Food for Thought: Long-Term Effects of Hyperglycemia on Dendrites in the CA1 Region of the Hippocampus

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This study reports significant damage to dendrites (neural information receivers in synapses) in the CA1 *stratum radiatum* region of the hippocampal due to hyperglycemia. Hyperglycemia is a disorder caused by abnormally high blood glucose levels (> 125 mg/dL) that puts 80% of extremely preterm babies born each year at risk for decreased brain mass, intraventricular hemorrhage, blindness, and possible damage to the hippocampus, which is the area of the brain responsible for memory and spatial reasoning. Fluorescent immunohistochemistry was selected to stain for greater sensitivity of dendrite visualization in tissues of the CA1 *stratum radiatum* region of the hippocampus from a hyperglycemic rat model. When some tissues stained poorly with the primary antibody anti-beta tubulin, chosen for its structural component of microtubules and critical role in axon growth and maintenance, a small-scale test was developed using the primary antibody microtubule associated protein-2 (MAP2) to isolate the staining problem. Results suggested that MAP2 was more effective in staining. Then, Adobe Photoshop CS6 image analysis was pioneered in the lab to quantify fluorescence from stained dendrites by fluorescence of tagged dendrites in pixels, rather than numbers of dendrites expressed relative to number of neurons (i.e. dendrites/neuron). To eliminate bias, Photoshop CS6 analyses were blinded. Adobe Photoshop CS6 worked well, effectively eliminating the need to manually count dendrites, thus decreasing error and time needed for analysis. Results showed significant damage to dendrites in tissues of the CA1 *stratum radiatum* region of the hippocampus due to hyperglycemia ($p = 0.024$). Kirkpatrick G, McFarland A. Food for thought: Long-term effects of hyperglycemia on dendrites in the CA1 region of the hippocampus. *Minnesota Academy of Science Journal of Student Research* 2015; **3:**12-18.

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Abbreviations: BSA, bovine serum albumin; CA1, CA3, Cornu ammonis 1, Cornu ammonis 2; DAPI, 4',6-diamidino-2-phenylindole; DEPC, Diethylpyro-carbonate; GLUT1, GLUT3 glucose transport protein 1, glucose transport protein 3; MAP2, microtubule-associated protein 2; MGSV, mean gray-scale value; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Bcl-2, b-cell lymphoma 2; PARP1, poly (ADP-ribose) polymerase 1; PBS, phosphate buffer solution; qPCR, quantitative polymerase chain reaction; RHEG, recurrent hyperglycemia; STZ, streptozotocin

INTRODUCTION

Each year, over 80% of extremely preterm babies (< 28 weeks of gestation) with very low birth weights (< 1000 g) are diagnosed with hyperglycemia, a disorder caused by abnormally high blood glucose levels (> 125 mg/dL)[2,3]. Recent studies have suggested that hyperglycemia puts these babies at risk for decreased brain mass, intraventricular hemorrhages, blindness, and damage to the hippocampus, which is the area of the brain responsible for memory and spatial reasoning[1]. The purpose of our study was to

determine if hyperglycemia results in long-term damage to hippocampal tissue.

Preterm hyperglycemia occurs because, like type-1 diabetes, insulin production is insufficient. While infants start producing insulin during the second half of pregnancy, the receptors to which insulin binds in the peripheral tissue (liver and skeletal muscle) have not completely formed[2,4]. This is problematic because, immature insulin receptors cannot remove excess glucose from the blood[4]. As a result, the excess blood glucose is transported into the brain via glucose transport proteins (GLUT). Two types of GLUT proteins, GLUT1 and GLUT3, transport glucose across the blood-brain barrier and into neurons, respectively[5,6]. Since maintaining brain-glucose homeostasis is critical to brain function, clinical studies have suggested that preterm hyperglycemia may disrupt normal neuroplasticity, neuron growth, and neural communications[7].

Although there has been some recent research on infant hyperglycemia, most prior research has focused on infant hypoglycemia. Hypoglycemia, which is the inverse of hyperglycemia, is caused by abnormally *low* blood-glucose levels (< 50 mg/dL)[8]. Work by Won *et al.*[8] showed that infant hypoglycemia leads to oxidative stress, which results in long-term damage to dendrites (neural information receivers in synapses). Furthermore, Won *et al.* found that hypoglycemia leads to abnormal reduction in the *Cornu Ammonis* area one (CA1) of the hippocampus (Fig. 1)[8].

Although Won *et al*. studied hypoglycemia in diabetic rat model, the study is applicable to our work on hyperglycemia because, prior to inducing hypoglycemia, one group of rats in the Won *et al.* study was initially treated with streptozotocin (STZ) to destroy the insulin-producing beta cells in the pancreas. As a result, the STZ-treated group developed hyperglycemia before receiving insulin injections to induce hypoglycemia. The STZ-treated group showed decreased dendritic density in the CA1 region of the hippocampus[8]. Based on these results, we chose to investigate dendritic damage in tissue from the CA1 *stratum radiatum* region of the hippocampus (Figs. 1 and 2) obtained from a hyperglycemic rat model. Based on the Won *et al.* study, we hypothesized that hyperglycemia would result in long-term damage to dendrites in the CA1 region of the hippocampus.

MATERIALS AND METHODS

Hippocampus tissue was analyzed from a Sprague-Dawley rat (*Rattus norvegicus)* model that had been collected in 2008 from a previous study. Tissue was obtained from six female rats born from pregnant dams purchased from Harlan Sprague Dawley (Indianapolis, IN). From postnatal day 3 through postnatal day 12, rat pups in the repetitive hyperglycemic group (RHEG) were fasted for two hours and then injected with a single dose of 100 mg/kg of octreotide (Sandostatin, Novartis, Annandale, NJ) to suppress endogenous insulin secretion. After pups were injected with octreotide, they were given two injections of 30% dextrose (Hospira Inc., Lake Forest, IL) separated by one hour. Littermates in the control group were similarly fasted and injected with octreotide; however, they were given an equivalent volume of 0.9% saline in place of dextrose. On day 30, rats were euthanized using sodium pentobarbital (100 mg/kg), and rats selected for histochemistry were perfused *in situ* with saline. All brain tissue harvested from rats was preserved in formaldehyde[10].

The antibodies, "anti-beta III tubulin" and microtubule associated protein-2 (MAP2), were purchased from Millipore (Temecula, CA). The secondary antibody, Alexa Fluor 555, was purchased from Life Technologies (Carlsbad, CA). Triton X-100, Tween 20, and microscope cover glasses were purchased from Fisher Scientific (Fair Lawn, NJ). Parafilm was purchased from Pechiney Plastic Packaging (Chicago, IL). Sodium chloride (NaCl), potassium chloride (KCl), sodium hydrogen phosphate (Na_2HPO_4), potassium monophosphate (KH_2PO_4), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Vector hardset with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories Inc. (Burlingame, CA).

Sectioning tissue: Pre-prepared tissue slides were visually examined. The best preserved slides from the four rats in the hyperglycemic group and four rats in the control group were selected. Slides were thawed.

Selection of staining method: Pros and cons of fluorescent immunohistochemical staining and immunoperoxidase staining were examined. Fluorescent immunohistochemical staining was chosen because the secondary antibody fluorescently tags the primary antibody, which provides greater sensitivity for visualization of dendrites than immunoperoxidase staining that involves the use of enzymes and a substrate of hydrogen peroxide[11].

Antibody Selection: After exploring five primary antibodies, anti-beta III tubulin antibody was initially chosen because beta III tubulin is a structural component of microtubules and plays a crucial role in axon growth and maintenance[12]. When the anti-beta III tubulin antibody did not produce successful stains, a small-scale test was developed to assess if staining problems arose from poor tissue quality or ineffective staining. Tissue from test groups that had previously stained using anti-beta III tubulin were selected based on tissue quality. Pre-prepared slides from the control and from the hyperglycemic groups, judged to be of "high" and "poor" quality, were stained with microtubule-associated protein two (MAP2). MAP2 was chosen because it had been in the Won *et al.*[8] study to stain for dendrites. The antibody MAP2 was not initially selected because it requires an antigen retrieval step before staining[13]. Slides were stained with MAP2 using a standard fluorescent immunohistochemistry protocol and visualized using a Nikon Eclipse E300 (Tokyo). Poor staining results suggested that tissue quality was the problem. Furthermore, when compared to anti-beta III tubulin slides, MAP2 staining showed even better staining in high-quality tissue but not in poor-quality tissue; therefore, only the slides with high-tissue quality were stained with MAP2 for the remainder of the study, and test groups that stained poorly with both anti-beta III tubulin and MAP2 staining were eliminated from the study. Alexa Fluor 555 was chosen as the second antibody for MAP2 based on the host animal for anti-beta III tubulin (rabbit) and because it reacts with the antibody to give off green fluorescence[14].

Antigen retrieval: Since MAP2 requires an antigen retrieval step, a standard laboratory antigen retrieval procedures was run with the following modifications. Tissue slides were rinsed with 1X phosphate buffer solution (PBS) [8 g of NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 made to 1 L with dH_2O] for two five-minute periods. A step to agitate the slides in each immersion using an Orbit 300 (Edison, NJ) was added based on a reference by Chemicon International that indicated agitation facilitates dissolution of hydrogen bonds and better staining[11]. Then, slides were immersed twice for five minutes in 95 °C citric acid buffer solution [1.92 g of citric acid anhydrous, 0.5 mL of Tween 20, made to 0.05% with dH_2O] and then were immersed in a cold water bath. Slides were stored in PBS.

Fluorescent Immunohistochemistry: Tissue slides were rinsed with PBS for five minutes with shaking on the Orbit 300 (Edison, NJ). Then, slides were incubated with 3% bovine serum albumin (BSA) and shaken on the Orbit 300 for 30 minutes. The MAP2 antibody was diluted to a ratio of 1:100 with BSA, and then added to each slide. Each slide was covered with Parafilm for overnight incubation at 4 ºC. Slides were rinsed in PBS twice for five minutes and then incubated for one hour with the secondary antibody, Alexa Fluor 555, which was diluted to 1:200 with diethylpyrocarbonate (DEPC) H_2O. Slides were then rinsed in PBS with Triton twice for five minutes. Vector hardset with DAPI was pipetted onto slides, and then slides were cover-slipped and stored at 4 °C.

Analysis: A densitometry analysis technique outlined by Matkowskyi, Schonfield, & Benya[15] was updated to quantify density of dendrites with Adobe Photoshop CS6, which eliminated the need to manually count individual dendrites. Slides were photographed using a Nikon Eclipse E300 (Tokyo) with 20X and 40X objectives, and then, three sections on each slide were analyzed using Adobe Photoshop CS6 to quantify fluorescence from stained dendrites. To ensure uniform brightness, photo

brightness was adjusted to match one photo that was selected as a standard. The rectangular marquee tool in Adobe Photoshop CS6 was used to measure the mean gray-scale value (0-255) at three locations in each photo[16]. Analyzing three locations in each slide (for a total of six measurements for each test subject) was done to eliminate error that could result since mean gray-scale value (MGSV) measures fluorescence of the tagged dendrites in pixels, rather than numbers of dendrites expressed relative to number of neurons (i.e., dentrites/neuron). Additionally, to eliminate bias, analyses were blinded. The averages for mean gray-scale values were found; then, the hyperglycemic and control groups were compared using an unpaired Student *t*-test, with significance set at $p < 0.05$.

RESULTS

Figure 1 shows average mean gray-scale values from Adobe Photoshop CS6 analysis of dendrite density in the CA1 *stratum radiatum* regions of the hippocampus. Dendrite density in the hyperglycemic group was 33.5 ± 4.1 pixels whereas dendrite density in the CA1 *stratum radiatum* region of the hippocampus for the control group was 37.9 ± 2.6 pixels. There was significantly less fluorescence of dendrites in hyperglycemic tissue (RHEG) than in control tissue ($p = 0.024$).

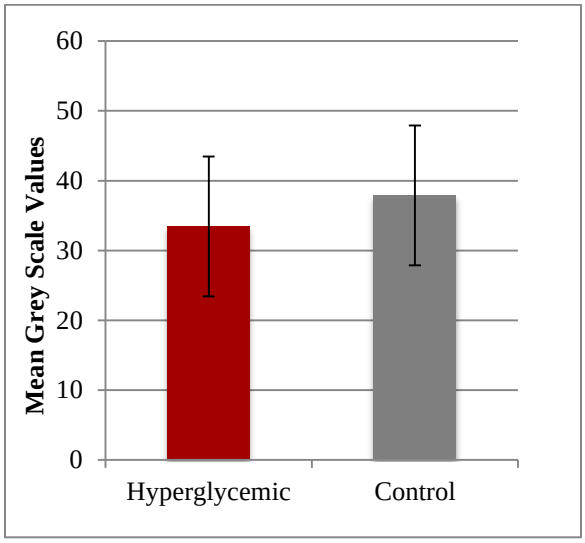

Figure 1. A two-tailed unpaired Student's *t*-test showed a significantly lower density of dendrites in the CA1 *stratum radiatum* region of the hippocampus in hyperglycemic (RHEG) than control tissue ($p = 0.024$). Bars show one standard error from the mean. Significance was set at $p < 0.05$ ($n = 4$).

DISCUSSION

Results from blinded measurements showed significant damage to dendrites in the CA1 *stratum radiatum* region of the hippocampus due to hyperglycemia. Rats were sacrificed at day 30 (eighteen days after the last dextrose injection), which is equivalent to 1.4 human years[17]. These results support our hypothesis that hyperglycemia results in long-term damage to the CA1 region of the hippocampus. These results also are consistent with the findings of Won *et al*. that showed significant long-term damage to dendrites in the CA1 area of the hippocampus in hyper-to-hypoglycemic induced rats[8].

Recognition of the effects of octreotide, which is an analogue of the naturally occurring growth hormone somatostatin, in the rat model from which we obtained tissue is important, since octreotide not only affects insulin production but also inhibits production of pituitary and gastroenteropancreatic growth hormones[18]. Since both the control and hyperglycemic groups received the same dose of octreotide, the lower dendritic density in the hyperglycemic group cannot be attributed to reduce secretion of growth hormone.

Our results are supported by a study by Tayman *et al.*[19] that came out after we completed our study. Tayman *et al*. used histopathologic and immunohistochemical analyses to determine concentration of the proteases caspase-3, -8 and -9 in the CA1 area of the hippocampus, which indicated neuronal death. Tayman *et al*. also found evidence of severe damage to the CA1 area of the hippocampus due to hyperglycemia[19]. Based on our literature review, our study is the first to identify hyperglycemic-induced dendritic damage in the CA1 region of the hippocampus based fluorescent immunohistochemical staining.

We also pioneered the use of Adobe Photoshop CS6 in our laboratory to analyze the results of fluorescent immunohistochemical staining. Adobe Photoshop CS6 effectively eliminated the need to manually count each dendrite, thus decreasing counting errors and time needed for analysis. However, use of Adobe Photoshop CS6 to analyze the fluorescence intensity required slides to be subjectively brightened or darkened to obtain a uniform color, which may have introduced error. Despite this possible limitation, Adobe Photoshop CS6 shows promising potential in analyzing fluorescent immunohistochemical stains.

A major limitation of our work was the age of the tissue samples we used. We began our study by focusing on quantitative polymerase chain reaction (qPCR) to quantify gene expression in hippocampus due hyperglycemia; however, we were unsuccessful in producing qPCR results after multiple trials, likely due to the old age of the tissue samples[20]. The probes we selected for qPCR that should be tried in future work on fresher tissues include postsynaptic density protein-95 (PSD-95), myelin-based protein, and brain-derived neurotrophic factor. PSD-95 is essential for synapse maturation and plasticity[21] and should support our fluorescent immunohistochemical results by confirming changes in the density of dendrites in the CA1 region of the hippocampus. Furthermore, myelin-basic protein should be used to measure gene expression of myelin, the insulating sheath that surrounds axons that is essential for normal impulse conduction and synaptic transmission[22,23]. Brain-derived neurotrophic factor is another important factor for synapse formation[21], which may also show the effects of hyperglycemia on synaptogenesis.

Given that the study by Won *et al*. that suggested dendrite damage from hypoglycemia was due to oxidative stress, future work should also include investigating the role of antioxidants in hyperglycemia, including the possibility of using vitamin C and/or glutathione in reducing oxidative effects on the developing brain[24]. If work shows that antioxidants are effective against preterm hyperglycemia, ways in which to block pathways by which oxidants cause brain injury should be explored.

Lastly, since cell apoptosis due to hypoglycemia has been shown to occur because of over activation of poly ADP-ribose polymerase-1 (PARP-1)[10], the role of PARP-1 in cell apoptosis should be investigated in hyperglycemia. PARP-1 is a nuclear enzyme responsible for DNA maintenance; however over activation can lead to depletion of NAD+/ATP and release of apoptosis inducing factor[10]. This work will involve using qPCR, Western blots, and fluorescent immunohistochemistry fluorescence to look at the expression of PARP-1 and its byproducts, which include apoptosis inducing factor, caspase 3, NF-κB, and bcl-2. Research in each of these areas will allow for ways in which to effectively treat hyperglycemia and explain why dendritic injury occurs in the hippocampus.

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