Citation	Sonneville R, den Hertog HM, (2013), Increasing glucose load while maintaining normoglycemia does not evoke neuronal damage in prolonged critically ill rabbits. Clin Nutr. 2013 Dec;32(6):1077-80. Epub 2013 Jan 12.
Archived version	Author manuscript: the content is identical to the content of the published paper, but without the final typesetting by the publisher
Published version	http://dx.doi.org/10.1016/j.clnu.2013.01.003
Journal homepage	http://www.journals.elsevier.com/clinical-nutrition
Author contact	your email greet.vandenberghe@med.kuleuven.be your phone number + 32 (0)16 344021
IR	https://lirias.kuleuven.be/handle/123456789/391623

(article begins on next page)

Increasing glucose load while maintaining normoglycemia does not evoke neuronal damage in prolonged critically ill rabbits

Romain Sonnevile,^{1,2,3} Heleen M. den Hertog,^{1,4} Sarah Derde,¹ Fabian Güiza,¹ Inge Derese,¹ Greet Van den Berghe,¹ and Ilse Vanhorebeek¹

¹ Clinical Department and Laboratory of Intensive Care Medicine, Division of Cellular and Molecular Medicine, KU Leuven, Belgium; ² Department of Intensive Care Medicine, EA4342, Raymond Poincaré University Hospital, Garches, and Université de Versailles-Saint Quentin, France; ³ Histopathologie Humaine et Modèles Animaux, Département Infection et Epidémiologie, Institut Pasteur, Paris, France; ⁴ Department of Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands

Short title: Glucose intake and the brain in critical illness

Non-standard abbreviations: GAPDH: glyceraldehyde-3-phosphate dehydrogenase, GFAP: glial fibrillary acidic protein, GLUT-4: glucose transporter-4, IV: intravenous, MnSOD: manganese superoxide dismutase

Address for correspondence:

Prof. Ilse Vanhorebeek, PhD

Laboratory of Intensive Care Medicine, Division of Cellular and Molecular Medicine

KU Leuven, B-3000 Leuven, Belgium

Phone: +32 16 330532; Fax: +32 16 344015; E-mail: ilse.vanhorebeek@med.kuleuven.be

ABSTRACT

Background & Aims: Preventing severe hyperglycemia with insulin reduced the neuropathological alterations in frontal cortex during critical illness. We investigated the impact of increasing glucose load under normoglycemia on neurons and glial cells.

Methods: Hyperinflammatory critically ill rabbits were randomized to fasting or combined parenteral nutrition containing progressively increasing amounts of glucose (low, intermediate, high) within the physiological range but with a similar amount of amino acids and lipids. In all groups, normoglycemia was maintained with insulin. On day 7, we studied the neuropathological alterations in frontal cortex neurons, astrocytes and microglia, and MnSOD as marker of oxidative stress.

Results: The percentage of damaged neurons was comparable among all critically ill and healthy rabbits. Critical illness induced an overall 1.8-fold increase in astrocyte density and activation status, largely irrespective of the nutritional intake. The percentage of microglia activation in critically ill rabbits was comparable with that in healthy rabbits, irrespective of glucose load. Likewise, MnSOD expression was comparable in critically ill and healthy rabbits without any clear impact of the nutritional interventions.

Conclusions: During prolonged critical illness, increasing intravenous glucose infusion while strictly maintaining normoglycemia appeared safe for neuronal integrity and did not substantially affect glial cells in frontal cortex.

Key Words

Critical illness, normoglycemia, glucose load, astrocyte, microglia, neuron

INTRODUCTION

Persistent hyperglycemia is a common complication of critical illness that may contribute to acute brain injury and dysfunction and has been associated with long-term adverse cognitive sequelae in survivors.^{1,2} Maintaining blood glucose levels normal with insulin infusion was suggested to be neuroprotective. Indeed, studies in human and animal brain specimens showed that strict blood glucose control during critical illness attenuated neuropathological alterations at the level of neurons, astrocytes and microglia in vulnerable areas of the brain.² We also demonstrated clinical benefit, as maintenance of normoglycemia lowered intracranial pressure levels and the risk of seizures in patients with isolated brain injury and reduced the incidence of neuromuscular complications of critically ill patients admitted to the surgical or medical intensive care unit.³ However, maintaining normoglycemia with insulin remains a controversial intervention. Indeed, several other mostly small studies failed to reproduce these clinical benefits while uniformly observing an increased risk of severe hypoglycemia, though methodological quality may be an important issue.⁴ Importantly, long-term follow-up of critically ill children randomized to strict glycemic control versus usual care showed that this intervention did not increase the incidence of poor outcomes and did not compromise, but rather improved, neurocognitive development four years after ICU admission.⁵

In theory, glucose toxicity during critical illness could have a dual origin. Indeed, high blood glucose levels per se may be detrimental, but also the amount of glucose intake may play a role. As such, high intake of glucose could theoretically overload the brain cells. Supplementation of enteral nutrition with parenteral nutrition to achieve the caloric target has been recommended to counteract the hypercatabolic state of critical illness. Interestingly, however, early initiation of supplemental parenteral nutrition when enteral nutrition is insufficient has recently been shown to delay recovery of critically ill patients in our setting.⁶ The impact of this intervention, while maintaining normoglycemia, on the central nervous

74 system is so far not known.

75 In the present experimental neuropathological study, we addressed the impact of glucose
76 intake on neurons and glial cells during critical illness. Therefore, we varied the intravenous
77 glucose load under normoglycemia in a rabbit model of sustained hyperinflammatory critical
78 illness.

79

MATERIALS AND METHODS

Study Design

The KU Leuven Ethical Review Board for Animal Research approved the study (Protocol P05110). Animals were treated according to the Principles of Laboratory Animal Care (US National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Our catheterized, fluid-resuscitated third-degree burn injury rabbit model of prolonged critical illness has been extensively described previously.^{7,8} Male New Zealand white rabbits were instrumented under general anesthesia induced with an intramuscular injection of 30 mg/kg ketamine and 0.15 ml/kg medetomidine and maintained by 1.5% isoflurane inhalation. A catheter for repeated blood sampling was placed in the left carotid artery and another one for continuous intravenous nutrition and insulin administration was placed in the left jugular vein. After performing a paravertebral block with 5 ml 1% Xylocaine (Astra-Pharmaceuticals, Brussels, Belgium), a full-thickness third-degree burn wound of 15-20% total body surface area was inflicted on the flanks (painless because cutaneous nerve ends are destroyed), which was covered dry and clean by a home-made jacket. Continuous fluid resuscitation was started with Hartmann solution (Baxter, Lessines, Belgium) supplemented with 5% glucose at 16 ml/hour. Glycemia was controlled to normal fasting levels (80-110 mg/dl, 4.4-6.1 mmol/l) under continuous intravenous insulin infusion (Actrapid, Novo Nordisk, Bagsvaerd, Denmark). In the evening, a subcutaneous 0.2 mg/kg piritramide injection (Dipidolor, Janssen-Cilag, Beerse, Belgium) was given to prevent post-operative pain from surgery and inflammation around the burn injury. The critically ill rabbits were randomly allocated by sealed envelopes to four groups to receive different feeding regimens from the next morning onwards. The animals were deprived from oral feeding, but received parenteral nutrition

according to randomization to assure uptake of the exact amount of administered nutrients and had free access to water and a small amount of hay. A fasted group received only minimal glucose to prevent/treat hypoglycemia (Table 1). The other three groups received parenteral nutrition based on Clinomel N7 (Baxter; Clinitec, Maurepas Cedex, France), containing a same amount of amino acids and lipids but with a low, intermediate or high amount of glucose, infused at 10 ml/h. In all groups, blood glucose control to 80-110 mg/dl was continued until the end of the study. On day 7, animals were anesthetized and sacrificed. Brain tissue samples were harvested at the level of frontal cortex and were embedded in paraffin or stored at -80°C until analysis. The study was continued until in each group at least ten rabbits survived until day 7. For establishment of healthy reference ranges, data and brain biopsies were collected from healthy rabbits.

Histology

Neuropathological studies were performed as previously described.² The percentage of damaged neurons (shrunken eosinophilic cytoplasm, pyknotic nuclei after hematoxylin-eosin staining) was determined by counting of the cells with ImageJ 1.36b® software (Wayne Rasband, NIH, Bethesda, MD). Reactive astrocytes were stained for Glial Fibrillary Acidic Protein (mouse monoclonal anti-GFAP, 1/400, Millipore, Brussels, Belgium). Astrocytes were considered activated when an increase in the size of GFAP-positive cells was observed and when they presented with longer, thicker processes. Density of GFAP-positive astrocytes and cross-sectional area of GFAP staining were evaluated with Image J 1.36b®. Microglia were visualized with isolectin-B4 (5 µg/ml, Sigma, Bornem, Belgium). Microglia were classified as activated when they had an amoeboid shape (phagocytic phenotype), a rod shape (non-phagocytic phenotype) or encircled degenerating neurons (neuronophagia). Two independent observers (R.S., H.d.H.) evaluated all stainings, blinded for study groups. Images

were acquired with identical exposure on a Leica DM3000 microscope with DFC420C digital camera (Wetzlar, Germany). For each section, 3-5 different fields were evaluated and average values from all fields were determined.

Western blot

Western blot was performed on rabbit brain cortex homogenates using anti-manganese superoxide dismutase (MnSOD) (1:10000; Abcam, Cambridge, UK) or anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH, 1:30,000, Applied Biosystems/Ambion, Austin, TX). GAPDH was used as control for equal loading. Data are normalized to the median level in healthy rabbits.

Statistical analysis

Differences among groups were analyzed with the Chi-square test for comparison of proportions and Kruskal-Wallis/Mann-Whitney-U test for continuous data. Differences were considered statistically significant when two-tailed p -values were ≤ 0.05 . Statistical analyses were performed using Statview 5.0.1 (SAS Institute, Cary, NC).

RESULTS

According to the protocol, intravenous glucose load differed significantly between the 4 critically ill groups (Table 1). With increasing glucose load, insulin doses required to achieve normoglycemia as well as the corresponding plasma insulin levels were higher. Four fasted animals experienced an episode of severe hypoglycemia versus 2 in the low-glucose-load, none in the intermediate-glucose-load and one in the high-glucose-load group.

The percentage of damaged neurons was not increased by critical illness while maintaining normoglycemia, and was not affected by increasing the glucose load (Figure. 1). Critical illness induced an overall 1.8-fold increase in the density of GFAP-positive astrocytes as compared with the healthy rabbits, largely irrespective of the glucose load. Similar results were obtained for astrocyte activation status, as evaluated by GFAP-stained area per astrocyte. Critical illness and the different nutrient regimens had no effect on the activation status of microglia. Neuropathological scores were comparable for critically ill animals that experienced an episode of severe hypoglycemia and those that did not (data not shown). Expression of MnSOD as marker of oxidative stress was comparable with healthy levels in all critically ill groups, with no clear impact of increasing glucose load.

DISCUSSION

In an *in vivo* model of critical illness, increasing the intravenously administered amount of glucose within the physiological range, while blood glucose levels were maintained normal with insulin infusion, did not have a substantial impact on neuronal damage or the number and activation status of astrocytes and microglia.

Several aspects of dysregulated glucose homeostasis may contribute to “glucose neurotoxicity” during critical illness, including persistent hyperglycemia, episodes of severe hypoglycemia and increased glucose variability, but also excessive intake of glucose may be detrimental. Previous studies in deceased critically ill patients as well as in the same parenterally fed *in vivo* model of critical illness as used in the present study demonstrated that persistent hyperglycemia aggravates neuronal damage and histopathological features of impaired astrocyte reactivity, increased microglia activation and increased neuronal and glial cell apoptosis in hippocampus and/or frontal cortex.² Hyperglycemia also exacerbated blood-brain-barrier dysfunction after cerebral ischemia-reperfusion.⁹ Hyperglycemia-induced oxidative stress may play a crucial role in such toxicity to the brain.^{2,9} Also consumption of excessive amounts of glucose may increase oxidative stress, whereas too low amounts increase the risk of hypoglycemia. We increased the intravenous infusion of glucose from minimal amounts to the upper limit of physiological intake, while maintaining normoglycemia. We did not observe any clear impact of these dietary interventions on neuronal damage, number of GFAP-positive astrocytes or their activation status, activation of microglia, or on MnSOD expression as oxidative stress marker. Neuropathological findings were similar whether or not the rabbits had experienced a brief episode of hypoglycemia, confirming previous findings in this animal model.²

191 Altogether, these data identify high circulating blood glucose levels as culprit of brain
192 damage, whereas increasing the glucose load within the physiological range does not cause
193 detectable harm as long as blood glucose levels are maintained normal. This suggests that the
194 increasing amounts of infused glucose are safely consumed or stored in depots, and thus
195 disabled to cause any harm to the brain. Analysis of tissue biopsies harvested from patients
196 who took part in the first landmark randomized study on strict blood glucose control with
197 intensive insulin therapy during critical illness suggested that this intervention lowered blood
198 glucose levels mainly through stimulation of skeletal muscle glucose uptake.¹⁰ These studies
199 showed that maintenance of normoglycemia better preserves the metabolic insulin signaling
200 pathway in muscle. In addition, both the glucose transporter responsible for insulin-stimulated
201 glucose uptake (glucose transporter-4, GLUT-4) and the rate-limiting enzyme for intracellular
202 insulin-stimulated glucose metabolism (hexokinase-II) in muscle are increased at mRNA level
203 with this therapy. In contrast, the liver remained refractory to insulin. Interestingly, a recent
204 study on adipose tissue revealed a larger number of small adipocytes with an increased ability
205 to take up circulating glucose and triglycerides in biopsies from critically ill patients than in
206 those from healthy volunteers.¹¹ As such, adipose tissue may be biologically active as a
207 functional storage depot for potentially toxic metabolites in this condition.

208 Increasing intravenous glucose infusion within the physiological range during critical illness,
209 under normoglycemia, previously appeared able to attenuate catabolism as compared with
210 fasting, although the highest dose showed a lower efficacy.⁸ None of the glucose loads caused
211 organ failure or lethality, provided normoglycemia was maintained.⁸ The current study
212 extends these findings to safety for the brain. Nevertheless, although the model closely
213 mimics the characteristic human stress response to severe trauma⁷ and has been repeatedly
214 used for parenteral nutrition studies,^{7,12} caution is warranted when extrapolating experimental
215 animal data to the human setting. Optimal intake of carbohydrates and other nutrients for

216 critically ill patients still has to be determined. In particular, it remains unclear whether
217 increasing the glucose load under normoglycemia is safe in the setting of severe
218 traumatic/ischemic brain injury.

219 In conclusion, increasing intravenous glucose infusion while maintaining normoglycemia in
220 hyperinflammatory critically ill rabbits did not reveal any dose-effect in the neuropathological
221 studies and thus appeared safe for neuronal integrity and for the other brain cells.

222

223

ACKNOWLEDGEMENTS

Statement of authorship: RS, GVdB and IV designed the study. RS, HdH, SD and ID performed the experiments and collected the data. RS, HdH, FG, GVdB and IV analyzed the data. RS, GVdB and IV wrote the manuscript. All authors read and approved the final manuscript.

Sources of funding: The work was supported by the Research Foundation (FWO), Flanders, Belgium (G.0585.09). HdH received grants from the Erasmus Trustfonds and the Dr. Jan Meerwaldt Stichting. GVdB via the University of Leuven receives long-term structural research financing via the Methusalem program, funded by the Flemish Government (METH/08/07). The study sponsors had no role in the study design; collection, analysis and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

Conflict of interest statement: The authors have no duality of interest to declare. We thank Tyco Healthcare (Mechelen, Belgium), Fresenius-Kabi (Wilrijk, Belgium), Lameris (Aartselaar, Belgium), and Baxter (Lessines, Belgium) for technical and nutritional supplies.

REFERENCES

1. Heymann A, Sander M, Krahne D, Deja M, Weber-Carstens S, MacGuill M, Kastrup M, Wernecke KD, Nachtigall I, Spies CD. Hyperactive delirium and blood glucose control in critically ill patients. *J Int Med Res* 2007; 35: 666-677.
2. Sonnevile R, den Hertog HM, Güiza F, Gunst J, Derese I, Woutes PJ, Brouland JP, Polito A, Gray F, Chrétien F, Charlier P, Annane D, Sharshar T, Van den Berghe G, Vanhorebeek I. Impact of Hyperglycemia on Neuropathological Alterations during Critical Illness. *J Clin Endocrinol Metab* 2012; 97: 2113-2123.
3. Van den Berghe G, Schoonheydt K, Becx P, Bruyninckx F, Wouters PJ. Insulin therapy protects the central and peripheral nervous system of intensive care patients. *Neurology* 2005; 64: 1348-1353.
4. Hirsch IB. Understanding low sugar from NICE-SUGAR. *N Engl J Med* 2012; 367: 1150-1152.
5. Mesotten D, Gielen M, Sterken C, Claessens K, Hermans G, Vlasselaers D, Lemiere J, Lagae L, Gewillig M, Eyskens B, Vanhorebeek I, Wouters PJ, Van den Berghe G. Neurocognitive development of children 4 years after critical illness and treatment with tight glucose control: a randomized controlled trial. *JAMA* 2012; 308: 1641-1650.

6. Casaer MP, Mesotten D, Hermans G, Wouters PJ, Schetz M, Meyfroidt G, Van Cromphaut S, Ingels C, Meersseman P, Muller J, Vlasselaers D, Debaveye Y, Desmet L, Dubois J, Van Assche A, Vanderheyden S, Wilmer A, Van den Berghe G. Early versus late parenteral nutrition in critically ill adults. *N Engl J Med* 2011; 365: 506-517.
7. Weekers F, Giulietti AP, Michalaki M, Coopmans W, Van Herck E, Mathieu C, Van den Berghe G. Metabolic, endocrine, and immune effects of stress hyperglycemia in a rabbit model of prolonged critical illness. *Endocrinology* 2003; 144: 5329-5338.
8. Derde S, Vanhorebeek I, Ververs E-J, Vanhees I, Darras VM, Van Herck E, Larsson L, Van den Berghe G. Increasing intravenous glucose load in the presence of normoglycemia: effect on outcome and metabolism in critically ill rabbits. *Crit Care Med* 2010; 38: 602-611.
9. Kamada H, Yu F, Nito C, Chan PH. Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction. *Stroke* 2007; 38: 1044-1049.
10. Vanhorebeek I, Langouche L. Molecular mechanisms behind clinical benefits of intensive insulin therapy during critical illness: glucose versus insulin. *Best Pract Res Clin Anaesthesiol* 2009; 23: 449-459.

- 291 11. Langouche L, Vander Perre S, Thiessen S, Gunst J, Hermans G, D'Hoore A, Kola B,
292 Korbonits M, Van den Berghe G. Alterations in adipose tissue during critical illness:
293 An adaptive and protective response? *Am J Respir Crit Care Med* 2010; 182: 507-516.
294
- 295 12. Derde S, Vanhorebeek I, Güiza F, Derese I, Gunst J, Fahrenkrog B, Martinet W,
296 Vervenne H, Ververs EJ, Larsson L, Van den Berghe G. Early parenteral nutrition
297 evokes a phenotype of autophagy deficiency in liver and skeletal muscle of critically
298 ill rabbits. *Endocrinology* 2012; 153: 2267-2276.
299

FIGURE LEGENDS

Fig. 1 Quantification of neuropathological findings in frontal cortex of critically ill rabbits. Neuronal damage is expressed as the percentage of damaged neurons relative to the total number of neurons, counted in 5 fields at x40 magnification per rabbit. The number of GFAP-positive cells and astrocyte complexity (GFAP area/cell) as a marker of astrocyte activation were evaluated in 5 fields at x40 magnification and the percentage of activated microglia in 3 fields at x40 magnification. Data are presented as box plots, with the central line indicating the median, the box the interquartile range, and the whiskers the 10th and 90th percentiles. The Mann-Whitney U *P*-value represents the comparison between all critically ill rabbits and the healthy rabbits. The Kruskal-Wallis *P*-value represents the overall comparison among the 4 critically ill groups.

Table 1 Nutritional intake and metabolic control of critically ill rabbits

Group	Fasted (n=11)	Low- glucose- load (n=12) ^A	Intermediate- glucose- load (n=10)	High- glucose- load (n= 11)	<i>P</i> -value
Parenteral nutrition					
IV glucose, kcal/day	14 (1)	178 (13)	235 (6)	301 (15)	<0.0001
IV amino acids, kcal/day	0	14 (1)	14 (1)	14 (1)	<0.0001
IV lipid, kcal/day	0	31 (2)	31 (1)	31 (2)	<0.0001
IV total, kcal/day	14 (1)	223 (16)	280 (7)	346 (17)	<0.0001
Blood glucose levels					
Baseline, mg/dl	157 (18)	155 (21)	152 (21)	172 (33)	0.40
Mean of all blood glucose measurements, day 1-7, mg/dl	91 (16)	104 (9)	103 (6)	105 (7)	0.003
SD of all blood glucose measurements, mg/dl	68 (5)	74 (7)	74 (4)	75 (6)	0.048
Insulin					
Dose administered, IU/day	1.8 (0.6)	6.3 (1.2)	12.5 (5.6)	13.4 (6.1)	<0.0001
Plasma levels, mIU/l	20 (13)	56 (15)	122 (116)	181 (188)	<0.0001
Hypoglycemic events (≤ 40 mg/dl)					
n/total	4/11	2/12	0/10	1/11	0.13
n of episodes per animal	1-2	1	0	2	
Other metabolic parameters					
pH	7.38 (0.04)	7.39 (0.04)	7.39 (0.05)	7.39 (0.05)	0.91
PaCO ₂ , mmHg	28 (5)	30 (7)	36 (3)	26 (7)	0.005
PaO ₂ , mmHg	99 (17)	103 (13)	106 (30)	99 (11)	0.65
Hemoglobin, g/dl	10 (1)	10 (1)	10 (1)	11 (1)	0.47
Lactate, mmol/l	0.5 (0.4)	1.3 (1.0)	0.9 (0.3)	1.0 (0.6)	0.02

Data are shown as mean (SD) unless indicated otherwise. The four critically ill groups were compared by Kruskal-Wallis test. ^A In the low-glucose-load group, 13 surviving rabbits had been included, but for one of these rabbits no usable sample was available. IV: intravenous.

Figure 1