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Prehabilitative exercise hastens recovery from isoflurane in diabetic and non-diabetic rats

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Abstract

Diabetes has been demonstrated to be one of the strongest predictors of risk for postoperative delirium and functional decline in older patients undergoing surgery. Exercise is often prescribed as a treatment for diabetic patients and regular physical activity is hypothesized to decrease the risk of postoperative cognitive impairments. Prior studies suggest that anesthetic emergence trajectories and recovery are predictive of risk for later postoperative cognitive impairments. Therapeutic strategies aimed at improving emergence and recovery from anesthesia may therefore be beneficial for diabetic patients. Wistar (n = 32) and Goto-Kakizaki (GK) type 2 diabetic (n = 32) rats between 3–4 months old underwent treadmill exercise for 30 min/day for ten days or remained inactive. Pre-anesthesia spontaneous alternation behavior was recorded with a Y-maze. Rats then received a 2-h exposure to 1.5–2 % isoflurane or oxygen only. The time to reach anesthetic emergence and post-anesthesia recovery behaviors was recorded for each rat. Postsynaptic density protein-95 (PSD-95), an important scaffolding protein required for synaptic plasticity, protein levels were quantified from hippocampus using western blot. Spontaneous

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Author contribution
CGS helped design and perform the experiments, analyze and interpret the data, and write the manuscript.
AO helped perform the experiments and write the manuscript.
ANS helped perform the experiments and write the manuscript.
DCL helped perform the experiments, analyze and interpret the data, and write the manuscript.
RSA helped perform the experiments and write the manuscript.
MTP helped design the experiments, interpret the data, and write the manuscript.
PSG helped design the experiments, interpret the data, and write manuscript.

Declaration of Competing Interest
The authors report no declarations of interest.
alteration behavior (p = 0.044) and arm entries (p < 0.001) were decreased in GK rats. There was no difference between groups in emergence times from isoflurane, but exercise hastened the recovery time (p = 0.008) for both Wistar and GK rats. Following 10 days of exercise, both Wistar and GK rats show increased levels of PSD-95 in the hippocampus. Prehabilitation with moderate intensity exercise, even on a short timescale, is beneficial for recovery from isoflurane in rats, regardless of metabolic disease status.

**Keywords**
General anesthesia; Exercise; Prehabilitation; Anesthetic recovery; PSD-95; Diabetes mellitus

1. **Introduction**

Diabetes Mellitus (DM) is a major public health problem, with approximately 9.4% of the U.S. population affected [1]. DM has been demonstrated to be one of the strongest predictors of risk for postoperative delirium and functional decline in aged patients undergoing surgery [2]. Exposure to volatile anesthetics have been shown to induce memory deficits in both the streptozotocin-induced rat model of type I DM and the Goto-Kakizaki (GK) rat, a spontaneously occurring, non-obese model of type II diabetes [3,4].

Brain activity patterns seen during the emergence from anesthesia can be predictive of the risk for delirium in patients in the postanesthesia care unit (PACU) [5]. The process of emergence can be influenced by modulation of neuronal subpopulations involved in the regulation of arousal [6,7]. The subsequent “recovery” from anesthesia describes the restoration of cortical integration of complex information [8]. Disruption of the recovery process is a potential mechanism for incidents of postoperative delirium [9]. Patients experiencing postoperative delirium are at an increased risk for long-term cognitive decline [10], indicating the potential for therapeutic strategies modulating the emergence and recovery from anesthesia to improve both immediate and long-term cognitive function.

Exercise is known to be beneficial in managing blood glucose levels in patients with type 2 DM [11] and to attenuate cognitive decline after surgery with general anesthesia in rats with metabolic syndrome [12]. Exercise also increases levels of postsynaptic density protein 95 (PSD-95) [13], a scaffolding protein required for structural changes during synaptic plasticity [14]. PSD-95 expression is dramatically decreased in rats experiencing cognitive impairments following exposure to sevoflurane [15]. Treatments increasing PSD-95 levels in medial prefrontal cortex and hippocampus have previously been shown to be improve performance on cognitive tests following anesthesia exposure [16,17]. These results suggest that prior exercise may modify the duration of emergence and recovery from anesthesia. Exercise represents a promising prehabilitative intervention that may reduce postoperative cognitive complications for patients with type 2 DM. The goal of this work was to investigate the influence of moderate exercise on both anesthetic emergence as well as post-anesthesia recovery in a type 2 diabetic rodent model (GK rat) and in non-diabetic Wistar control rats. We hypothesized that GK rats receiving isoflurane would show delayed
anesthetic emergence and recovery, while GK rats undergoing exercise training would display no differences on these tests when compared with Wistar control rats.

2. Materials and methods

Animals -

Male Wistar (n = 34) and GK (n = 37) rats derived from an established breeding colony were used for data collection at 3–4 months of age. The rats were pair-housed and kept on a 12-h light/dark cycles with ad libitum access to standard rodent chow and water. Experimental groups were randomly assigned such that no cagemates were assigned to the same group. All experiments were performed at the Atlanta VA Health Care System Animal Facility with approval from the Institutional Animal Care and Use Committee and followed guidelines provided by the National Institutes of Health and the Association for the Assessment and Accreditation of Laboratory Animal Care.

Glucose Tolerance Test -

The glucose tolerance test was performed to verify the presence of hyperglycemia and impaired glucose metabolism in our GK rats. Wistar and GK rats were food deprived for 6 h and blood glucose levels were monitored by test strip (FreeStyle Lite, Abbott Diabetes Care Inc., UK). Blood glucose levels were recorded at baseline and at 15, 30, 60 and 120 min after an intraperitoneal injection of glucose (2 mg per kilogram body weight in sterile water).

Treadmill Exercise -

Active rats completed a 30-minute exercise routine consisting of forced treadmill running for 10 days (Exer 3/6 Animal Treadmill, Columbus Instruments, Columbus, OH, USA), similar to previous reports [18]. Rats were trained to run on treadmills up to a speed of 15 m per minute. Treadmills were equipped with shock detection from an electric grid (1 Hz, 0.46 mA) if rats left the treadmill during exercise sessions. If 10 shocks were received during any single session, the session was aborted. Failure to train to under 10 shocks received per session within the first 5 days of training resulted in removal of that rat from the study. Seven rats (2 Wistar & 5 GK) were removed for failure to complete training. Inactive rats were placed on a stationary treadmill to control for environment.

Y-maze Continuous Spontaneous Alternation Test -

The spontaneous alternation test (SAT) was used to assess activity and spatial working memory [19]. Rodents introduced to a Y-maze display a tendency to alternate arm entries during maze exploration. This tendency towards alternation is thought to result from a preference towards investigation of new environments, and the SAT takes advantage of this behavior as an assessment of spatial working memory [20]. Rats were recorded while freely exploring the Y-maze (San Diego Instruments, San Diego, CA, USA) for 8 min. The total arm entries and the path of arm exploration for each rat were scored by a blinded observer. If at any time the rat remained stationary for >60 s during the session, movement was motivated by briefly grasping and releasing the rat from the base of the tail.
A successful alternation was scored when the rat completed three consecutive arm entries via turns in the same direction. Alternation percentage was determined using the following equation [19]:

\[
\text{Alternation percentage} = \frac{\# \text{of Alternations}}{\text{Total \# of Arm Entries} - 2} \times 100
\]  

(1)

**Isoflurane Anesthesia -**

Our anesthesia protocol was modified from our previous work [21]. Rats were anesthetized in an induction chamber pre-charged with 2% isoflurane in oxygen (O\(_2\)) and maintained for 2 h via a nose cone delivering 1.5–2 % isoflurane in O\(_2\) at a rate of 1 L/minute. Respiration rates were monitored for each rat in 5-minute intervals. Body temperature was maintained at 38±0.5 °C via heating pad and monitored via rectal thermometer. After 1 -h & 50-minutes of general anesthesia, the temperature probe was removed and a small piece of adhesive tape (≈0.5cm × 3cm) was affixed on the rat’s left forepaw. At the two-hour mark, the isoflurane concentration was reduced to 0% and a stopwatch was started simultaneously to record the latency to the first attempt to remove the adhesive tape [22]. Sham general anesthesia (2 h of exposure to O\(_2\) at 1 L/minute) was delivered in an induction chamber to half of the rats in the study.

**Anesthetic Emergence & Recovery -**

We chose to distinguish between the period of emergence from isoflurane anesthesia and the period of recovery from the effects of isoflurane anesthesia. Anesthetic emergence was defined as the period between cessation of isoflurane delivery (ISO OFF) and the return of righting reflex. Anesthetic recovery was defined as the period from the return of righting reflex until the first attempt to remove the adhesive tape from the left forepaw. The modified sticky dot test used in these experiments requires perception of the adhesive tape and initiation of actions to remove the adhesive tape following the regaining of consciousness [7]. The tape was positioned so that the rat’s forepaw was enclosed, with the remaining length of tape facing towards midpoint of the rat’s body. In this way, the length of tape was easily reachable by the rat with either the mouth or opposite forepaw. Time to notice the sticky dot was defined as the first attempt to displace the tape from the forepaw, either by vigorous shaking of the taped paw or biting of the tape. Rats receiving sham anesthesia were acclimated to handling and placement of the adhesive tape in a separate training session. On the date of sham anesthesia, the adhesive tape was positioned as previously described and observed until completion of the test.

**Western Blot -**

Hippocampal tissue was collected from rats receiving isoflurane anesthesia via perfusion with ice cold saline [Wistar, n = 13 (7 active, 6 inactive); GK, n = 14 (6 active, 8 inactive)]. Hippocampi from a subset (N = 5) of rats: [Wistar (1 active, 2 inactive) and GK (2 active, 0 inactive)] were not included due to their use in separate experiments. Samples were analyzed for levels of PSD-95. Tissue was homogenized in lysis buffer [137 mM NaCl, 20 mM tris-HCl (pH = 8), 1 % Igepal, 10 % glycerol, 1:100 Phosphatase Inhibitor Cocktails]
and 2 (Sigma-Aldrich) and 1:1000 Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA) and protein concentrations were determined by a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). 20 μg of each sample was separated by SDS-page on a 7.5 % gradient, stain-free Tris-glycine gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and total protein images were obtained prior to transfer. Next, samples were transferred to a PVDF membrane (Bio-Rad) and blocked with 5% non-fat dry milk for 1 h. The membrane was incubated overnight at 4 °C in primary antibody for PSD-95 [1:5000; Cell Signaling (Product #3450)]. Following 1 h of incubation in secondary antibody [goat anti-rabbit peroxidase labeled IgG (Vector Laboratories, Burlingame, CA, USA)], immunoreactivity was assessed using a chemiluminescence substrate (Thermo Fisher Scientific). Both total protein and immunoreactivity was quantified using the ChemiDoc MP Imaging System (Bio-Rad).

**Data Analysis -**

Statistical testing was performed using Prism 7.0 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics V26 (IBM Corporation, Armonk, NY, USA). This manuscript adheres to the applicable ARRIVE guidelines. A sample size of 8 rats per group was justified using a power analysis in G*Power 3.1.9.4 with α = 0.05, β = 0.80 and an anticipated effect size of 0.5 based on a previous publication on the effect of a volatile anesthetic on GK rats [4]. The Shapiro-Wilk test was used to assess normality in the datasets. Shapiro-Wilk test results were compared with visual inspection of the data for distributions with large numbers of observations (Glucose Tolerance Test and Y-Maze test performance). The Mann-Whitney U Test, Welch’s t-test and Two-way ANOVA with Sidak’s post hoc comparisons were used for statistical analysis where indicated. Values are reported as mean±SD for parametric tests and as medians for non-parametric tests, with p < 0.05 taken as the level for significant difference.

**3. Results**

Fasted blood glucose levels in GK rats were significantly different from controls (Median = 79.0 mg/dL, n = 32 for Wistar, Median = 118.5 mg/dL, n = 32 for GK, p < 0.001) (Table 1). Following intraperitoneal injection of glucose, blood glucose levels were consistently higher for GK rats when compared with controls throughout the 2-hs of blood glucose monitoring (Table 1).

A summary of the experimental design and timeline is included in Fig. 1. The continuous spontaneous alternation test was performed in a Y-maze for all rats 7 days prior to anesthesia exposure. There were no statistical differences in test performance as a result of prior treadmill training for either strain, so data from inactive and active rats from each strain were pooled together for comparison of Y-maze performance. GK rats displayed decreased spatial working memory when compared with Wistar controls (p = 0.044) (Fig. 2A). Spontaneous alternation percentage was higher for Wistar rats (73.06 ± 9.46, n = 32) than GK rats (67.63 ± 12.5, n = 32) as measured using an unpaired Student’s t-test. There was also a notable decrease in exploratory behavior between strains during the continuous spontaneous alternation test. GK rats completed significantly fewer arm entries than Wistar rats during
Maze exploration (23.78 ± 6.833, n = 32 for Wistar, 12.88 ± 3.687, n = 32 for GK, p < 0.001) (Fig. 2B). Overall, GK rats display a decrease in spatial working memory in addition to a decrease in maze exploration, similar to a previous report [23].

There were no significant main effects of genotype (31.2 ± 26.3 s for Wistar, n = 16; 32.8 ± 14.2 s for GK, n = 16) or activity (36.6 ± 24.9 s for Inactive, n = 16; 35.4 ± 17.1 for Active, n = 16) in sticky dot test performance from rats receiving sham anesthesia (Fig. 3A). Similarly, there were no significant main effects of genotype (208.4 ± 106.0 s for Wistar, n = 16; 302.1 ± 158.5 for GK, n = 16) or activity (247.75 ± 151.1 for Inactive, n = 16; 262.75 ± 134.5 for Active, n = 16) in time to emergence from isoflurane as determined by two-way ANOVA (Fig. 3B). The recovery period was defined for each rat as the difference in time from return of righting reflex to the first sticky dot removal attempt. Active rats (983.4 ± 236.2 s, n = 16) showed a significant reduction in time to notice the sticky dot compared with inactive rats (1303.1 ± 365.1 s, n = 16) following isoflurane anesthesia (two-way ANOVA, F (1,28) = 8.197, p = 0.008, Fig. 3C). Synaptic protein levels in the hippocampus showed a significant interaction between diabetes and exercise (Two-way ANOVA (F(1,23) = 34.47, p < 0.001). Western blots are pictured in Fig. 4A. Treadmill exercise increased hippocampal PSD-95 levels for both Wistar (p < 0.001) and GK rats (p = 0.016). Wistar rats displayed a larger increase in PSD-95 levels as a result of exercise than GK rats (p < 0.001) (Fig. 4B).

4. Discussion

Our results highlight a positive effect of exercise to reduce the duration of recovery from isoflurane anesthesia. Ten days of moderate exercise hastened recovery after isoflurane anesthesia. At baseline, GK rats displayed higher fasted blood glucose levels and impaired glucose metabolism when compared with Wistar rats. GK rats also demonstrated decreased spatial working memory performance and overall activity levels at 3 months of age, consistent with previously published work demonstrating memory and activity deficits at comparable ages [24,25]. Despite prior differences in metabolic status and behavioral alterations between GK and Wistar rats, prehabilitative exercise training decreased the time to recovery from general anesthesia for both strains. While our initial hypothesis was that exercise may hasten recovery in diabetic rats, it was surprising that exercise also hastened recovery time in adult control rats. These results suggest that 10 days of treadmill exercise has a larger effect size than initially expected, even in normally developing Wistar rats (M1 = 1303.06, M2 = 983.44, s = 307.49, Cohen’s d = 1.039, large effect), and that a potential opportunity exists to improve anesthetic recovery through prehabilitative exercise.

In humans, exercise interventions have been shown to increase performance on learning and memory tasks. These benefits have been hypothesized to result from an increase in factors promoting neurogenesis and synaptogenesis following exercise, including upregulation of neurotrophins such as brain derived neurotrophic factor (BDNF) [18] and increased proteins regulating synapse growth and stability such as PSD-95. The binding of BDNF to TrkB receptors has been shown to regulate the transportation of PSD-95 to the synapse and, in addition, the formation of PSD-95-TrkB complexes is important for intact BDNF signaling [26]. Studies in rats using similar treadmill designs with low or moderate intensity exercise
have demonstrated increases in hippocampal BDNF and PSD-95 levels that correlate with improved performance on memory tasks [13]. Previous investigations into PSD-95 levels in GK rats have demonstrated decreased levels of the protein in hippocampus from 5 to 20 weeks of age when compared with Wistar controls [24]. This difference was primarily driven by a developmental increase in PSD-95 levels seen in Wistar rats that is lacking in GK rats. We did not demonstrate a difference in PSD-95 levels for inactive Wistar and GK rats after isoflurane, however there was a significant increase for both groups after treadmill exercise. However, following exercise there were between strain differences in hippocampal PSD-95 levels are suggest agreement with the Matsunaga et al. findings suggesting that insulin receptor signaling in the GK rats may impact synaptic development. Overall, our results demonstrate increased PSD-95 levels in hippocampus following treadmill exercise, suggesting that our exercise regimen was effective for increasing factors that regulate synapse integrity in rats.

The recovery from anesthesia involves the restoration of attention and awareness of self and surroundings. This occurs as the information content of neural signaling becomes more complex. The anesthetic effect wanes and this information is integrated between brain regions [9]. The modified sticky dot removal task has traditionally been used to measure sensorimotor integration and function in models of stroke [7]. There were no sensorimotor deficits demonstrated between Wistar and GK rats following sham anesthesia on the time to investigate the sticky dot. Completion of the modified sticky dot task following anesthesia requires both the perception of the sticky dot on the forepaw and completion of movements to investigate or attempt to remove the sticky dot. Exercise decreased the duration of time necessary for completion of this task. Future studies into the recovery from anesthesia would benefit from a comparison with the time to regain normal grooming behaviors or a correlation with EEG activity.

There was no effect of genotype or prior exercise on emergence between Wistar and GK rats. This result might be expected for the inactive groups given prior results demonstrating that rats with a low aerobic oxidative capacity show no change in isoflurane-mediated suppression of movement when compared to normal rats (measured by minimum alveolar concentrations (MAC)) [27]. While we did not directly assess aerobic capacity at the cessation of exercise training, We feel it is unlikely that this treadmill exercise paradigm increased aerobic capacity sufficiently to induce group differences in MAC that would impact the duration of emergence from isoflurane. GK rats are a non-obese model and share little physiologic similarities with the low capacity runner rat model of metabolic syndrome as studied in other contexts. Previous work has demonstrated that uncontrolled diabetes in the streptozotocin-induced Type 1 diabetes rat model changed the MAC for isoflurane by 17 %, and that this change was reversible via insulin treatment [28]. We did note more variability in the emergence times from GK rats compared with WT rats for both inactive and active rats, even though statistical comparisons between groups were not significant. Complex pharmacologic interactions among neuronal networks mediate movement to noxious stimuli, emergence, and recovery. It seems unwise to assume that hastening emergence is always correlated with suppression of movement while anesthetized (or vice-versa). Additional study is needed to determine the extent to which metabolic syndrome impacts recovery of behaviors after anesthesia and to resolve the contrasting
findings from different rodent models (Type 1 vs. Type 2, controlled vs. uncontrolled diabetes, obese vs. non-obese).

All studies have limitations and the present study did not assess blood glucose or insulin levels following treadmill exercise in the Wistar or GK rats. A previous report using a higher intensity, 8-week treadmill exercise regimen in the GK rat demonstrated no change in glucose concentrations in blood plasma after training, however there was a significant decrease in insulin concentrations [29]. Insulin signaling in the brain has been shown to be an important regulator of synapse growth and stability. For example, activation of insulin receptors has been shown to increase PSD-95 synthesis in hippocampus through the PI3-kinase-Akt-mTOR pathway [30]. It is possible that the more modest increase in PSD-95 levels in the GK rats following exercise is due to hyperglycemia and/or insulin resistance in the GK rats. Both groups recovered faster following exercise regardless of differences in glucose metabolism, but further study is needed to determine if changes in PSD-95 levels in these strains are dependent on alterations in insulin signaling following exercise. While it is known that both inhaled anesthetics and diabetes can interfere with PSD-95 levels [15,31], the combined effects have not been well characterized. It will also be necessary to assess how exercise impacts PSD-95 levels and neurotrophic factors in other brain areas. This study also limited assessment of cognitive performance and improvement to assays of hippocampal function. Hippocampal development has been previously demonstrated to be impaired in Wistar and GK rats [24,25], and our results are suggestive of generalized benefits of exercise on PSD-95 levels in hippocampus. Further study of the effects of exercise on anesthetic recovery would benefit from assessments of function in additional brain areas such as frontal and association cortices, which are prominently implicated in mechanisms underlying impaired cognitive performance in the postoperative period.

The results of this study show that exercise is effective for reducing recovery time following general anesthesia with isoflurane. Altogether these observations suggest that prehabilitative exercise may help to improve recovery in patients after general anesthesia.

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References


Fig. 1.
Illustration of experimental timeline and design. A) Full experimental timeline for each rat in the study. Acclim = Treadmill Acclimation, GTT = Glucose Tolerance Test, SAT = Continuous Spontaneous Alternation Behavior Test, GA = General Anesthesia, Sham = Sham Anesthesia. B) Experimental groups in $2 \times 2 \times 2$ study design.
GK rats perform worse than Wistars on a test of spatial working memory. A) Wistar (W, n = 32) rats were more likely than GK rats (n = 32) to alternate between maze arms during Y-maze exploration (Welch’s t-test, p = 0.044). B) GK rats perform significantly fewer arm entries during maze exploration when compared with Wistar rats (Welch’s t-test, p < 0.001). Results are represented as group mean with standard deviation.
Fig. 3.
Exercise hastens recovery from isoflurane anesthesia. A) For rats receiving sham anesthesia, there was no effect of genotype or exercise on time to notice the sticky dot. B) For rats receiving isoflurane anesthesia, there was no effect of genotype or exercise on the time to emergence, defined as the time from ISO OFF to return of the righting reflex. C) Exercise decreased the time to notice the sticky dot for both Wistar and GK rats ($F(1,28) = 8.197, p = 0.008$).
Fig. 4. Exercise increased hippocampal PSD-95 levels in both Wistar and GK rats. A) Western blots for PSD-95 from hippocampal tissue are shown (W/Inactive: n = 7, W/Active: n = 6, GK/Inactive: n = 6, GK/Active: n = 8). Representative ladders are displayed below. B) Exercise increases hippocampal PSD-95 levels (Interaction effect, F(1,23) = 34.47, p < 0.001). Hippocampal PSD-95 levels increased in both Wistar (p < 0.001) and GK rats (p = 0.016), but Wistar rats demonstrate a greater overall increase in PSD-95 from pre-exercise levels (p < 0.001).
Table 1
Blood glucose levels as measured by test strip for GTT.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Wistar (n = 32)</th>
<th></th>
<th>Median Blood Glucose (mg/dL)</th>
<th></th>
<th>Median Blood Glucose (mg/dL)</th>
<th>Median Rank</th>
<th>U</th>
<th>p</th>
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<tr>
<td>Baseline</td>
<td>79.0</td>
<td>16.25</td>
<td>118.5</td>
<td></td>
<td>48.50</td>
<td>65.5</td>
<td>&lt;0.001,***</td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>140.5</td>
<td>17.25</td>
<td>243.5</td>
<td></td>
<td>48.50</td>
<td>73.0</td>
<td>&lt;0.001,***</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>148.0</td>
<td>16.50</td>
<td>308.0</td>
<td></td>
<td>48.50</td>
<td>5.0</td>
<td>&lt;0.001,***</td>
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</tr>
<tr>
<td>60 min</td>
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<td>16.75</td>
<td>312.0</td>
<td></td>
<td>48.50</td>
<td>0</td>
<td>&lt;0.001,***</td>
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</tr>
<tr>
<td>120 min</td>
<td>84.5</td>
<td>16.50</td>
<td>219.0</td>
<td></td>
<td>48.50</td>
<td>0</td>
<td>&lt;0.001,***</td>
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