux of GHB from rat brain endothelial RBE4 cells was not significantly altered by D-mannitol. The *in vitro* findings are not surprising, given the role of D-mannitol in increasing BBB permeability via opening of tight junctions rather than facilitating intracellular transport. GHB ECF concentrations did trend higher in the presence of D-mannitol compared to GHB alone, consistent with increased paracellular

transport of GHB into the brain. However, based on our current studies, we were not able to confirm a substantial effect of mannitol on GHB TK/TD.

GHB overdose results in coma, sedation, respiratory depression, and death, most likely due to its actions at GABA_B receptors in the brain (16). One potential treatment for GHB overdose is the administration of L-lactate to increase GHB's renal clearance. Data here suggest that if sufficiently high L-lactate concentrations are reached, GHB-induced sedative/hypnotic effects can be overcome through the inhibition of renal reabsorption as well as alterations in GHB transport at the BBB. The highest L-lactate concentrations obtained in this study (5 mM) are physiologically relevant values, since endogenous L-lactate plasma concentrations of 10 mM are reached after at least 20 minutes of moderate-intensity exercise (33). The exogenous administration of L-lactate has been demonstrated to have both inhibitory and stimulatory effects on respiration and may also result in electrolyte imbalances (34). As respiratory depression is the primary factor resulting in GHB fatality, it is critical to understand the impact of L-lactate on this measure. Previous work in our laboratory has demonstrated that L-lactate administered alone targeting an increase of 2 mM in plasma L-lactate concentrations results in no measurable effects on respiration (17) or electrolyte balance (15). Similarly, no adverse effects were measured when sodium lactate (1/6 M solution) was administered to humans co-administered GHB (35). These results suggest that treatment with L-lactate may represent a safe and efficacious therapeutic strategy for the treatment of GHB overdose.

In summary, we demonstrated that L-lactate dose-dependently increased GHB renal clearance and reduced GHB sedative/hypnotic effects. The use of microdialysis *in vivo* demonstrated that brain ECF GHB concentrations provided a direct correlation with the offset of GHB's sedative/hypnotic effect (RRR). Studies of GHB transport *in vitro* were consistent with the *in vivo* data suggesting alterations in BBB transport with high L-lactate concentrations. The findings here further support the role of L-lactate as a potential treatment for GHB overdose.

Acknowledgments

This work was supported by the National Institutes of Health National Institute on Drug Abuse [grant DA023223]. NV was funded by a fellowship from Pfizer Global Inc.

Abbreviations

aCSF	artificial cerebrospinal fluid
AUC	area under the plasma concentration-time curve
BBB	blood-brain barrier
CHC	α -cyano-4-hydroxycinnamate
Cl_r	renal clearance
ECF	extracellular fluid
GABA	gamma-aminobutyric acid
GHB	γ -hydroxybutyrate
MCT	monocarboxylate transporter
TK/TD	toxicokinetics/toxicodynamics

References

1. Andriamampandry C, Taleb O, Kemmel V, Humbert JP, Aunis D, Maitre M. Cloning and functional characterization of a gamma-hydroxybutyrate receptor identified in the human brain. *Faseb J*. 2007; 21(3):885–95. Epub 2007/01/02. [PubMed: 17197387]
2. Andriamampandry C, Taleb O, Viry S, Muller C, Humbert JP, Gobaille S, et al. Cloning and characterization of a rat brain receptor that binds the endogenous neuromodulator gamma-hydroxybutyrate (GHB). *Faseb J*. 2003; 17(12):1691–3. [PubMed: 12958178]
3. Gallimberti L, Spella MR, Soncini CA, Gessa GL. Gamma-hydroxybutyric acid in the treatment of alcohol and heroin dependence. *Alcohol*. 2000; 20(3):257–62. [PubMed: 10869867]
4. Dyer JE. gamma-Hydroxybutyrate: a health-food product producing coma and seizurelike activity. *The American journal of emergency medicine*. 1991; 9(4):321–4. [PubMed: 2054002]
5. Halkitis PN, Palamar JJ, Mukherjee PP. Poly-club-drug use among gay and bisexual men: a longitudinal analysis. *Drug Alcohol Depend*. 2007; 89(2–3):153–60. Epub 2007/02/03. [PubMed: 17267140]
6. Varela M, Nogue S, Oros M, Miro O. Gamma hydroxybutyrate use for sexual assault. *Emerg Med J*. 2004; 21(2):255–6. [PubMed: 14988371]
7. Okun MS, Boothby LA, Bartfield RB, Doering PL. GHB: an important pharmacologic and clinical update. *J Pharm Pharm Sci*. 2001; 4(2):167–75. [PubMed: 11466174]
8. Wood DM, Nicolaou M, Dargan PI. Epidemiology of recreational drug toxicity in a nightclub environment. *Subst Use Misuse*. 2009; 44(11):1495–502. Epub 2009/11/27. [PubMed: 19938928]
9. Mason PE, Kerns WP 2nd. Gamma hydroxybutyric acid (GHB) intoxication. *Acad Emerg Med*. 2002; 9(7):730–9. [PubMed: 12093716]
10. Wang Q, Lu Y, Yuan M, Darling IM, Repasky EA, Morris ME. Characterization of monocarboxylate transport in human kidney HK-2 cells. *Molecular pharmaceutics*. 2006; 3(6):675–85. Epub 2006/12/05. [PubMed: 17140255]
11. Wang Q, Morris ME. The role of monocarboxylate transporter 2 and 4 in the transport of gamma-hydroxybutyric acid in mammalian cells. *Drug metabolism and disposition: the biological fate of chemicals*. 2007; 35(8):1393–9. Epub 2007/05/16. [PubMed: 17502341]
12. Fishbein WN, Merezhinskaya N, Foellmer JW. Relative distribution of three major lactate transporters in frozen human tissues and their localization in unfixed skeletal muscle. *Muscle Nerve*. 2002; 26(1):101–12. [PubMed: 12115955]
13. Leino RL, Gerhart DZ, Drewes LR. Monocarboxylate transporter (MCT1) abundance in brains of suckling and adult rats: a quantitative electron microscopic immunogold study. *Brain Res Dev Brain Res*. 1999; 113(1–2):47–54.
14. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *The Biochemical journal*. 1999; 343(Pt 2):281–99. [PubMed: 10510291]
15. Wang Q, Wang X, Morris ME. Effects of L-lactate and D-mannitol on gamma-hydroxybutyrate toxicokinetics and toxicodynamics in rats. *Drug metabolism and disposition: the biological fate of chemicals*. 2008; 36(11):2244–51. Epub 2008/08/23. [PubMed: 18719239]
16. Carter LP, Koek W, France CP. Behavioral analyses of GHB: receptor mechanisms. *Pharmacol Ther*. 2009; 121(1):100–14. Epub 2008/11/18. [PubMed: 19010351]
17. Morse BL, Vijay N, Morris ME. gamma-hydroxybutyrate (GHB)-Induced Respiratory Depression: Combined Receptor-Transporter Inhibition Therapy for Treatment in GHB Overdose. *Mol Pharmacol*. 2012 Epub 2012/05/09.
18. Roiko SA, Felmlee MA, Morris ME. Brain uptake of the drug of abuse gamma-hydroxybutyric acid in rats. *Drug metabolism and disposition: the biological fate of chemicals*. 2012; 40(1):212–8. Epub 2011/10/25. [PubMed: 22019629]
19. Felmlee MA, Roiko SA, Morse BL, Morris ME. Concentration-effect relationships for the drug of abuse gamma-hydroxybutyric acid. *The Journal of pharmacology and experimental therapeutics*. 2010; 333(3):764–71. Epub 2010/03/11. [PubMed: 20215411]
20. Morris ME, Hu K, Wang Q. Renal clearance of gamma-hydroxybutyric acid in rats: increasing renal elimination as a detoxification strategy. *The Journal of pharmacology and experimental therapeutics*. 2005; 313(3):1194–202. [PubMed: 15722403]

21. Rapoport SI. Osmotic opening of the blood-brain barrier: principles, mechanism, and therapeutic applications. *Cell Mol Neurobiol.* 2000; 20(2):217–30. Epub 2000/03/04. [PubMed: 10696511]
22. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates.* New York: Academic Press; 1986.
23. de Lange EC, de Boer AG, Breimer DD. Methodological issues in microdialysis sampling for pharmacokinetic studies. *Adv Drug Deliv Rev.* 2000; 45(2–3):125–48. Epub 2000/12/08. [PubMed: 11108971]
24. Bouw MR, Hammarlund-Udenaes M. Methodological aspects of the use of a calibrator in in vivo microdialysis-further development of the retrodialysis method. *Pharmaceutical research.* 1998; 15(11):1673–9. Epub 1998/12/02. [PubMed: 9833986]
25. Felmler MA, Wang Q, Cui D, Roiko SA, Morris ME. Mechanistic Toxicokinetic Model for gamma-Hydroxybutyric Acid: Inhibition of Active Renal Reabsorption as a Potential Therapeutic Strategy. *The AAPS journal.* 2010 Epub 2010/05/13.
26. Ronaldson PT, Lee G, Dallas S, Bendayan R. Involvement of P-glycoprotein in the transport of saquinavir and indinavir in rat brain microvessel endothelial and microglia cell lines. *Pharmaceutical research.* 2004; 21(5):811–8. Epub 2004/06/08. [PubMed: 15180339]
27. Smith JP, Drewes LR. Modulation of monocarboxylic acid transporter-1 kinetic function by the cAMP signaling pathway in rat brain endothelial cells. *J Biol Chem.* 2006; 281(4):2053–60. Epub 2005/11/23. [PubMed: 16301311]
28. Bhattacharya I, Boje KM. GHB (gamma-hydroxybutyrate) carrier-mediated transport across the blood-brain barrier. *The Journal of pharmacology and experimental therapeutics.* 2004; 311(1):92–8. [PubMed: 15173314]
29. Morse BL, Felmler MA, Morris ME. gamma-Hydroxybutyrate blood/plasma partitioning: effect of physiologic pH on transport by monocarboxylate transporters. *Drug metabolism and disposition: the biological fate of chemicals.* 2012; 40(1):64–9. Epub 2011/10/07. [PubMed: 21976619]
30. Wang Q, Darling IM, Morris ME. Transport of gamma-hydroxybutyrate in rat kidney membrane vesicles: Role of monocarboxylate transporters. *The Journal of pharmacology and experimental therapeutics.* 2006; 318(2):751–61. Epub 2006/05/19. [PubMed: 16707723]
31. Roux F, Couraud PO. Rat brain endothelial cell lines for the study of blood-brain barrier permeability and transport functions. *Cell Mol Neurobiol.* 2005; 25(1):41–58. Epub 2005/06/21. [PubMed: 15962508]
32. Morris ME, Felmler MA. Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse gamma-hydroxybutyric acid. *The AAPS journal.* 2008; 10(2):311–21. Epub 2008/06/05. [PubMed: 18523892]
33. Stanley WC, Wisneski JA, Gertz EW, Neese RA, Brooks GA. Glucose and lactate interrelations during moderate-intensity exercise in humans. *Metabolism.* 1988; 37(9):850–8. Epub 1988/09/01. [PubMed: 3138512]
34. Tappy L, Cayeux MC, Chioloro R. Effects of sodium lactate on ventilation and acid-base balance in healthy humans. *Clin Physiol.* 1996; 16(4):393–401. Epub 1996/07/01. [PubMed: 8842575]
35. Morris ME, Morse BL, Baciewicz GJ, Tessena MM, Acquisto NM, Hutchinson DJ, et al. Monocarboxylate transporter inhibition with osmotic diuresis increases gamma-hydroxybutyrate renal elimination in humans: a proof-of-concept study. *J Clin Toxicol.* 2011; 1:105.10.4172/2161-0494.1000105

Figure 1.

The experimental timeline to study the effects of L-lactate and D-mannitol on GHB TK/TD in rats. All L-lactate and D-mannitol treatments were administered 5 minutes after the GHB dose.

A.

B.

Figure 2.

GHB plasma (A) and brain ECF (B) concentration-time profiles after GHB administration in the presence and absence of L-lactate and D-mannitol treatments. L-lactate was administered 5 minutes after GHB dosing either as a low dose (bolus 66 mg/kg, infusion 300 mg/kg/hr) or a high dose (bolus 330 mg/kg, infusion 605 mg/kg/hr). A separate group of rats received D-mannitol 500 mg/kg 5 minutes after GHB dosing with or without the low dose of L-lactate. Data shown are mean $\pm$ SD, $n = 4$ for GHB alone, $n = 3$ for L-lactate and mannitol treatments.

Figure 3.

(A) Effects of L-lactate (1, 5, and 10 mM) on the uptake of GHB (10 mM) in RBE4 cells. L-lactate 5 and 10 mM reduced the uptake of GHB to 74% and 64% of control, respectively ($p < 0.05$). Data represent mean $\pm$ SEM of three sets of studies conducted in triplicate. (B) Effects of D-mannitol (10 and 100 mM) on the uptake of GHB (10 mM) in RBE4 cells. D-mannitol was either applied acutely with GHB (acute) or cells were pre-incubated for 30 minutes at 37°C with mannitol (chronic). No significant differences in the uptake of GHB were observed. Data represent mean $\pm$ SEM of three sets of studies conducted in triplicate.

Figure 4.

(A) Time-course of GHB efflux in RBE4 cells. Cells were incubated with GHB (10 mM) for 30 minutes at 37°C. Efflux was characterized at room temperature by the removal of GHB and application of uptake buffer for the specified time points. Data represent the mean $\pm$ SEM of 3 experiments conducted in triplicate, plotted as intracellular GHB remaining after efflux. (B) Effects of GHB (3 and 15 mM), L-lactate (10 mM), D-mannitol (100 mM), and CHC (2.5 mM) on the efflux of GHB (10 mM) at 0.5 and 2 minutes. At 0.5 minutes, D-mannitol, L-lactate, GHB, and CHC, as well as L-lactate combined with D-mannitol or GHB increased the efflux of GHB compared to control. At 2 minutes, only L-lactate or GHB alone or L-lactate combined with D-mannitol or GHB increased the efflux of GHB. Data

represent the mean $\pm$ SEM of 3 experiments conducted in triplicate, plotted as intracellular GHB remaining after efflux.

Table I

Plasma and brain ECF concentration-time profiles and toxicodynamic endpoints (sleep time, GHB concentrations in plasma and ECF at RRR) for rats administered 600 mg/kg GHB i.v alone or with L-lactate, D-mannitol, or L-lactate and D-mannitol combined administered 5 minutes after the GHB dose. Lactate was administered 5 minutes after GHB dosing as a bolus of 66 mg/kg and infusion of 300 mg/kg/hr. High-dose lactate was a bolus of 330 mg/kg and infusion of 605 mg/kg/hr). A separate group of rats received a bolus of D-mannitol 500 mg/kg 5 minutes after GHB dosing with and without L-lactate (low dose). Data shown are mean $\pm$ SD, n = 4 for GHB alone, n = 3 for lactate and mannitol treatments.

GHB Dose (mg/kg)	Toxicokinetics				Toxicodynamics		
	Plasma AUC (mg*min/ml)	Brain ECF AUC (mg*min/ml)	Overall partition coefficient (Brain AUC/Plasma AUC)	GHB Renal Clearance (ml/min/kg)	F _e (mean $\pm$ SD)	Sleeptime (min)	GHB C _p at RRR (μ g/ml) GHB C _{ecf} at RRR (μ g/ml)
600	123.7 $\pm$ 23	8.7 $\pm$ 3	0.070 $\pm$ 0.02	1.40 $\pm$ 0.1	0.29 $\pm$ 0.07	137.6 $\pm$ 12	373.2 $\pm$ 46 38.8 $\pm$ 16
600 + Lactate (low dose)	79.6 $\pm$ 16 ^a	5.7 $\pm$ 3	0.069 $\pm$ 0.02	2.42 $\pm$ 0.2 ^a	0.32 $\pm$ 0.03	90.7 $\pm$ 16 ^a	339.4 $\pm$ 26 34.3 $\pm$ 15
600 + Mannitol	116.0 $\pm$ 9	13.2 $\pm$ 6	0.112 $\pm$ 0.04	1.60 $\pm$ 0.3	0.30 $\pm$ 0.04	128.7 $\pm$ 12	385.8 $\pm$ 59 62.9 $\pm$ 26
600 + Lactate (high dose)	78.9 $\pm$ 9 ^a	2.1 $\pm$ 0.6 ^a	0.027 $\pm$ 0.08 ^a	4.68 $\pm$ 0.5 ^{a,b}	0.60 $\pm$.04 ^a	55.3 $\pm$ 5 ^{a,b}	544.7 $\pm$ 96 ^a 23.3 $\pm$ 6
600 + Lactate (low dose) + mannitol	107.4 $\pm$ 5	9.3 $\pm$ 3	0.087 $\pm$ 0.03	2.60 $\pm$ 0.4	0.45 $\pm$ 0.07	99.3 $\pm$ 7 ^a	425.2 $\pm$ 30 55.4 $\pm$ 9

^a p < 0.05 when compared to control,

^b p < 0.05 when compared to low dose lactate treatment.