THE MECHANISM OF GLUCOSE METABOLISM IN STREPTOZOTOCIN-INDUCED DIABETIC CHICKEN EMBRYOS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Mechanism of Glucose Metabolism in Streptozotocin-Induced Diabetic Chicken Embryos.
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PURPOSE. The physiological and biochemical processes of the body are regulated by the circadian oscillators, the endogenous biological timing systems. The molecular mechanism of the circadian clock is composed by a specific set of genes, the “clock genes”, and their protein products through the interlocking transcription-translation feedback loops. There are many circadian oscillators in various tissues and organs most of them are regulated by the central clock (the “master clock”) found in the brain. However, in the retina, the circadian clock is not controlled by the brain. Past studies have found that disruption of the circadian clock can lead to metabolic syndromes, such as diabetes. This study will examine the diurnal profiles of retinal ERK and plasma glucose in streptozotocin-induced diabetes in chicken embryos.

METHODS. Diabetes was induced in chicken embryos (12 hour-12 hour light/dark cycle) with streptozotocin. Control and diabetic embryos were killed every 4 hours throughout the light-dark cycle. Retinal ERK and plasma glucose content was measured by Western blot.

RESULTS. Our results suggest that diabetic conditions alter the rhythm of the clocks in retinal cells. The diurnal phases of the phosphorylation of ERK were reversed and shifted towards the

light period in STZ-injected embryos when compared to the control embryos.

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CHAPTER I

INTRODUCTION

Circadian oscillators are biological clocks that control the rhythmic patterns of biological processes, physiology and organismal behavior in almost all living organisms. These circadian oscillators are self-driven with a cycle about a day (circa dian) in duration, and they function to synchronize the internal biological processes with the 24 h cycle of the external environment (reviewed in Pittendrigh *et al.* 1993). While the molecular mechanism of circadian oscillators, "molecular clock", is species dependent, the canonical model involves a specific set of the "clock" genes and their protein products. In mammals, the molecular clock consists of two interlocking transcription-translation feedback loops in which two basic helix-loop-helix-PAS (period-Arnt-Single-Minded) transcription factors, CLOCK (circadian locomotor output cycle kaput) and BMAL (brain and muscle, ARNT-like) 1 (Mop3) play a crucial role (reviewed in Ko *et al.* 2009). CLOCK and BMAL 1 heterodiamerize and bind to downstream genes containing E-box *cis* regulatory enhancer sequences in their promoting regions initiating transcription (Gekakis *et al.* 1998). The clock genes and their products work together to regulate transcription and translation in a rhythmic pattern thus playing a crucial role in establishing the circadian rhythm (reviewed in Ko *et al.* 2009).

In higher vertebrates, the 'master clock' located in the suprachiasmatic nuclei (SCN) of the brain functions to regulate most other peripheral oscillators in other tissues and brain areas (Bell-Pedersen *et al.* 2005; Balsalobre *et al.* 2000; Cheng *et al.* 2002). However, not all organs are regulated by the SCN. The oscillators in the retina work independently of the SCN allowing the

retina to anticipate daily light-dark cycling (Reme *et al.* 1991; Tosini and Menaker *et al.* 1996; Sakamoto *et al.* 2000) This anticipation is crucial for detecting ambient light, since the amount of light changes throughout the day creating a need for an adaptive mechanism (Chae *et al.* 2007).

Research in the past has found a correlation between disruption of the circadian clocks and diabetes incidence (Woon *et al.* 2007; Monteleone *et al.* 2008; Scott *et al.* 2008; Sookoian *et al.* 2008; Dupuis *et al.* 2010). Several transgenic mice studies have observed the occurrence of diabetes when the clock genes were wiped out (Shimba *et al.* 2011; Marcheva *et al.* 2010; Turek *et al.* 2005). Additionally, research has shown shift-workers to have an increased risk of cancers, cardiovascular diseases, and metabolic syndromes, which include diabetes (Knutsson *et al.* 1988; Vinogradova *et al.* 2009;Scheer *et al.* 2009). These shift workers are more active during the night and least active during the day. This causes their internal circadian cycle to become out of synchronization with the external environmental cycle leading to detrimental effects on the workers' health (Knutsson *et al.* 1988; Vinogradova *et al.* 2009;Scheer *et al.* 2009). Even though it is known that mutations in the clock genes trigger the onset of diabetes, it is not known whether diabetes might also have a detrimental effect on the circadian clocks.

The hypothesis of this study is that under the diabetic condition, the circadian clocks in the retina are affected adversely, which then leads to diabetic retinopathy. This study will examine the diurnal profiles of retinal ERK and plasma glucose in streptozotocin-induced diabetes in chicken embryos. Studying the effects of diabetes in the retina will eventually lead to an understanding of the mechanisms behind diabetic retinopathy. Diabetic retinopathy is a major complication in diabetes and is a dual disorder with vascular complications and retinal degeneration. It is one of

the leading causes of vision loss. (Lang *et al.* 2012).

CHAPTER II

METHODS

Chick embryos were maintained in 12:12 hr light-dark cycles starting from embryonic day 8 (E8). Diabetes was induced in chick embryos at E11 or E12 through administration of streptozotocin into the eggs. At E18, the retina tissues from both control and diabetic embryos were collected every 4 hours throughout the course of a day, and processed for protein analysis using Western immunoblotting, as previously described (Ko *et al.* 2007). The Zeitgheber time (ZT) 0 is the time when the lights are on, and ZT 12 is the time when the lights are off. Briefly, whole-cell extracts of retinas were lysed in 2× Laemmli buffer and boiled for 5 minutes. The samples were separated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes. Membranes were probed with primary antibodies specific for total AKT, pAKT, total ERK, pERK, and secondary anti-mouse or anti-rabbit antibodies, conjugated to horseradish peroxidase. An ECL detection system (Pierce, Rockford, IL, USA) was used to visualize the blots, and protein bands were quantified using Scion Image (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) (Ko *et al.* 2007). All experiments were repeated twice.

CHAPTER III

RESULTS

There was no significant difference of the body weights between the control embryos and the diabetic embryos (Figure 1). This is mainly because we measured the embryos only 7 days after the induction of diabetes. Approximately 64% of the diabetic embryos displayed cataracts, while approximately 8% of the control embryos displayed cataracts (Figure 2). Cataracts are a hallmark of embryonic diabetes, such as in the type I diabetes of human infants (Perucho-Martinez *et al.* 2007). We used the cataracts as an indicator of successfully induced diabetes in chicken embryos. The plasma glucose levels were higher in the diabetic group compared to the control group across different time points of the day (Figure 4). The plasma glucose levels for the control group were around 120 to 140 mg/dl, while the plasma glucose levels for the diabetic group were around 160 to 180 mg/dl. In conjunction with the data collected on cataracts, these results provide evidence that diabetes was successfully induced in the chicken embryos. As for the plasma glucose levels, both control and diabetic groups reached the highest plasma glucose levels at ZT 12. While the control group reached the lowest glucose level at ZT 8, the diabetic group reached the lowest glucose level at ZT 4. Previously, our lab demonstrated that the phosphorylation of ERK is under the circadian control in the retina (Ko *et al.* 2009). This indicates that the activity of ERK is under the circadian regulation. Therefore, the circadian rhythm of phosphorylated ERK, the pERK, is a good indication of whether the circadian rhythm was altered after STZ treatment. The two Western blots in Figure 5 for total ERK serve as our loading control, since the total amount of ERK should remain constant (Figure 5). Figure 6 illustrates the phosphorylation of ERK. This was determined by plotting the ratio of pERK over

total ERK. Phosphorylation of ERK was reversed and shifted towards the light period in STZ-injected embryos when compared to the control embryos.

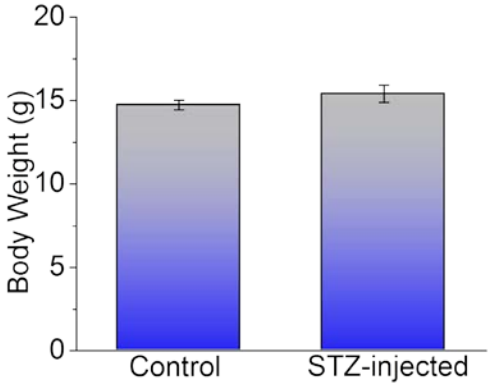

Figure 1. Embryonic Weight

	Cataracts
Control	3 / 36
STZ-injected	23 / 36

Figure 2. Cataracts

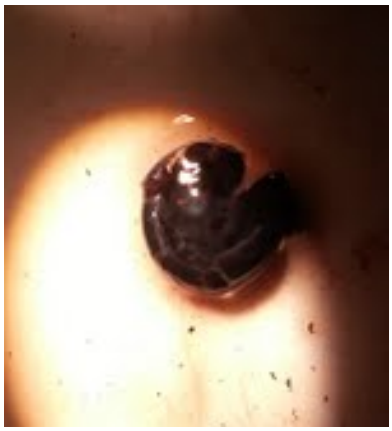

Figure 3. STZ-injected chicken eye

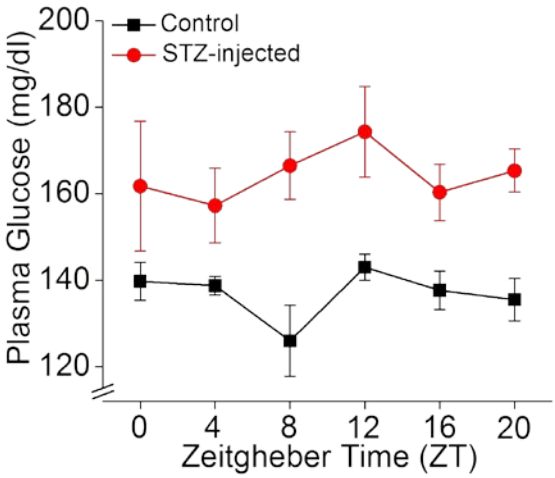

Figure 4. Plasma Glucose

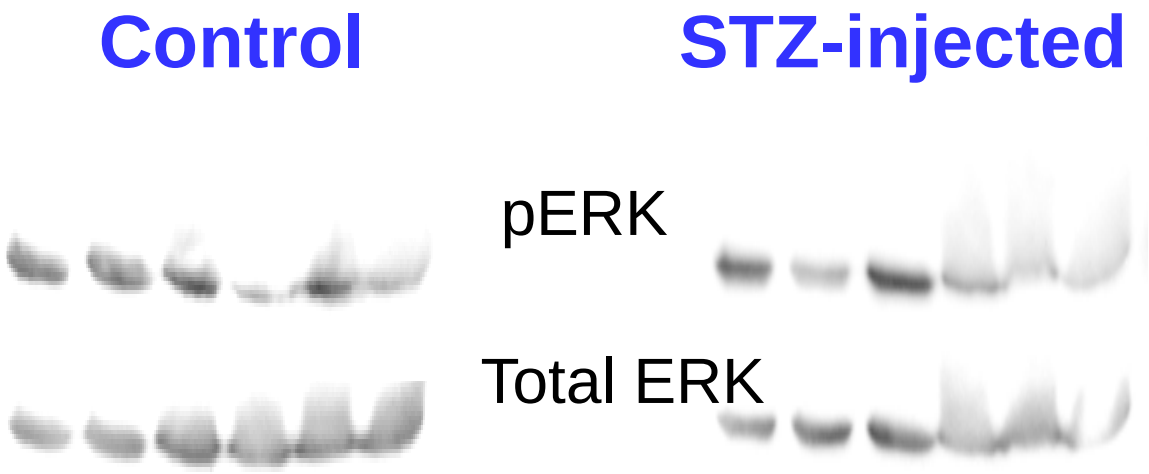

Figure 5. Western Immunoblots

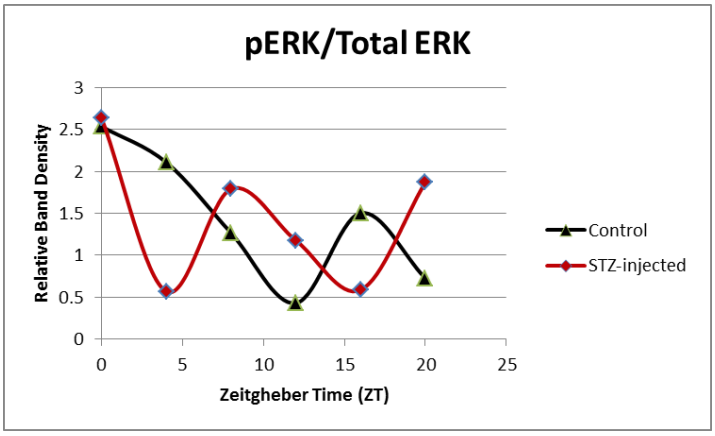

Figure 6. Phosphorylated ERK

CHAPTER IV

CONCLUSIONS

The results indicate that diabetes was successfully induced in the STZ-injected embryos. Cataracts were more frequently present in the STZ-injected embryos than in the control embryos and plasma glucose levels were, overall, higher in the STZ-injected embryos than in the control embryos. Furthermore, the results demonstrate that diabetic conditions alter the pattern of plasma glucose levels. Finally, our research suggests that diabetic conditions alter the rhythm of the clocks in retinal cells. The findings in this study are relevant to the current obesity trends in the United States. Diabetes has been on the rise since the last decade in the United States. In regards to adolescent diabetes, the country has seen a 23% increase from 2001 to 2009 (Mayer-Davis *et al.* 2012). It is not completely understood what triggers the disease or why the prevalence of the disease has been increasing. With the number of children diagnosed rising, one thing is for certain, that there is a need now more than ever to understand the disease and prevent it. By providing evidence for a cause and effect relationship between a dysfunctional clock gene and diabetes as bidirectional this study contributes to the scientific community's understanding of the disease.

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