THE EFFECTS OF POSTNATAL HYPERTHYROIDISM ON CORONAL SUTURE COMPLEXITY IN RABBITS WITH FAMILIAL, DELAYED-ONSET CRANIOSYNOSTOSIS

by

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Craniosynostosis results in abnormal biomechanical forces transmitted across the developing sutures and can be seen as increased interdigitation (sutural complexity). The present study was designed to examine a gene - environmental interaction by testing the hypothesis that postnatal thyroid hormone administered to rabbits with delayed-onset coronal suture synostosis (DOS) would result in an accelerated suture fusion and an increased sutural complexity compared to wild-type and in-colony normal rabbits.

138 coronal sutures were obtained from 69 rabbits, 13 wild type controls; 25 in-colony "phenotypically" normal rabbits, and; 31 rabbits with DOS. The three phenotypes were each divided into 3 treatment groups: untreated controls; vehicle controls, and; rabbits who received a 14 day course of treatment with 0.2 mg/kg of Triiodo thyronine (T3) (Sigma) in saline from 25 to 39 days of age. Longitudinal body weight and blood serum levels of T3 were taken and sutures were extirpated at 42 days of age. Suture images were captured digitally, sutural interdigitation was traced and measured using Image J, and a suture complexity index ((length/interdigitation length) x100) was calculated. Mean values were analyzed using a 3x3 (phenotype x treatment) ANOVA.

Rabbits treated with T3 showed significantly ($p<0.01$) decreased body weight and increased ($p<0.01$) T3 blood serum levels by 42 days of age. DOS rabbits showed significantly more suture complexity in all three treatment groups compared to controls (F Group= 3.15; $p<0.05$). Only wild-type rabbits with T3 treatment showed more complexity compared to their own phenotypic controls, however, no treatment or treatment by group effects were noted (F Treatment = 0.415;NS; F Group x Treatment = 1.93;NS).

Postnatal T3 exposure resulted in increased suture complexity only in wild-type control rabbits compared to rabbits with familial craniosynstosis. Results suggest that there was no

statistically significant gene - environmental interaction between elevated postnatal T3 levels and craniosynostosis in this model.

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PREFACE

To my dad, Mohammad Hosseinian who is my true inspiration in life. Thank you for being my role model all through these years and teaching me to be strong and passionate in life. Thank you for being a dad who every girl in the world envies to have. What I have learned from you in life is the reason I have gotten this far in my endeavors now and what I will reach in the future.

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1.0 INTRODUCTION

1.1 CRANIOSYNOSTOSIS

Craniosynostosis is defined as early closure of one or more cranial sutures. This closure will affect the skull shape. Timing and the number of sutures that are closed prematurely also affect the form of bones in the calvaria. Some examples can be observed in sagittal, coronal, or metopic synostosis which involve different sutures of the skull. This early suture fusion can happen before birth or after birth. The onset may be during infancy or childhood, but the effect of this suture closure on growth and development is more significant when it occurs earlier in life (Cohen 2000, 2005).

The cranial vault consists of the temporal, frontal, parietal, occipital, and sphenoid bones. The vault bones undergo intramembranous ossification from the mesenchymal tissues. These bones are connected to one another by fibrous connective tissue. The fibrous connective tissue junctions are called sutures, and in the vault, are called cranial sutures (Gray, 2000).

The skull cannot enlarge perpendicular to the fused suture. A compensatory mechanism is its growth in a direction parallel to the closed sutures. It compensates by growing more in the direction parallel to the closed sutures. This growth pattern results in enough space for the brain but causes an abnormal head shape and compensatory facial deformations. If the space for brain

growth is not enough, craniosynostosis will result in increased intracranial pressure, which may lead to visual and sleeping impairment (Slater, 2008).

Sutures serve as the most important centers of growth in calvaria in the very first years of life. The patency of the sutures and the brain growth are dependent to each other. The neurocranium grows when the brain pulls the two sides of the sutures apart from each other (Panchal 2003).

The sutures in the infant's skull are comprised of the coronal sutures, sagittal suture, metopic suture, and lambdoid sutures (Slater, 2008). The metopic suture closes during the first year of life (Cunningham, 2007). The coronal, sagittal and lambdoid sutures close between the second and fifth year of life (Cunningham, 2007, Cohen, 2000 Table-1). The closure of spheno-occipital synchondrosis which is considered another growth location of cranium occurs between ages 15-20 (Powell, 1963; Cendekiawan. 2010); the intersphenoid synchondrosis closes immediately after birth, and the spheno-ethmoidal synchondrosis closes approximately at 6 years of age birth (Cendekiawan, 2010).

Although cranial sutures are part of the synarthrotic group of joints they only allow for limited movement. Fontanelles which are defined as the confluence of two or more sutures in an infant have an essential role during vaginal birth. During this process the fontanelles which lack rigidity allow for a small shift in the skull bone), while the passage of the baby through the birth canal is achieved (Gray, 2000). Any movement at the joint is prevented when the fusion of the cranial sutures occur. The term synostosis (syn- no movement; ostosis- between boney segments) is used to describe a fused cranial suture; and the definition of craniosynostosis is a lack of movement between the two boney segments of a cranial suture (Gray, 2000; Kabbani, 2004; Delashaw, 1989).

After the physiologic growth for all cranial sutures is completed closure occurs. At age 6, the time for high peak of brain growth, development and maturity of brain is completed. We would observe various clinical consequences if suture fusion happens pathologically before the brain develop to the maximum growth level (Gray, 2000). Moreover, not only clinical symptoms of craniosynostosis are expressed phenotypically in many levels, a combination of genetic and environmental factors may result in the expression of craniosynostosis. In addition, craniosynostosis can be observed in isolated cases or part of a syndrome (Gray, 2000).

Craniosynostosis has deleterious effects on neural physiology and function since cranial sutures are replaced by bone before complete growth of craniofacial, skeletal and cranial mater components (Most, 1998). Mental retardation is a significant result of early fusion of cranial sutures which is a result of lack of growth of cranial mater which is observed in many syndromes which have the feature of early cranial suture fusion (Cohen 2000, 2005).

According to Most et al., the pathways underlying suture fusion and mechanisms that are involved in its development are not well known. It is possible to improve the knowledge of craniosynostosis by better understanding the mechanisms and factors involved in premature suture fusion (Most et al., 1998).

One of the ways that craniosynostosis can be categorized is to designate it into primary or secondary condition. Secondary craniosynostosis is when suture closure is secondary to a known condition. For example, in thalassemia or in hyperthyroidism, suture obliteration is a secondary condition to a known cause (Cohen, 1980). In addition, in secondary craniosynostosis environmental interactions affect the expressivity of fused sutures. Furthermore, Secondary craniosynostosis may result from teratogen exposure such as exposure to large doses of Vitamin

D, cocaine, or sodium valproate during pregnancy (Friedman and Mills, 1969; Gardner et al., 1998; Lajeunie et al., 2001).

Table 1. Suture Closure in humans (Cohen, 2000)

Cranial suture	Closure begins Year
Metopic[b]	2
Sagittal	22
Coronal	24
Lambdoid	26
Squamosal	35-39
Sphenofrontal	22
Sphenoparietal	29
Sphenotemporal	28-32
Masto-occipital	26-30
Facial suture	**Closure begins Year**
Palatal	30-35
Frontomaxillary	68-71
Frontozygomatic	72
Zygomaticotemporal	70-71
Zygomaticomaxillary	70-72
Frontonasal	68
Nasomaxillary	68

1.2 SUTURE MORPHOLOGY

"A suture is a craniofacial articulation in which contiguous margins of bone approximate each other and are united by a thin layer of fibrous tissue. During development, a presumptive suture becomes a definitive suture with interdigitation. Patent sutures cranial and facial eventually close with bony bridging" (Cohen, 2000).

1.2.1 Suture Function

Cranial sutures allow calvarial bones to have an "adjustive overlap" during birth. Human head can change form and compresse during passage through the birth canal. The skull decompresses

and will begin to have its normal shape by widening and expansion of sutures in the first week after birth (Cohen 2000). Sutures might inhibit external forces such as trauma that result in calvarial bone separation. In addition, sutures allow small movement and work as an absorber for the mechanical stresses of head trauma during in early childhood and infancy (Cohen, 2000).

Research on the calvaria of mouse has shown that histologically sutures develop at first by "wedge-shaped proliferation of cells at the periphery of the extending bone fields", which has been defined as the osteogenic front. The osteogenic fronts are believed to determine the suture morphology and its architecture. Osteogenic fronts have different ways of approximation. They can either overlap each other with an "intervening zone of Immature fibrous tissue" which will create overlapping suture or they can lead to an end-to-end type of suture which is the approximation of osteogenic fronts in the same (Cohen, 2000).

End-to-end sutures are found in the midline. Evidence shows that in humans, end-to-end type sutures are found in the sagittal and midpalatal suture. Beveled type, overlapping sutures are interestingly away from the midline and have an uneven biomechanical forces acting on them. Ridging is produced more often in Sagittal and metopic calvarial sutures (midline synostosis) rather than coronal and lambdoid sutures (non-midline synostosis).

Growing sutures are the site of continuous bone deposition and resorption. When sutures form at first they are straight edges of bone on both sides which are connected by a connective tissue layer (a sutural ligament). When sutures start to mature, interdigitations form and become more detectable. Herring has designed a grading system that categorizes sutures as straight, slightly interdigitated, interdigitated, and very interdigitated (Cohen, 2000).

The main reason underlying sutural interdigitation is to allow for flexible movements and also to contribute to stress reduction. The design and form of interdigitations might be due to the

distribution of forces which also depend on the type of forces as well. Interdigitated sutures have been observed in various complex patterns and complex architecture. The suture interdigitation pattern depends on the type of forces present when the suture was forming. Forces such as compressive, shear, and/or tensile forces all effect sutural bone, and connective tissue (Cohen, 2000).

1.2.2 Joint classification in the body

All joints in the body are categorized to three kinds: synarthrosis, which is immovable; amphiarthrosis, slightly movable; diathrosis, freely movable. Cranial sutures are also considered a type of anatomic joint. The bony joints in the skull mostly lack movement and are considered synarthrotic, these are the spaces in between two bones adjacent to each other with a band of connective tissue or hyaline cartilage. synarthrotic joints are subcategorized in four groups: sutura, schindylesis, gomphosis, and synchondrosis. Sutura are the types that only exist in the skull and a thin fibrous connective tissue separates the two bones adjacent to each other. Furthermore, in the category of sutura there are more subtypes. True sutures are sutures where the margins of two bones are interlocked together by indentations and processes. There are three variants of true sutures: sutura serrate, sutura dentate, and limbosa. The joint between the two frontal bones is called the metopic suture and demonstrates small, fine saw serrated teeth shape indentations and is called sutura serrate .The sagittal suture, which is formed between the parietal bones displays tooth like projections and is called a sutura dentate. In sutura limbosa, inter-lockings of the bones occur with a degree of overlap of the entire bone. This type of suture is the coronal suture seen between the frontal and parietal bones. False Sutures contains rough borders of two bones which are placed in front of one another, but lack the interlocking processes. False

sutures are categorized to two kinds, there are two kinds: sutura squamosa, and sutura harmonia ,the former is found between temporal and parietal bones and displays overlapping of the two bones; the latter is found between the two maxillae (and palatine bones) and demonstrates continuous rough surfaces. (Gray, 2000)

1.3 TERMINOLOGY AND CLASSIFICATION OF CRANIOSYNOSTOSIS

Terminology and classification of craniosynostosis were first based on the anatomy and therefore they still exist in the current literature. The first system of classification was first published by Virchow in 1851on the preceding work of Otto which described the morphologic nomenclature and was based on the variation forms of head shape (Cohen and Maclean, 2000). After the beginning of 20th century craniosynostosis was correlated with many genetic syndromes such as Apert and Crouzon, therefore a clinical genetic classification system became more and more prevalent in use. In the early 1990's, some of the genes for the most known craniosynostotic syndromes started to be identified. Therefore, another system called molecular genetic classification was established (Wilkie, 1997; Cohen and MacLean, 2000). Each nomenclature system has its own limitations thus; all three systems are used in current literature. A nomenclature needs to be established to correlate the molecular pathogenesis, phenotype, prognosis, and recurrence risk to the patient and family. Therefore, it is essential that specialists in the area of craniosynostosis be familiar with all three nomenclature systems (Jones, 2002).

1.3.1 Morphologic Nomenclature

Morphologic nomenclature is correlated with skull shape. The morphologic form is the result of fusion of sutures and the time and specific suture that is fused. The terms in this nomenclature are very descriptive. For example, Dolichocephaly means long head and is used for sagittal synostosis. Trigonocephaly means triangular- shaped head and is used for metopic synostosis and plagiocephaly means asymmetric head and is used for unilateral coronal synostosis (Jones, 2002).

Table 2. Current Morphologic Nomenclature of craniosynostosis (Jones, 2002)

Term	***Appearance***	***Affected Suture***
Dolicocephaly	Long head	Sagittal suture
Scaphocephaly	Keel-shaped head	Sagittal suture
Acrocephaly	Pointed head	Coronal, Coronal/Lambdoid, or all sutures
Brachycephaly	Short head	Coronal suture
Oxycephaly	Tower-shaped head	Coronal/lambdoid or all sutures
Turricephally	Tower-shaped head	Coronal suture
Plagiocephaly	Asymmetric head	Unilateral coronal, unilateral lambdoid, or positional
Kleeblattschadel	Clover-leaf skull	Multiple but not all sutures
Craniofacial dysostosis	Midface deficiency	Craniosynostosis with involvement of cranial base sutures

1.3.2 Clinical Genetic Classification

In case where craniosynostosis is associated with a group of other symptoms or conditions that consistently occur together such as Apert syndrome, an additional classification system is necessary; therefore the clinical genetic classification was formed. In the 1980's, there was an increased observation of cases of craniosynostosis in association with malformation of the limbs. Nearly 80% of syndromes associated with craniosynostosis involved abnormalities of the limbs. Questions were raised during that era whether the genes that are responsible for malformation of the limbs are the same as the genes associated with craniosynostosis. The same genes that caused limb defects or syndactaly in syndromes were questioned at being responsible for craniosynostosis (Jones, 2002).

Table 3. Clinical Genetic Classification (Jones, 2002)

Diagnostic Category	*Name of Disorder*	*Etiology*
Isolated craniosynostosis	Morphologically described	Unknown, uterine constraint or FGFR3 or EFNA4 mutation
Syndromic craniosynostosis	Antley-Bixler syndrome	POR
	Apert syndrome	Usually one of two common mutations in FGFR2
	Bacre-Stevenson syndrome	Mutation in FGFR2 or FGFR3
	Baller-Gerold syndrome	Mutation in TWIST heterogenous
	Carpenter Syndrome	RAB23
	Crouzon Syndrome	Numerous different mutations in FGFR2
	Muenke syndrome	Mutation in FGFR3
	Pfeiffer syndrome	Mutation in FGFR1 or numerous mutations in FGFR2

Table 4. Identified genes of genetic syndromes that may involve craniosynostosis (Jones, 2002)

Gene	*Mutation*	*Phenotype*
FGFR1	755C through G	Pfeiffer syndrome (milder phenotype)
FGFR2	Multiple	Apert, Bacre-Stevenson, Crouzon, Jackson-Weiss, Pfeiffer syndrome (severe phenotype)
FGFR3	Multiple	Bacre-Stevenson, Crouzonodermoskeletal, Muenke syndrome
MSX2	Pro148His	Boston-type synostosis
TWIST	Multiple	Baller-Gerold, Saethre-Chotzen syndrome

1.3.3 Molecular Genetic Classification

In the 1990's, specific genes began to be known in the syndromes such as Apert, Crouzon, and Pfeifer that are related to craniosynostosis. Mutations in TWIST were seen Baller-Gerold or Saethre-Chotzen syndromes. Mutations in FGFR1, 2 or 3 were seen in Pfeifer, Apert, Muenke, and other syndromes. Other genes have also been identified in other syndromes too. It is still unknown how mutations in different genes that are also seen in different syndromes can result in craniosynostosis. A classification that addresses these genes and syndromes were categorized as clinical genetic classification (Jones, 2002).

1.4 HERITABILITY OF CRANIOSYNOSTOSIS

The underlying causes of non-syndromic craniosynostosis in humans are not fully understood (Cohen and MacLean, 2000). Although the majority of cases seem to be sporadic, familial cases have been reported in the literature, suggesting a heritable component (Cohen et al., 1993; Lajeunie et al., 1995). Moreover, the discovery of fibroblast growth factor receptor (FGFR) activating mutations (Gripp et al., 1998; Renier et al., 2000) and mutations in the Msx2 homeobox domain (Jabs et al., 1993; Liu et al., 1995) in association with cases of apparently non-syndromic craniosynostosis support to a genetic hypothesis.

Segregation analyses in clinical cases show a variety of expression in phenotypes in craniosynostosis. This phenotypic expression can range from delayed-onset synostosis to complete early fusion of multiple sutures. Based on the available studies, the mode of transmission for simple, non-syndromic craniosynostosis is suggested to be autosomal dominant with slight to moderate reduced penetrance. Other genes and environmental influences also affect the penetrance of the gene. Reduced penetrance means that the major locus involved may be modified by these factors. (Lajeunie et al., 1995; Cohen et al., 1993).

1.5 EPIDEMIOLOGY OF CRANIOSYNOSTOSIS

1.5.1 Human Demographics and Epidemiology

Craniosynostosis can be syndromic or nonsyndromic (simple). Simple, nonsyndromic craniosynostosis has been estimated to occur in 300 per 1,000,000 live births (Cohen, 1986, 1989; Cohen and Kreiborg, 1992, Mooney et al., 1993). Coronal suture synostosis is observed approximately in 24% of the total cases of simple craniosynostosis. Coronal suture synostosis in infants has been linked with cranial vault abnormalities cranial base deformities and occasionally an increase in intracranial pressure (Cohen, 1989; Mooney et al., 1993).

Premature coronal suture synostosis is related to secondary deformities in the cranial vault and cranial base. It is also associated with midfacial growth abnormalities and pathologic

brachicephalization. Such severe skeletal anomalies result in wide-range, costly, and sometimes recurrent clinical and surgical management difficulties (Mooney et al., 1993).

1.5.2 Diagnosis of Craniosynostosis

Premature craniosynostosis can be observed as either simple synostosis with premature closure of one or more of the cranial sutures causing reduced or arrested growth perpendicular to the closed suture(s). This form of synostosis happens in ninety percent of cranial premature closures. Ten percent of the cased happen in more complex craniofacial syndromes. In these complex craniofacial anomalies hypoplasia of the maxilla is observed which a significant feature of the orbits' development. A high frequency of mental retardation is seen in patients with premature craniosynostosis. The underlying cause is caused by reduced intracranial volume which is due to an increased intracranial pressure. In these patient early intervention is essential in order to prevent secondary damage to the brain and achieve aesthetic results. Complex Craniofacial syndromes with facial hypoplasia entail a multi-disciplinary treatment. Patients with simple craniosynostosis should be diagnosed and treated within the first six months after birth.

The diagnosis of craniosynostosis usually happens when the physician or the parent suspects the normal morphology of the skull in the infant. Clinical examination of a patient with craniosynostosis includes different measurements. The head circumference, assessment of skull and limb deformities and the child's growth curve plots. Skull deformities can be assessed from a superior view (bird's eye view), posterior view, and/or anterior view of the skull. Asymmetry is most important factor in the diagnosis of abnormal growth. This asymmetry can be present in eyes, ears and nose in addition to overall head figure.

The gold standard for radiographic analysis and diagnosis of craniosynostosis is computed axial tomography (CT). 2 dimensional radiographs such as dorsoventral or lateral cephalogram radiographs can be used to diagnose single suture synostosis. CT scans are much more precise in diagnosis of craniosynostosis and identification and extent of deformity in the skull (Medina, 2000).

1.5.3 Genetic factors

Craniosynostosis has been associated with genetic factors. While syndromic forms of craniosynostosis are known to result from mutations in several genes (FGFR, TWIST1), the etiology of non syndromic craniosynostosis (NSCS) is still poorly understood. Although a large number of sporadic cases have been reported, genetic risk factors are clearly involved, as evidenced by excess familial aggregation in all three major forms of NSCS: coronal (Lajeunie et al. 1995), sagittal (Lajeunie et al. 1996) and metopic (Lajeunie et al. 1998). Mutations in several specific genes, including FGFR3 and MSX2, have been implicated in apparent NSCS suggesting some overlap with syndromic forms of the disease (Gripp et al., 1998; Renier et al., 2000; Jabs et al., 1993; Liu et al., 1995). However, these known mutations comprise only a very small portion of NSCS cases.

1.5.4 Environmental Factors

Numerous environmental risk factors and systemic disorders are also associated with NSCS (Cohen, 1980; Friedman and Mills, 1969; Gardner et al., 1998; Lajeunie et al., 2001; Shashi and Hart, 2002). Notable among these is maternal hyperthyroidism. Thyrotoxicosis is estimated to

occur in 1 in 500 pregnancies and can result from a several causes: maternal Graves Disease (Krude et al., 1997; Segni et al., 1999; Radetti et al., 2002;), maternal hyperthyroidism as a consequence of thyroid hormone replacement therapy (Daneman and Howard, 1980; Chiovato et al., 1991; Hirano et al., 1995; Zimmerman, 1999; Radetti et al., 2002), or the presence of a thyrotoxic goiter (Robinson et al., 1969; Riggs et al., 1972; Hollingsworth and Mabry, 1976; Radetti et al., 2002).

The medical literature is replete with reports of premature synostosis resulting from maternal hyperthyroidism (Menking et al., 1972; Penfold and Simpson, 1975; Chiovato et al., 1991; Krude et al., 1997; de Lima et al., 1999; Segni et al., 1999; Hashmi et al., 2012). In a recent major population-based cohort study, investigators at the Center for Disease Control identified maternal hyperthyroidism as a potential risk factor for craniosynostosis in offspring, with an odds ratio of 2.47 (Rasmussen et al., 2007). Although several investigators have shown evidence of increased Insulin-Like Growth Factor (IGF) expression at the suture site in developing mice following exposure to exogenous thyroid hormone, the pathogenetic mechanisms leading to hyperthyroid-induced secondary craniosynostosis remain unknown.

1.5.5 Vitamin D Deficiency and Rickets

Rickets is the failure of bone calcification due to impaired metabolism of vitamin D, phosphorus or calcium, potentially resulting in fractures and deformity. The main cause is a vitamin D deficiency, but lack of sufficient calcium in the diet may also lead to rickets. (Shashi and Hart, 2002).Vitamin deficiency can be a result of insufficient dietary intake, liver disease renal failure

or hypophosphatasia which are all linked to craniosynostosis (Coleman and Foote, 1954; Fraser, 1957; Reilly et al. 1964; McCarthy and Reid, 1980; Shashi and Hart, 2002).

Reily et al in 1964 observed that one third of 59 children with Rickets had premature fusion of sutures and the severity of Rickets was proportionally linked to severity of craniosynostosis. Willis and Beaty in 1997 described cases of X-linked hypophosphatemic rickets and its relation to craniosynostosis and they suggested that radiographic screening should be offered to all these patients. Therefore we can conclude that rickets is "causatively" linked to craniosynostosis (Shashi and Hart, 2002).

1.5.6 Teratogens

Several teratogens have been implicated in the causation of craniosynostosis. Phenytoin ingestion during pregnancy has been found to result in fusion of sagittal and coronal sutures (Char et al. 1978). Retinoic acid is another substance that has been found to result in craniosynostosis. In three out of eight cases, as one of the features of retinoic acid embryopathy, Lammer et al. reported that treatment of pregnant women with retinoids resulted in craniosynostosis (Lammer et al.1985).

Valporate, an anticonvulsant medication which is associated with cleft lip and palate has also been associated with metopic ridging. (Ardinger et al., 1988). In a study by Lajeunie et al. (2001) out of 1676 cases, 17 mothers were found to have undergone regular treatment with sodium valproate monotherapy at the time of their pregnancies, all 17 children exhibited trigonocephaly which is caused by premature fusion of metopic suture.

Exposure to aminopterin/methotrexate prenatally had an increased incidence of craniosynostosis (Milunsky et al., 1988). Fluconazole an antifungal medication was reported to

increase the risk of craniosynostosis in two out of four infants who had a specific phenotype that resembled an autosomal recessive condition, Antley- Bixler syndrome, in which craniosynostosis is common(Aleck and Bartley,1997). Mutchinick et al. (1992) and Enns et al. (1999) reported that Cyclophosphamide, an alkalating agent used in cancer chemotherapy, was found to have in two out of seven exposed infants. Moreover, it was observed that in rabbit whose mothers received large amounts of vitamin D during pregnancy, the most marked functional accompaniment of these abnormalities was premature closure of the cranial bones, severe malocclusion and peculiar faces (Friedman and Mills, 1969).

1.5.7 Hyperthyroidism

Hyperthyroidism has been associated with craniosynostosis in numerous studies. It has been related to congenital and auto-immune diseases such as Graves disease or as a consequence of thyroid replacement therapy, (Krude et al., 1997; Segni et al., 1999; Zimmerman, 1999; Shashi and Hart, 2002).

Thyrotoxicosis is estimated to occur in 1 in 500 pregnancies and can result from a several causes: maternal Graves disease (Krude et al., 1997; Segni et al., 1999).

According to Krude et al., craniosynostosis is a significant complication of infants that have "consistent hyperthyroidism" (Krude et al 1997). In addition, Zimmerman and Segni et al. designate craniosynostosis as one of the main symptoms of fetal and neonatal hyperthyroidism (Zimmerman, 1999; Segni et al, 1999).

In a study by Johnsonbaugh and colleagues, they found out that hyperthyroidism was also identified as a potential cause for craniosynostosis. They evaluated suture fusion radiographically in children with hyperthyroidism, adrenal hyperplasia, precocious puberty and normal children.

All subjects who had craniosynostosis, also were the group with hyperthyroidism, none of the children from the other groups had craniosynostosis. They concluded that the sutures may be very sensitive to excess thyroid hormone (Johnsonbaugh et al, 1978; Shashi and Hart, 2002).

According to Shashi et al, in a study that was conducted by Leonard et al. they found out that fetuses with an underlying craniosynostotic condition, that would otherwise not express the condition, may be more likely to develop the signs and expression of craniosynostosis if their mother has hyperthyroidism. It was reported that two infants developed craniosynosotosis (a 4 month old with bicoronal synostoses and a 15 month old with pansynostoses) whose mothers had Graves disease. The mothers' hyperthyroid condition was considered the reason for the premature suture fusion (Shashi and Hart, 2002).

In a recent major population-based cohort study, investigators at the Center for Disease Control identified maternal hyperthyroidism as a potential risk factor for craniosynostosis in offspring, with an odds ratio of 2.47 (Rasmussen et al., 2007).

Akita et al., studied fusing sutures in a hyperthyroid rat model (Akita et al 1994, 1996). Akita concluded that excess administration of thyroid hormone enhanced the cranial suture closure, increased local IGF-1, and that local IGF-I played an important role in the sutural closure (Akita et al. 1996).

Although several investigators have shown evidence of increased Insulin-Like Growth Factor (IGF) expression at the suture site in developing mice following exposure to exogenous thyroid hormone, the pathogenetic mechanisms leading to hyperthyroid-induced secondary craniosynostosis remain unknown. Further studies purpose is to understand the general osteogenic role of both Thyroid hormone and local IGF-1. (Rizzoli et al. 1986; Wolf et al. 1989;

Lakatos et al. 1993 and 2000; Thaller et al. 1993a and 1993c; Varga et al. 1994; Klaushofer et al. 1995; Wakisaka et al. 1998; Huang et al. 2000; Rizos et al. 2001; Conover and Rosen, 2002).

1.6 SUTURE COMPLEXITY AS A PHENOTYPIC OUTCOME OF CRANIOSYNOSTOSIS

One of the consequences of premature suture fusion is altered biomechanical forces transmitted across the remaining patent sutures. These altered forces are generated from the growing brain and cranial base acting on a skull vault that can no longer expand to accommodate this growth. In response to biomechanical forces, sutures adapt by changing their morphology. There is ample evidence that sutures become morphologically more complex, as measured by the degree of interdigitation, when they are subjected to loading forces. This increase in complexity is observed during normal skull growth, in response to rapid brain expansion in early childhood and the biomechanical stress of typical activities like mastication (Wu et al., 2007).

Experimental studies on animal models have shown that altering biomechanical stresses can result in changes in suture complexity in a predicable manner. Early studies by Moss (1957, 1961), for example, showed that when sutures are experimentally removed from their normal mechanical loading environment, they lose their interdigitation and revert to simple butt joints. The relationship between suture complexity and mechanical stresses is further supported by a wide variety of observational studies. Archeological studies of skulls from populations that practiced artificial cranial deformation indicate an increase in suture interdigitation compared to skulls from reference populations (Gottleib, 1978; Anton et al., 1992). The relationship between

increased masticatory muscle loading and increased cranial suture complexity has been shown in both laboratory animals (Byron et al., 2004) and wild populations (Byron, 2009).

Despite the fact that craniosynostosis clearly results in abnormal biomechanical forces transmitted across the developing sutures, little work has been done to characterize the degree of sutural interdigitation when synostosis is present. Hinton et al. (1984) showed an increase in interdigitation of the lambdoid suture in a series of lambdoid synostosis cases. Burrows et al. (1997) showed an increase in the frequency of sutural bones (another phenotypic marker for altered biomechanical forces) in the same sample of craniosynostotic rabbits described earlier. Preliminary reports have shown increased interdigitization (Kreithen et al., 1998) and altered bony trabecular orientation (Ozaki et al., 2000) postnatally in 25-42 day old delayed-onset rabbits with fusing coronal sutures.

1.7 AIM OF STUDY

The aim of this project was to identify the environmental influence of post-natal administration of tri-iodothyronine (T3) in rabbits that demonstrate familial delayed onset craniosynostosis when compared to in-colony normal and control rabbits. The hypothesis was tested in a well-established rabbit model of familial craniosynostosis with variable expression (Mooney et al. 1994a, 1994b, 1996, 1998, 2002). This rabbit model, similar to humans, demonstrates autosomal dominant transmission with incomplete penetrance (Mooney et al. 1996), and a broad range of phenotypic expression that includes: phenotypically normal animals that carry the mutation, unilaterally affected animals with postnatal or delayed-onset synostosis, animals presenting with

complete bilateral fusion with prenatal or early-onset, or animals with severe synostosis that do not survive (Mooney et al. 1998).

These rabbits possess a broad spectrum phenotype and provide a unique opportunity for investigating the relationship between circulating thyroid hormones and suture pathology. In the presence of excess tri-iodothyronine (T3), the pattern of craniosynostotic progression in affected and unaffected rabbits may be altered, such that changes in the timing and severity of sutural hyperostosis will be followed.

1.8 PURPOSE OF PRESENT INVESTIGATION

If increased sutural complexity is a consequence of altered biomechanical forces vis-à-vis premature synostosis, then any factor capable of exacerbating synostotic progression should also result in further pushing of the suture toward an abnormal morphological state. The present study tested the hypothesis that exogenous thyroid hormone administered at 25 to 42 days of age to rabbits with delayed-onset craniosynostosis will result in accelerated premature coronal suture fusion resulting in increased coronal suture interdigitation compared to both unexposed affected and control rabbits.

1.9 HYPOTHESIS

The present study was designed to examine a gene - environmental interaction by testing the hypothesis that postnatal thyroid hormone administered to rabbits with delayed-onset coronal

suture synostosis (DOS) would result in accelerated suture fusion and increased sutural complexity compared to wild-type and in-colony normal rabbits.

2.0 MATERIALS AND METHODS

138 coronal sutures were obtained from 69 rabbits were utilized in the present study. A 3 x 3 (phenotype x treatment) design was employed. The phenotype variable included: 1) wild type controls (n=13); 2) normal in-colony rabbits (n=25), and: 3) rabbits with delayed onset coronal suture synostosis (n=31). The treatment variable included: 1) untreated controls; 2) sham or vehicle treated controls, and: 3) rabbits treated with tri-iodothyronine (T3). Vehicle control treatments groups received buffered saline every three days from 25 days of age to 42. Surgical sham control groups only received amalgam markers with no further treatment. Thyroid hormone treatment group received 0.2mg/kg dose of tri-iodothyronine (T3) every 3 days beginning at 25 days of age to 42.

Numbers for the all groups of rabbits are as follows (See Table 5):

Table 5. Number of rabbits in each group

	Control	***Vehicle***	***T3***	***Total***
ICN	7	9	9	25
DOS	11	8	12	31
WT	4	4	5	13
Total	22	21	26	69

2.1 ACQUISITION OF SAMPLE

A total of 69 New Zealand white rabbits (Oryctolagus cuniculus) were utilized for this study. Following a standardized breeding protocol (Losken et al. 1993; Mooney et al. 1994b). 31 rabbits with delayed-onset craniosynostosis were obtained from an existing breeding colony with variably expressed familial, non-syndromic coronal suture synostosis and Twenty five (25) phenotypically normal in-colony rabbits were obtained from the same existing breeding colony, but without coronal suture synostosis and thirteen (13) wild type controls rabbits were purchased from a commercial breeder (Charles River,251 Ballardvale Street, Wilmington, Massachusetts 01887) to serve as out-colony wild type control rabbits. All rabbits were housed and maintained in the Physical Anthropology Laboratory and Vivarium (University of Pittsburgh, Department of Anthropology). All rabbits were fed a standard diet of rabbit chow and water for the duration of the experiment. Sample sizes of ten per group were based on power calculations from a previous study (Cray et al., 2012). With the calculated effect size of 0.93, and setting alpha at 0.05, a total of ten per group should result in a power of 94.6%. Statistical calculations were made using the SPSSPC, sample power program. Full IACUC approval was obtained for this study.

2.2 DETERMINING AFFECTION STATUS AND IMPLANTING AMALGAM MARKERS

At 10 days, radiopaque suture markers were implanted to monitor suture growth and help identify and diagnose the in-colony rabbits. First, rabbits were anesthetized with an intramuscular (IM) injection of a solution comprised of 91% Ketaset (ketamine hydrochloride, 100mg/ml) and 9% Rompun (xylazine hydrochloride, 20mg/ml) at a dose of 0.59ml/kg body weight. Following anesthetization, hair was removed from the scalp and prepared with betadine and a midline incision was made with a surgical knife in the skin overlying the calvaria. The skin was then undermined and reflected to facilitate visual inspection of the sutures. At this point, an initial visual diagnosis was attempted for all rabbits based on synostotic progression. A 0.4mm dental burr was used to make six holes in the periosteum and bone. Three sets of holes were made 2mm lateral to the mid-sagittal plane on the animal's left side: 2mm anterior and posterior to the coronal, frontonasal and anterior lambdoidal sutures. Each hole was then packed with silver amalgam to serve as radiopaque markers. Following marker implantation, the skin was closed with 4-0 resorbable vicryl sutures and the rabbits were administered antibiotics (Baytril—Bayer Health Care LLC, from Med Vet International) through IM injection in order to prevent post-operative infections. All rabbits were monitored closely to assure full recovery.

The delayed-onset synostosis (DOS) rabbit model presented with gross sutural abnormalities by 25 days of age (Mooney et al., 1994b). Following the initial attempt at 10 days to visually diagnose the rabbits, suture marker separation from radiographs was used to confirm initial observations and diagnose DOS. By plotting sutural growth against somatic growth curves, it was possible to diagnose rabbits as having slow growing or normal sutures by 25 days of age. Rabbits that had less than 2.2mm of bilateral coronal marker separation were given the

diagnosis of delayed on-set craniosynostosis. Those that had more than 2.2mm bilaterally were diagnosed as phenotypically normal, in-colony rabbits. A threshold of 2.2 mm of growth across the coronal suture was used to classify rabbits as either delayed-onset (falling below the threshold) or in-colony unaffected (at or above the threshold). The threshold value represents the lower bound of the 95% confidence interval for growth at the coronal suture in normal (wild type) rabbits at this same time point.

2.3 DEVELOPING THE EXPERIMENTAL MODEL

Following diagnostic confirmation at 25 days of age, rabbits within each of the two- phenotypes (In-colony normal and Delayed onset synostosis rabbits) were also randomly assigned to one of the three treatment groups. These three groups included: 1) no-treatment controls, 2) vehicle injection control group (sham), and 3) tri-iodothyronine (T3) treatment. T3 was administered for 17 days, beginning at 25 days of age. Rabbits in the treatment groups received subcutaneous injections every three days of tri-iodothyronine (half-life 2-3 days, Wiersinga, 2001) dissolved in water/ethanol solution, buffered with sodium hydroxide, at a dose of 200 μg/kg body weight similar to therapeutic hormone replacement dose for hypothyroidism (Tremblay et al. 1977; Banerjee, 1983; Chizzonite et al. 1984; Sadiq et al. 1985; Saeki et al. 1987; Seiden et al. 1989; Goto et al. 1990; Szymanska et al. 1991; Boerth and Artman, 1996; Jiang et al. 2000; Ozdemirci et al. 2001). Rabbits in the vehicle injection control (sham) group likewise received treatments for 17 days beginning at day 25, and every third day through 42 days. These injections only contained the saline solution without tri-iodothyronine. Serum T3 and T4 levels were taken at 42 days from the marginal ear vein with a 25 gauge butterfly needle, and analyzed by enzyme

immunoassay (AHDC Endocrinology Laboratory, Cornell University, Ithaca, NY). All treatment ended when the rabbits reached 42 days of age. The treatment period, between 25 and 42 days, was chosen because it is characterized by rapid somatic and craniofacial growth. Data collection stopped at 42 days of age due to craniofacial growth plateau at 42 days of age for New Zealand white rabbits.

2.3.1 Image Acquisition and Suture Measurement

At 42 days of age, the rabbits were euthanized with an IV (40mg/kg) injection of pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). A 5mm x 20mm block of calvaria containing both right and left coronal sutures were identified and extirpated. The specimens were preserved in 10% buffered neutral formalin.

Dorsoventral digital images of the extirpated coronal sutures were captured in a standardized fashion using a Leica MZ12 Stereo Zoom microscope with a Sony DKC-5000, 3 CCD digital camera attachment. Each image was imported in to Adobe Photoshop, adjusted for contrast and brightness, and a start point (at the bregma) and a stop point (at the lateral termination of the coronal suture) was marked with colored dot. Using the image analysis program Image J (NIH), the linear distance between the start and stop point was calculated; this measurement is the *linear suture length* (Figure 1). Then the entire suture length was measured from the start point to the stop point following the interdigitation pattern; this measurement is the *total suture length* (Figure 2). A *suture complexity index* was calculated by dividing the linear suture length by the total suture length. The lower the index, the more complex the suture is. A simple suture with no discernable interdigitation will have an approximate an index value of 1. The same procedure was carried out separately on the left and right portion of the coronal suture.

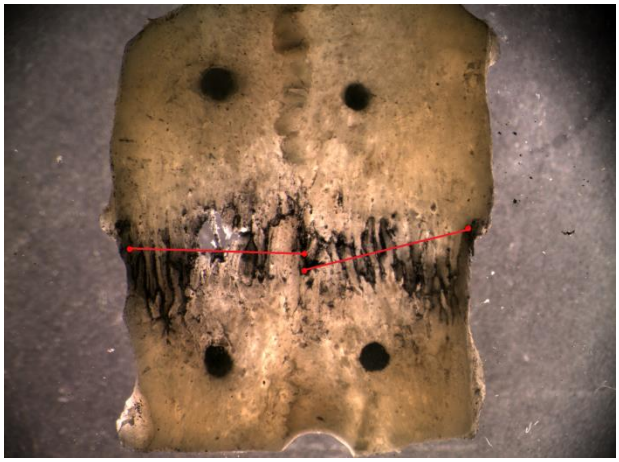

Figure 1. Linear suture length measurement

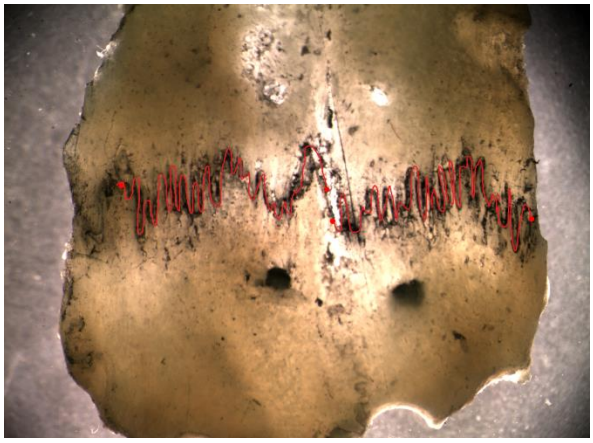

Figure 2. Total suture length measurement

2.4 DATA ANALYSIS

2.4.1 Morphometric Statistical Analysis

The mean coronal suture complexity index was compared among groups using a 3 x 3 (phenotype x treatment), analysis of variance (ANOVA). Significant intergroup differences were assessed with the LSD multiple-comparisons test. All data were analyzed using SPSS v. 19. Differences were considered significant if $p \leq 0.05$. Intra- and inter-rater reliability were measured as follows (Table 6).

Table 6. Intra-rater and Inter-rater reliability measurements

Suture length	Intra-rater Reliability	Inter-rater Reliability
Left Total Suture length	0.929	0.932
Left Linear Suture Length	0.995	0.632
Right Total Suture length	0.932	0.928
Right Linear Suture Length	0.989	0.515

3.0 RESULTS

3.1 THYROID HORMONE BLOOD LEVELS

Results of blood serum levels for tri-iodothyronine (T3) and thyroxine (T4) are represented in units of ng/ml and µg/ml respectively and are presented in Figure 3 and Figure 4. Results show that mean T3 levels were significantly elevated in the wild type, delayed on-set and in-colony normal rabbits treated with T3 when compared to the control no-treatment or vehicle-control groups ($F=13.48$; $P<0.001$). There was no statistical difference between levels of T3 between the treated groups (F treatment = 1.39; NS). Furthermore, there were no statistical differences in the control groups of either the wild type ,delayed on-set or in-colony normal rabbits, meaning the untreated groups were not different from the colony as a whole ($F=2.26$; p: NS).

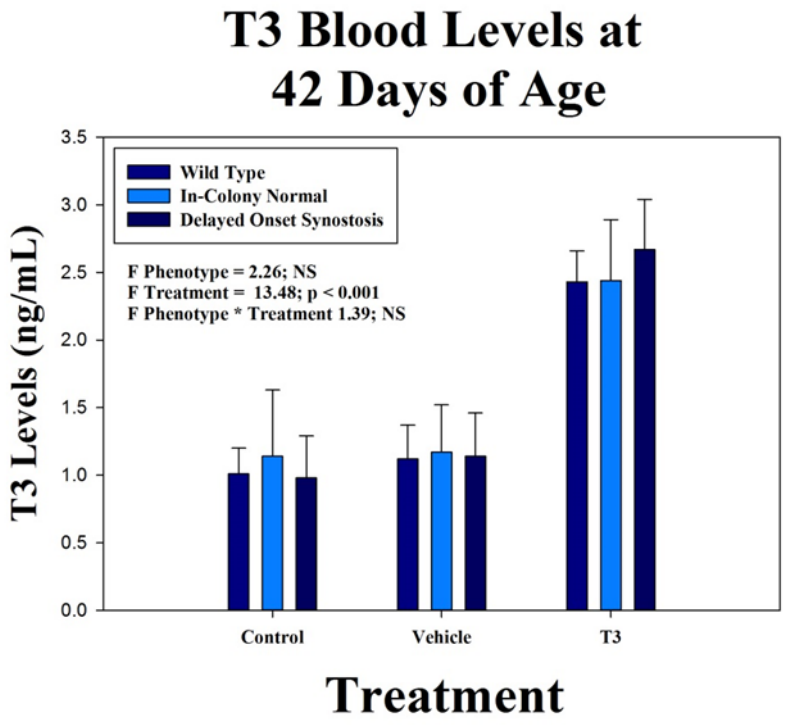

Figure 3.T3 levels significantly increased in treated rabbits

Mean levels of T4 were lowered in treatment groups (Figure 4). Wild type, delayed on-set rabbits or in-colony normal rabbits (F=48.73.P<0.001) .There were no statistical differences of T4 levels between the three treated groups (F = 0.55; p NS). There were no varying levels of T4 between the control groups of either phenotype, delayed on-set or in-colony normal (F = 1.52; p NS).

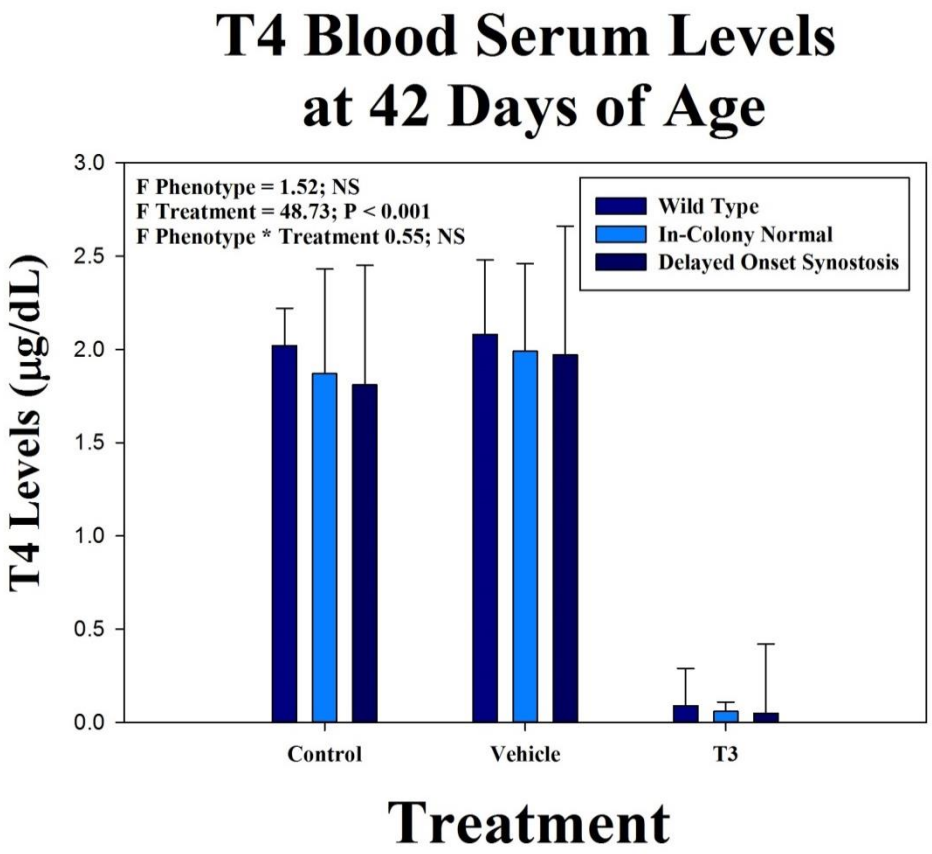

Figure 4. T4 levels significantly decreased in treated rabbits.

3.2 BODY WEIGHT

Figure 5 shows mean changes in body weight by phenotype and control group. At 42 days of age, all treatment groups of T3 show reduced but non-significant body weights of the rabbits when compared to the control rabbits (F = 0.53; NS). There were no statistical differences in rabbit body weights between control groups amongst the phenotype groups (F=1.12; NS), nor between the three treatment groups in either phenotype group (F=0.77; NS).

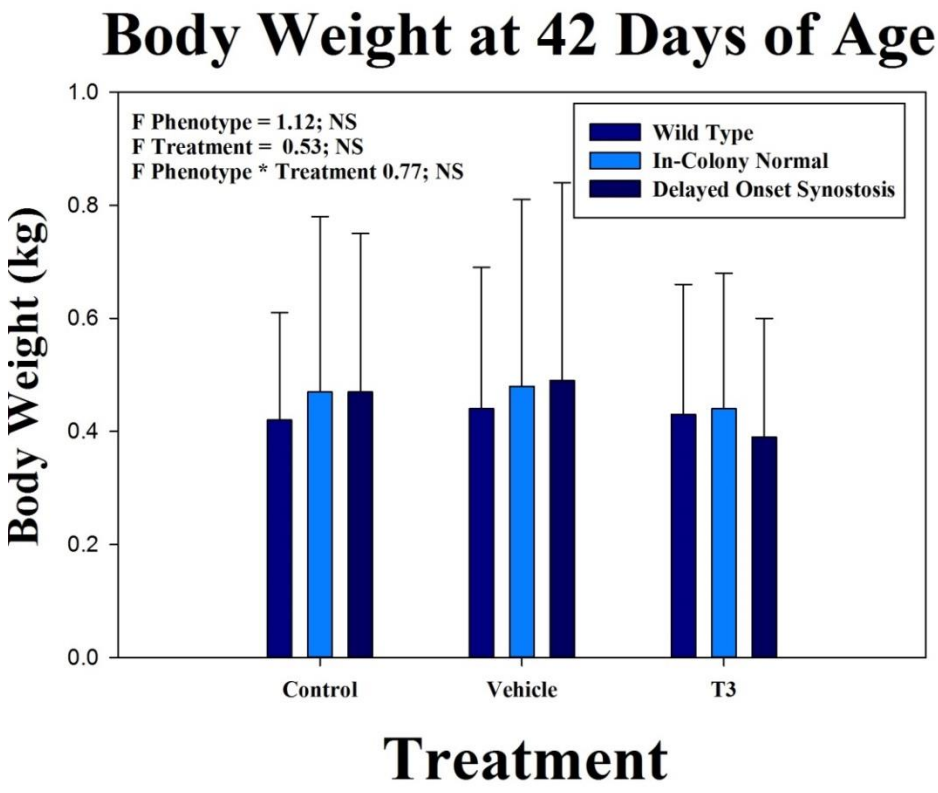

Figure 5. Treated rabbits with T3 show decreased body weights.

3.3 SUTURAL COMPLEXITY

3.3.1 Qualitative Suture Analysis

Figures 6, 7, and 8 depict the three different groups of sutures; wild type (Figure 6), in colony normal (Figure 7) and delayed onset groups (Figure 8). Each group is comprised of control with no treatment group, sham vehicle group, and thyroxin group. The figures show the microscopic photograph of the coronal suture which illustrates how the lineal suture length and total suture length were measured in each group. Amalgam markers are also observed in each figure. The interdigitation of sutures is detected. In wild type treatment group, the suture has a more complex suture pattern and an increased total suture length in comparison to the control and sham vehicle groups. In the in colony normal treatment group we observe the same amount of complexity in sutures when compared to phenotypic sham vehicle and control groups. Also, in the delayed onset groups we observe a non-significant difference in suture complexity when compared to phenotypic sham vehicle and control groups. In all groups the interdigitation of sutures at bony surfaces can be appreciated.

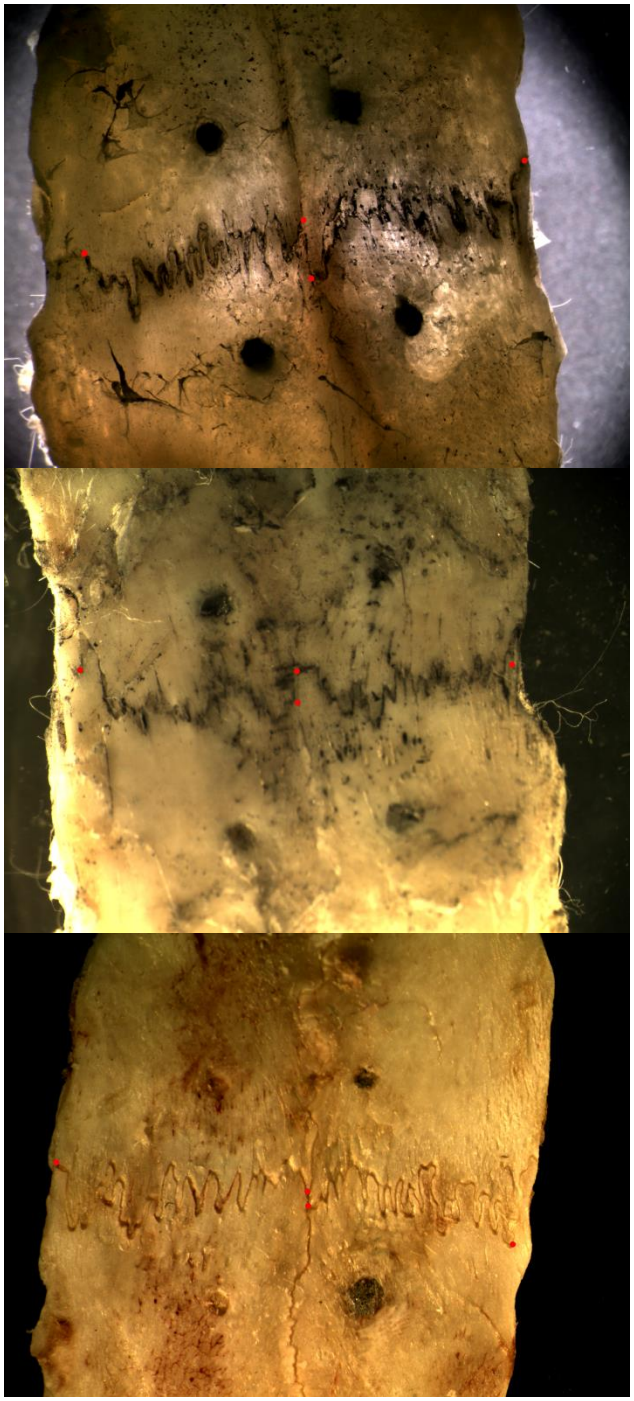

Figure 6. Sutures in Wild type groups. Top picture: Control with no treatment group, Middle picture: Sham vehicle group, Bottom picture: Thyroxin group

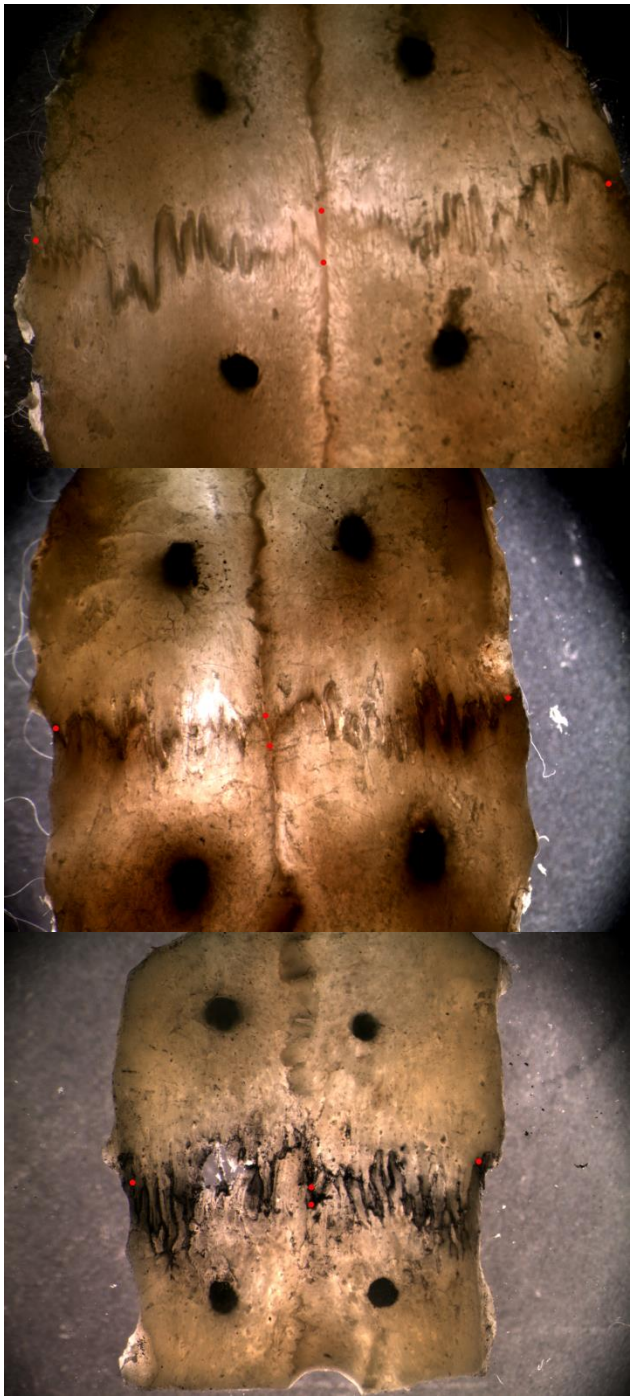

Figure 7. Sutures in In Colony Normal groups. Top picture: Control with no treatment group, Middle picture: Sham vehicle group, Bottom picture: Thyroxin group

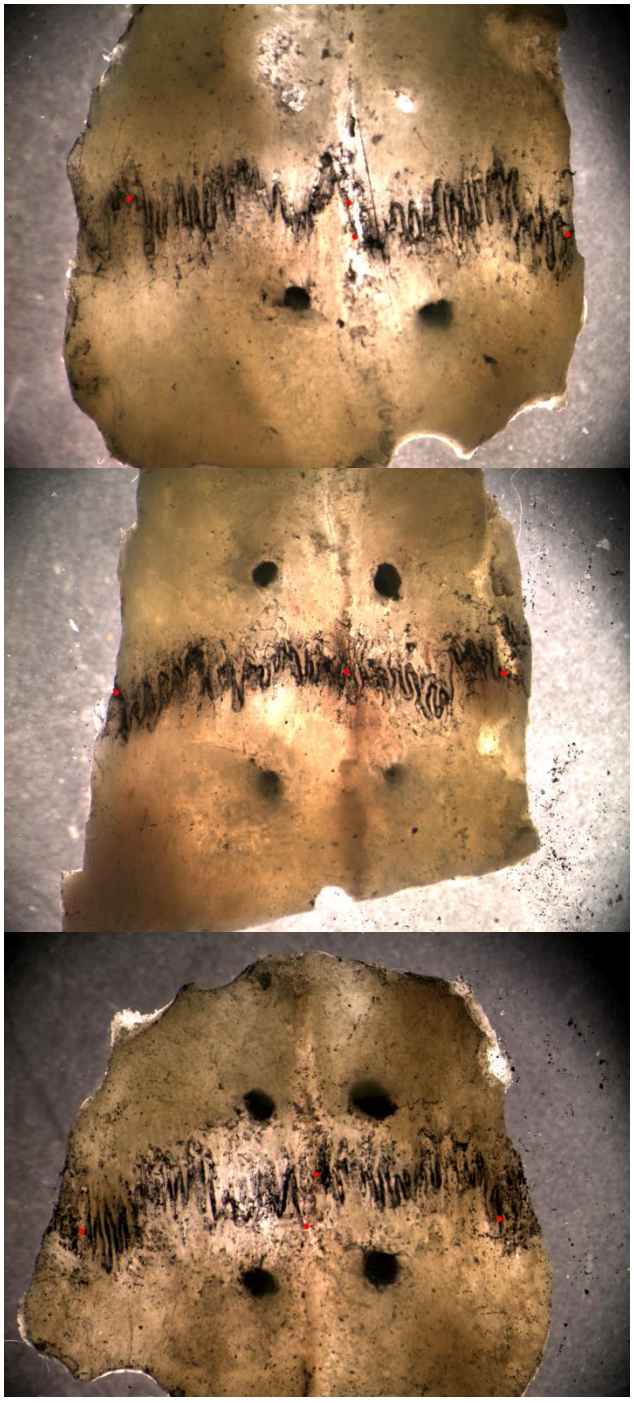

Figure 8. Sutures in in Delayed-onset groups. Top picture: Control with no treatment group, Middle picture: Sham vehicle group, Bottom picture: Thyroxin group

3.3.2 Quantitative Suture Analysis

Delayed- onset rabbits showed significantly more suture complexity in all three treatment groups compared to controls except for the T3 group (Figure 9) (F Group= 3.15; $p<0.05$). Only wild-type rabbits with T3 treatment showed more complexity compared to their own phenotypic controls. In Colony Normal Rabbits in T3 group did not show any difference in suture complexity compared to their own phenotypic controls. Moreover, no treatment or treatment by group effects were noted (F Treatment = 0.41; NS, F Group x Treatment = 1.93; NS).

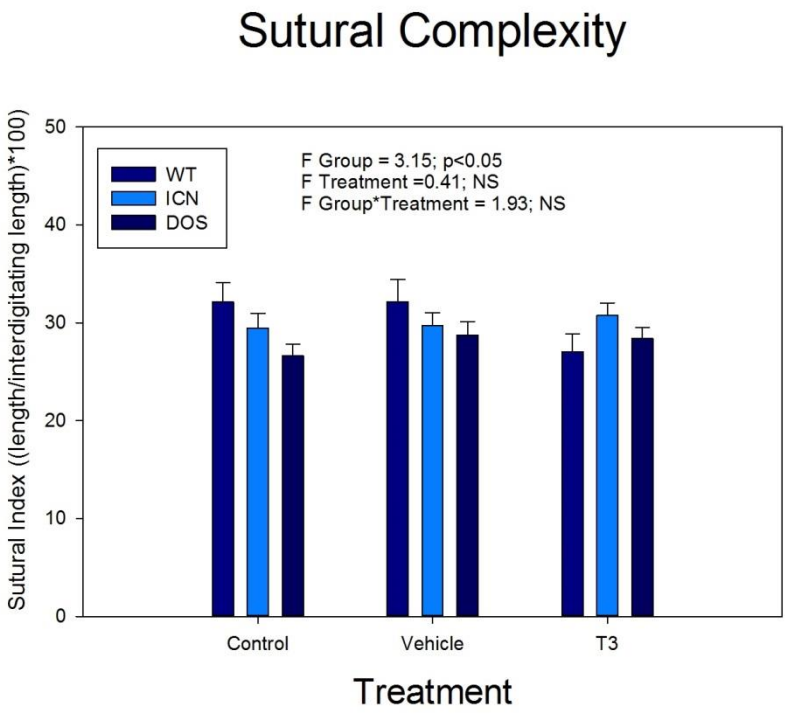

Figure 9. Comparison of Sutural complexity in wild type, In colony normal and delay onset groups

4.0 DISCUSSION

This study consisted of 69 rabbits from a colony that shows analogous phenotypic variability of craniosynostosis to humans. A total of 25 in-colony normal, 31 delayed- onset as well as 13 wild type rabbits were included in this study. The present study was designed to examine a gene - environmental interaction by testing the hypothesis that postnatal thyroid hormone administered to rabbits with delayed-onset coronal suture synostosis (DOS) would result in an accelerated suture fusion and an increased sutural complexity compared to wild-type and in-colony normal rabbits.

Results demonstrated that administration of tri-iodothyronine (T3) increased blood circulating levels of T3 treatment groups of delayed on-set and in-colony normal and Wild type rabbits compared to control rabbits. Increased level of T3 shows possible thyrotoxicosis. In order to categorize rabbits as "hyperthyroid" clinical assessment and measurements are required. Further studies which monitor appetite or physical activity and growth measurements would truly diagnose hyperthyroidism in these rabbits. Mean levels of T4 were also lowered in treatment groups. Decreased T4 levels in treated rabbits demonstrate that the rabbits' endocrine response received a negative feedback from administered (exogenous) T3 levels, which resulted in switching off the path way of producing endogenous T4. The result also supports the fact that the T3 treated rabbits demonstrated a level of thyrotoxicosis

Body weights were taken on different groups of rabbits. The rabbits that were treated with T3 showed decreased body weight compared to control rabbits .This might indicate that these rabbits might show signs of hyperthyroidism (decreased body weight is one of the sign of hyperthyroidism). It would be necessary to identify the somatic growth for each rabbit with the purpose of knowing for certain that decreased body weight in T3 treated rabbits is due to administered T3. The reason behind would be to exclude the decrease in weight because of normal variation in somatic growth rate of the rabbits

Although the effect of thyroid hormone had been determined to support the fact that excessive thyroid hormone might cause premature suture closure (Akita et al., 1994, Rasmussen et al., 2007), in our study we could not find any statistically significant gene - environmental interaction between elevated postnatal T3 levels and suture complexity.

Results of the study on suture complexity analysis showed that Delayed- onset rabbits showed significantly more suture complexity in all three treatment groups compared to controls except for the T3 group. Moreover, the study indicated that only wild-type rabbits with T3 treatment showed more complexity compared to their own phenotypic controls. One reason that might explain the results is the timing of administration of T3. What this means is that the synostosis in the rabbits could have occurred earlier than the T3 treatment. Therefore T3 could not have had any potential effect on the closure and consequently the complexity of the sutures. In other words, in order to have any effect of T3 hormone on suture complexity, the timing of synostosis of the sutures and exposure to T3 would have had to be earlier in these rabbits, before the suture was fused.

Another way to explain the results would be the fact that there is no gene –environment interaction between the synostosis genes and T3 hormone that are causing coronal synostosis in

this colony of rabbits. Growth factors, such as TGFβ1, TGFβ2, TGFβ3, and IGF-1, have been associated with suture development in humans, rats and rabbits (Opperman et al., 1997; 1999; 2000; Roth et al., 1997a; 1997b; Most et al., 1998; Bradley et al., 1999; Poisson et al., 1999; in review; Cohen, 2000b; Opperman and Ogle, 2002). Specifically, it was noted that TGFβ1, TGFβ2 and IGF-1 were associated with sutural fusion, while TGFβ3 may play a critical role in keeping sutures patent. How these signaling molecules may be affected by the presence of excess TH in this rabbit model is not completely clear. If exogenous T3 does not signal the synostosis pathway subsequently we would not see any change in accelerated premature coronal suture fusion. Therefore there would not observe an increased coronal suture interdigitation compared to both unexposed affected and control rabbits. This indicates that there was no gene-environment interaction of T3 on the gene(s).

In wild type group, T3 acts as a stimulatory hormone and its mechanistic effect on suture complexity is observed as increased suture interdigitation. T3 increases the osteoblastic activity in bony bridges and results in bone deposition at osteogenic fronts of the suture. The effect of T3 while the brain is growing on the suture complexity can also be explained mechanistically when tensile forces and stretching of the sutures triggers osteoblasts and results in bone deposition via pisoelectric effect at the site of tension (Cohen, 2000).

Comparing the in-colony normal group to the wild type group, the question comes into mind that if the animals were truly in-colony normal, the increased interdigitation should have been observed in treatment groups in the same pattern as we see in wild type treatment group. This means that T3 would increase the interdigitation of the sutures. In contrast, in the in-colony normal group we see a pattern closer to delayed onset group with slightly less interdigitation. One way to explain the observation is that in-colony normal rabbits have different phenotypes

than Delayed onset group, but they acquire the same genotype. This means that different level of gene expression is observed in these two groups. There might have been no gene(s),-environment interaction in regards to suture complexity. In addition, the Delayed onset group obtains more modifier genes since they are more inbred and hence they are more synostotic. Also, the timing of T3 administration might have been late for the synostosis in in-colony normal treatment group. In other words, the window of opportunity for the effect of T3 on suture synostosis has been missed.

Also due to the small sample size of each treatment group, the relative late administration of T3, and the small treatment effect, the significance of the treatment outcome could be compromised. It is possible that increasing the sample size, and thereby increasing the power of the study, would result in statistical significance. Future studies with a larger sample size are recommended to evaluate the effect of exogenous T3 hormone on suture complexity in rabbits. Also, the elevated amount of thyroid hormone necessary to produce any synostotic changes can be examined.

5.0 CONCLUSIONS

Postnatal T3 exposure in the dosage of 0.2mg/kg resulted in hyperthyroidism in the rabbits, as seen by the increased T3 blood serum levels, the decreased levels of circulating endogenous T4, and the decreased body weights following treatment.

Postnatal T3 exposure resulted in increased suture complexity only in wild-type control rabbits compared to rabbits with familial craniosynostosis. Results suggest that there was no statistically significant gene - environmental interaction between elevated postnatal T3 levels and craniosynostosis in this model study.

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