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Cell encapsulation as a potential non-dietary therapy for maternal phenylketonuria

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CELL ENCAPSULATION AS A POTENTIAL NON-DIETARY THERAPY FOR MATERNAL PHENYLKETONURIA

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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CHAPTER 1: INTRODUCTION

Phenylketonuria (PKU) is an inborn metabolic disorder that is caused by a deficiency of the enzyme phenylalanine hydroxylase. It is an autosomal recessive inherited disorder and is the most common amino acid metabolic disorder. Its prevalence is 1 in 15,000 live births in the United States. Several screening strategies are present in order to control the phenylalanine levels in patients affected with this disease. Recommended serum phenylalanine concentrations are in the range of 2-6mg/dL [1]. PKU is classified into classical PKU and mild hyperphenylalanemia based on individual serum phenylalanine levels. Although currently there is no complete cure for PKU, the most effective treatment strategy to control phenylalanine levels within the desired normal range is dietary therapy. Because many individuals affected with PKU fail to comply with the strict dietary regimen, alternative treatment methods are being sought. Some of the most widely studied and tested therapeutic strategies are gene therapy, enzyme replacement approach, and large neutral amino acid therapy [2].

Maternal PKU syndrome is characterized by the presence of high concentrations of maternal phenylalanine during pregnancy which can negatively affect the growth and development of the fetus. Strict adherence to a low phenylalanine diet prior to and throughout the time of pregnancy is essential to ensure the proper growth and development of the fetus. Because studies have demonstrated poor adherence to the diet in pregnancy, more efficient treatment strategies need to be devised [5]. In this study, cell encapsulation technology as a means of controlling phenylalanine levels during the gestational period were tested by encapsulating kidney and hepatic cell expressing phenylalanine hydroxylase to generate an artificial organ system. *In vitro* and *in vivo* studies utilizing cells encapsulated within polymeric microspheres were performed and analyzed to determine the efficacy of this treatment strategy.

CHAPTER 2: BACKGROUND

2.1 Phenylketonuria

Phenylketonuria (PKU) is an autosomal recessive metabolic disorder that is caused by the disruption of the metabolic pathway resulting in improper breakdown of phenylalanine. Mutation of the gene coding for the enzyme phenylalanine hydroxylase (PAH) is the primary cause of classical Phenylketonuria and this in turn leads to the accumulation of the amino acid phenylalanine and its metabolites within the body. Currently there is no cure for PKU, but early diagnosis and subsequent treatment can help control the abnormalities associated with this disorder [1].

2.1.1 History

Phenylketonuria is highly prevalent among Whites and Native populations and comparatively lower in the Black, Hispanic and Asian populations. In the United States, an average of 1 per 15,000 newborns is diagnosed with classical PKU. Various newborn screening strategies have been practiced to diagnose the disease at birth. Currently, individuals diagnosed with PKU are put under strict dietary control to maintain the proper phenylalanine levels. Besides dietary therapy other forms of therapy such as gene therapy and enzyme replacement therapy are also being tested to control the disease and prevent the development of any anomalies [2].

Phenylketonuria was discovered in the year 1930 by the Norwegian physician Asbjorn Fölling. In order to determine the cause of their mental retardation, Dr. Fölling conducted a urine test on two children of Norwegian origin who had severe motor problems, and skin abnormalities. Despite consultations with several physicians, no treatments proved successful. Although the routine urine tests performed on these children were normal, the addition of aqueous ferric chloride caused the urine to turn into a dark green color which was very different from the expected red-brown color. This test indicated the presence of phenylketone [3]. Additional biochemical tests done on repeated collection of urine samples from these patients for

a period of approximately 2 months led to the discovery of the compound phenylpyruvic acid in the urine. The breakdown of this substance to phenylacetic acid gave the urine a musty odor a common characteristic in PKU patients. As a result of these findings, biochemical testing was carried out on a larger population of institutionalized children in which urine samples from over 400 patients were collected. Phenylketones were observed in eight mentally retarded patients displaying similar characteristics as the other two children. It was later discovered that elevated levels of phenylpyruvic acid was due to the severe impairment of phenylalanine metabolism [3].

Further PKU studies conducted by Dr. Fölling led to the discovery that heterozygous carriers of the disease are less efficient in metabolizing phenylalanine compared to their homozygous non-carrier counterparts. To test this hypothesis, he injected himself with a dose of phenylalanine (containing both the l-form and d-form) and observed high levels of phenylpyruvic acid in his urine, a characteristic of PKU carriers. Similar results were observed in other humans and animals. To further confirm his theory, Dr. Fölling injected a pure sample of L-form phenylalanine (as the d-form cannot be metabolized by the body) in several subjects and observed elevated levels of phenylpyruvic acid in urine of those who were affected with the disease. Hence, due to the characteristic appearance of the phenylketone (phenylpyruvic acid) this disease was named as Phenylketonuria by the geneticist Dr. Lionel Penrose [3].

2.1.2 Disease

Phenylketonuria, is one of the most common amino acid metabolic disorders and is characterized by the improper breakdown of the amino acid phenylalanine to tyrosine. Phenylalanine is an essential amino acid which is commonly found in diet containing proteins and in some artificial sweeteners. Normal phenylalanine levels in the blood usually range from 2-6mg/dL. Hyperphenylalaninemia is a condition which results from impaired phenylalanine metabolism caused due to the deficiency of the enzyme phenylalanine hydroxylase. Classical PKU is the most severe form of this disorder and is characterized by the complete absence or severe deficiency of the enzyme PAH and the phenylalanine levels in the blood are usually

present at greater than 20mg/dL. This leads to an accumulation of phenylketones – the main cause of the abnormalities associated with this condition [4].

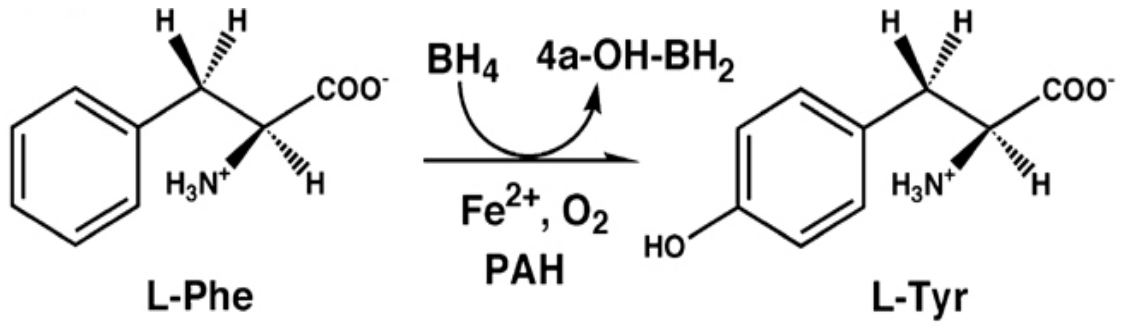

Figure 1: Phenylalanine metabolic pathway [1]

The enzyme phenylalanine hydroxylase is predominantly found in the liver and kidneys and is responsible for the irreversible catalysis of phenylalanine to tyrosine in the presence of the nonprotein cofactor tetrahydropbiopterin (BH4), Fe^{2+} and oxygen are also required in this reaction (Figure 1) [5]. The gene coding for PAH is located on chromosome 12. More than 400 different mutations of this gene that result in classical PKU have been identified. Mutations of the PAH gene coding for this enzyme include deletions, missense mutations, nonsense mutations and splicing mutations. These mutations block the phenylalanine metabolic pathway and in turn lead to the accumulation of phenylalanine in blood. The two most common mutations which are responsible for the manifestation of classical PKU are the substitution of Arginine to Tryptophan at position 408 on the PAH gene and single base substitution of GT (guanine-thymine) to AT (adenine-thymine) which causes truncation of the protein by 52 amino acids. Truncation of the protein in turn abolishes protein activity due to protein instability [6] [7].

Another form of hyperphenylalanemia is caused due to the defect in the biosynthesis of the BH4. BH4 is an essential cofactor which plays a key role in the degradation of phenylalanine by acting as a chaperone on PAH. BH4 is also involved in the biosynthesis of several

neurotransmitters including dopamine, melatonin, and epinephrine. BH4 deficiency is diagnosed by the BH4 loading test at a very early age. Deficiency of this cofactor can result in elevated phenylalanine levels which can be toxic to the body and can result in severe complications such as mental retardation, seizures, developmental and behavioral problems [8].

Biologically, L-phenylalanine is broken down to tyrosine within the body in the presence of the enzyme phenylalanine hydroxylase. In classical PKU, phenylalanine levels are elevated due to the deficiency of the enzyme. Phenylalanine is a large neutral amino acid (LNAA) that crosses the blood brain barrier via a L-type amino acid carrier similar to that of tryptophan and other large amino acids. Accumulation of phenylalanine within the body saturates these carriers blocking the passage of other essential amino acids into the brain [9] and leading to impaired brain development. Additionally, in PKU patients accumulated phenylalanine is metabolized to abnormally high levels of phenylpyruvic acid which inhibits the enzyme pyruvate decarboxylase in the brain resulting in defective myelin synthesis. Myelin is vital for the proper functioning of the nervous system as it serves as an insulating material for the axons. Hence, impaired myelin synthesis can in turn affect the brain development in PKU patients [10].

The breakdown of phenylalanine to tyrosine is an important process as it is the only source of tyrosine within the body. Tyrosine is a non-essential amino acid which can also be obtained through high-protein diets and supplementation. Tyrosine serves as a precursor to several neurotransmitters. It is converted to levodopa by the enzyme tyrosine hydroxylase in the brain. As dopamine, which is an important neurotransmitter in the central nervous system, does not readily cross the blood brain barrier, L-dopa serves as a precursor to dopamine. Tyrosine is also converted through a series of steps into other catecholamines such as epinephrine and norepinephrine which are both important hormones and neurotransmitters. Besides the neurotransmitters, tyrosine also serves as a precursor for the thyroid hormones thyroxine and triiodothyronine. The enzyme tyrosinase helps in the conversion of tyrosine to various forms of the skin pigment melanin and is inhibited by high phenylalanine concentrations in the blood.

Hence, reduced tyrosine due to impaired phenylalanine metabolism leads to mental retardation, seizures, microcephaly, hypopigmentation, and eczema [9].

2.1.3 Screening Strategies

In order to control the abnormalities associated with PKU, early diagnosis of the disease is essential. Several newborn screening strategies have been in practice in most developed countries since the early 1960s to diagnose PKU at infancy. Neonatal blood samples are collected on newborn screening Guthrie cards to check for elevated phenylalanine levels in the blood. Individuals affected with PKU are recommended to maintain diet restrictions by consuming phenylalanine-free food in order to control any of the severe neurological and other manifestations associated with the disease.

In the United States, the three main screening methods are Guthrie Bacterial Inhibition Assay (BIA), fluorometric analysis and tandem mass spectrometry. Blood samples are collected from the babies typically 2-7 days after birth by a neonatal heel prick. The Guthrie assay helps determine the elevated phenylalanine levels in the blood by checking the ability of phenylalanine to facilitate bacterial growth in a culture medium. Currently the most widely practiced test is tandem mass spectrometry which measures both the concentrations of Phenylalanine and Tyrosine in the blood allowing for determination of the Phe/Tyr ratio.

It is recommended that individuals with PKU have lifelong monitoring of their blood phenylalanine concentration. Maintenance of Phe levels between 2-6mg/dL is recommended for neonates through 12 years of age and between 2-15mg/dL after 12 years of age to ensure normal physiological development. Individuals diagnosed with PKU are also recommended to be monitored once weekly during the first year, twice monthly for the first two years of age and monthly after 12 years of age as per the guidelines set forth by the American Academy of Pediatrics. Early diagnosis in conjunction with frequent monitoring and dietary compliance, can reduce the complications associated with PKU such as impaired mental development and overall

growth. Currently, research is underway to develop treatment strategies that would help eliminate the abnormalities associated with classical PKU [2].

2.2 Maternal Phenylketonuria

Maternal PKU syndrome is characterized by the teratogenic effects passed on to the fetus from the mother during pregnancy. Maternal PKU syndrome results in multiple congenital defects in the offspring including microcephaly, congenital heart disease, intrauterine growth and mental retardation. 75 percent of females diagnosed with PKU are not in dietary control before pregnancy and tend to develop the maternal PKU syndrome where the blood phenylalanine levels are higher than normal desired ranges (2-6 mg/dL) of phenylalanine [11]. For women with PKU, appropriate treatment by continually adhering to the diet prior to conception and continuous monitoring of their conditions helps in contributing to better fetal development [12].

The inheritance of PKU was first observed in the year 1937 by Jervis when two children born to women with PKU also displayed PKU symptoms. Almost 20 years later, in the year 1956, during a Ross Pediatric conference, it was mentioned that children born to women with PKU had mental retardation. In the year 1963, reports on 31 children born to 7 women indicated that 22 children survived infancy with 15 of them displaying severe mental retardation and 7 children showing borderline intelligence with brain defects. In the subsequent years it was observed that children born to mothers with exceedingly high phenylalanine levels at the time of pregnancy had microcephaly, impaired brain development, intrauterine growth retardation, congenital heart defects and impaired postnatal growth [13].

In maternal PKU syndrome, the child does not necessarily have the defective form of the enzyme PAH, but the elevated phenylalanine levels in mothers with PKU causes the teratogenic effects seen in these children. Experimental studies have also shown that the more immature the brain is the more susceptible it is to phenylalanine intoxication. Hence at the time of pregnancy, high phenylalanine concentration in the maternal blood tends to cross the placental barrier more readily and in turn causes birth defects in the child (Figure 2).

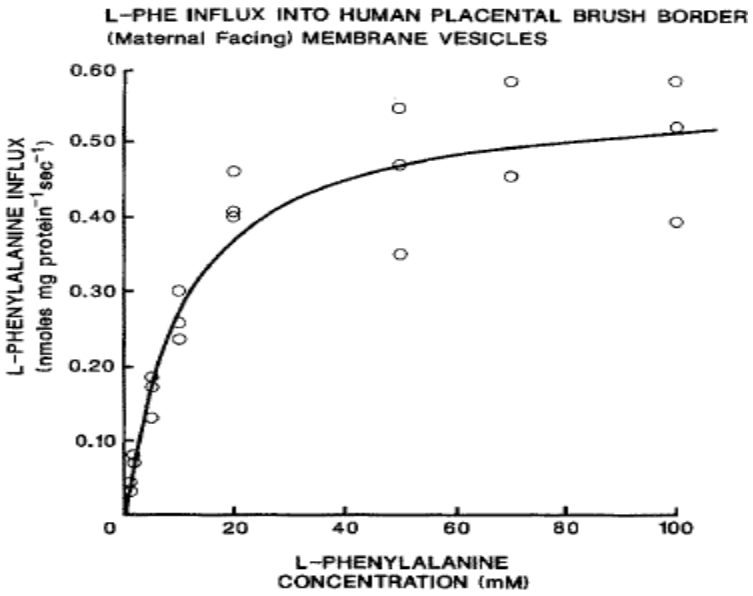

Figure 2: Effect of phenylalanine concentration on placental membrane vesicles [13]

Consequently the membrane bound vesicles of the placenta are saturated and an increase in phenylalanine can in turn inhibit the uptake of L-tryptophan and L-tyrosine which are important precursors of several neurotransmitter syntheses (Figure 3). Some of the other commonly observed effects include microcephaly, intrauterine growth retardation and vertebral anomalies. To control the teratogenic effects and promote proper development of the fetus, pregnant females diagnosed with PKU are recommended to adhere to strict diet control to maintain the blood phenylalanine levels below 6mg/dL at least 3 months prior to conception [14].

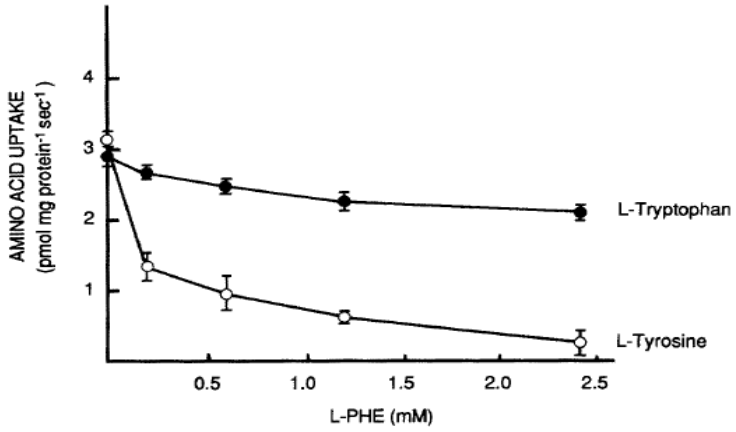

Figure 3: Influence of phenylalanine on amino acid transport [14]

Although a combination of dietary therapy and newborn screening has been helpful in controlling the complications associated with the disorder, some children still suffer mild cognitive defects. Due to the foul taste associated with the dietary supplements many pregnant women with PKU have trouble tolerating the restricted diet. The American Academy of Pediatrics guidelines recommends that women and girls of childbearing age with PKU be counseled about the risks of PKU at the time of pregnancy. Birth control is recommended for individuals who are unwilling to adhere to dietary control in order to maintain normal phenylalanine levels. Women whose phenylalanine levels are between 4-6 mg/dL are counseled about possible fetal problems and are encouraged to be monitored closely to ensure proper overall development of the fetus. Women with phenylalanine levels greater than 14.9 mg/dL are recommended to terminate the pregnancy due to the severe teratogenic effects to the fetus [15].

The maternal PKU Collaborative Study (MPKUCS) launched in the year 1984 by the National Institute of Child Health and Human Development was aimed at determining the effectiveness of dietary control when started prior to conception on the development of the offspring. Results from the study indicated that the cognitive development of the offspring depended on the mother's phenylalanine levels and a normal average IQ of 105 was observed in

offspring whose mothers had phenylalanine levels less than 6mg/dL. When phenylalanine levels were greater than 17.5 mg/dL, the offspring had a severely lower IQ of 56. Results from this study were in agreement with the earlier observations made by Lenke and Levy in 1980 (Table 1) that dietary control prior to conception promoted better development of the offspring. Normal fetal outcomes were also observed in women who achieved control of their phenylalanine levels by 8 weeks of pregnancy [16]. Hence, in order to promote the development of the offspring and to help control the phenylalanine levels in pregnant women, better strategies are critical.

Offspring abnormality	**Maternal Phe >1200μM (> 20mg/dL)**	**Maternal Phe 1000-1200μM (16.7 - 20mg/dL)**	**Maternal Phe 600-1000μM (10 – 16.7 mg/dL)**	**Maternal Phe 200-600μM (.3 – 10 mg/dL)**
Mental retardation	92%	73%	22%	21%
Microcephaly	73%	68%	35%	24%
Congenital Heart Disease	12%	15%	6%	0%

Table 1: Teratogenic effect and their frequencies relative to the degree of maternal hyperphenylalanemia in offspring

2.3 Current therapies

To promote the normal development of individuals with PKU, the overall goal is to maintain the phenylalanine levels within the desired range of 2-6mg/dL. Dietary therapy is the most widely practiced treatment option for PKU patients as it helps prevent the severe abnormalities associated with the disease and promotes in the normal development of the individual when individuals adhere to it strictly throughout life. Other treatment strategies that

have been tried with PKU patients are enzyme replacement therapy, gene therapy, and the use of large neutral amino acids [17].

Individuals diagnosed with PKU are advised to adhere to low phenylalanine diet starting at the time of diagnosis to ensure proper growth and development. Several studies were conducted to understand the effectiveness of dietary therapy in controlling PKU. Untreated adults with PKU showed several abnormalities such as seizures, neurological and behavioral disorders, musty odor, eczema and mental retardation prior to the inception of dietary therapy. Following the introduction of the phenylalanine-free diet, reduction in plasma phenylalanine levels as well as physical manifestations associated with the disease were observed. Sixty-seven percent of the patients showed improved behavioral patterns within 2 months after the diet was initiated. Dietary therapy helped improve the overall development of the PKU patients tremendously [17].

Similarly, it is recommended that women with PKU who wish to conceive adhere strictly to Phe-free diet at least 3 months prior to conception to reduce the teratogenic effects. The nutritional supplements are quite foul tasting and expensive. Hence, many women fail to adhere to the diet throughout pregnancy. Non-compliance with the prescribed diet is the main cause of the abnormalities observed in the offspring. Additionally, low-phenylalanine dietary products can also lead to imbalances in trace minerals such as zinc, iron, copper and chromium and cause disturbances in fatty acid and lipid profiles. Thus in order to help promote the overall well-being of the offspring, a more practical approach would be beneficial in treating the maternal PKU syndrome [18].

Another treatment strategy that has been tested in treating PKU is gene therapy. In this approach, a recombinant adenoviral vector containing human PAH cDNA was constructed and transferred to a PAH-deficient mouse model (PAHenu2). Within 1 week post administration of these vectors in mice, normalization of the hyperphenylalaninemic phenotype was observed and results indicated that approximately 10-20 percent of normal enzymatic activity was sufficient to normalize plasma phenylalanine levels. The major drawback with this treatment strategy was

that vectors were easily degradable and the results were not persistent for a long duration. In addition, immune responses to these vectors along with variable reductions in Phe levels have not made this a successful treatment method. This treatment cannot be re-administered effectively due to development of the neutralizing antibodies that are present within the body against these recombinant adenoviral vectors after the first administration. These antibodies could interfere with the efficacy of this treatment strategy [18]. Although the use of immunosuppressants has helped prolong the efficacy of these vectors, it cannot be widely used in treating the maternal PKU syndrome as immunosuppressants not only increase the chances of hypertension and renal dysfunction in pregnancy but also affect the fetal development and the immune system. An alternative strategy that was tested using the vector system employed a recombinant retrovirus to effectively transduce the PAH cDNA into PAH deficient hepatocytes. But the amount of PAH expressed in the liver through these systems was not sufficient enough to reduce the phenylalanine levels [19].

Additionally, enzyme replacement therapy has been tested strategy to treat PKU. In this method, a functional enzyme is inserted into the body through cells to facilitate the conversion of phenylalanine to tyrosine in PAH deficient patients. For this therapy, PAH has been replaced with phenylalanine ammonia lyase (PAL) to eliminate the need for the BH4 cofactor. PAL is a bacterial enzyme which converts phenylalanine to transcinnamic acid and prevents the buildup of phenylalanine and other metabolites. When administered by itself, PAL works effectively in the presence of immunosuppressants. In a short term study conducted by Sarkissian *et al*. subcutaneous administration of unPEGylated PAL showed significant clearance of plasma Phe levels but the effect reduced after 8 days [20] [21].

In the same study, PAL was conjugated with the PEG (polyethylene glycol) to reduce the usage of immunosuppressants. PEGylated PAL was administered into PAHenu mice models at varying doses and also through different routes of administration. Reduction in plasma Phe levels was observed at higher dose and lower frequency. Results indicated that male mice had an overall greater and sustained response over time and dose. Reversal of hypopigmentation was

observed around 7-10 days following the administration of PEG-PAL and the mice showed enhanced and immediate weight gain. Mice models with PEG-PAL showed a significant weight gain over a period of 47 days compared to the vehicle controls where the maximum weight gain was observed after 112 days. Overall healthy conditions were observed in the mice treated with PEGylated-PAL compared to those treated with unPEGylated PAL. For a more effective and prolonged response, repeated PAL administrations were necessary [21].

In hyperphenylalanemia, a less severe form of PKU, the phenylalanine metabolic pathway could be impaired due to the defective or improper recycling of the co-factor BH4. Studies were done to determine the effectiveness of BH4 administration in participants who responded positively to the BH4 loading test. Analysis was done to understand the effect of BH4 levels on PAH gene expression in human hepatic cells which is the site of natural expression of PAH and the enzymes responsible for the biosynthetic pathway of BH4 [8]. Sepiapterin was used as a precursor for the BH4 synthetic pathway and results indicated that in sepiapterin-supplemented cells there was a strong increase in the expression of PAH and its activity. The chaperon-like effect of BH4 also reduced the rate of degradation of the PAH proteins. Hence, oral BH4 supplementation has been a useful therapy in treating certain milder forms of PKU. The main drawbacks associated with this therapy are that the effectiveness depends on the genetic mutation of the patient because BH4 has the tendency to be eliminated rapidly, oral dosing would have to be given 2-3 times daily [22].

Another synthetic formulation of BH4 cofactor, called sapropterin for the treatment of PKU has been developed by BioMarin pharmaceuticals. This FDA approved drug has sapropterin dihydrochloride as its component and when used in combination with phenylalanine restricted diet has shown to be quite effective in controlling Phe levels in tetrahydrobiopterin (BH4)-responsive PKU [23]. Sapropterin is available in the form of 100 mg tablets and the recommended starting dose is 10mg/kg/day. Clinical trials of sapropterin have been carried out in a total of 579 patients. Results indicated that 20 percent-56 percent of PKU patients responded to sapropterin. Although sapropterin is recommended for use by people of all age groups, there

are serious side effects associated with the drug and there is not sufficient data to validate their efficacy in treating maternal PKU. Some of the common side effects associated with the use of sapropterin are headache, diarrhea, abdominal pain, upper respiratory tract infection, vomiting and nausea. Other adverse reactions reported by the patients were gastritis, spinal cord injury, streptococcal infection and urinary tract infection. Approximately 4 percent of the patients also reported mild to moderate neutropenia [24]. Due to the side effects associated with sapropterin and also the need for dietary therapy to control Phe levels, a more practical approach is necessary in treating the maternal PKU effectively.

2.4 Cell encapsulation

Cell encapsulation technology has been proposed to be a solution for the treatment of several debilitating diseases by reducing the dependency on immunosuppression for long term survival and functionality. Cell encapsulation is a process by which cells and other therapeutic agents are trapped within semipermeable polymeric membranes thereby isolating the cells from the external environment without eliciting an immune response in the host body. The controlled targeted release of therapeutic products within the host by these immunoisolated cells has been attained through several forms of encapsulation such as microcapsules, hollow fibers, and macrocapsules. This method has been tested in treating several endocrinal disorders, neurodegenerative disorders by the use of different cell lines and proteins.

The concept of cell encapsulation technology has been prevalent for several decades and has been widely developed into different applications. One of the earliest applications of this technology was performed by Bisceglie *et al*. in 1933 when they replaced the endogenous pancreas by insulin producing encapsulated tissues in a semipermeable membrane. Their efficacy was tested in rats by transplanting them into the abdominal cavity and results from this experiment indicated that there was no immune response elicited in the absence of vascularization[25]. In 1943, Algire *et al*. demonstrated the significance of biocompatibility and recognized that graft rejection of allogeneic and xenogenic tissues could be reduced by

encapsulating them in protective membranes prior to transplantation. Thirty years later, this technology was further developed by Chang by utilizing encapsulation as a means of protecting the transplanted cells from the host environment. Chang demonstrated that aqueous solutions of enzyme-loaded microcapsules ranging from 1-100 um in diameter could be made by interfacial polycondensation. His experiments proved that enzymes and other proteins encapsulated within these polymeric membranes retained biological activity both *in vivo* and *in vitro* [26].

The main aim of cell encapsulation is to protect the transplanted cells and other therapeutic agents from the host environment and to aid in long term survivability and functionality without the use of immunosuppressive agents. Encapsulation of cells within semipermeable polymeric membranes protects the cells from the host immune system and allows for the diffusion of important nutrients, oxygen, and growth factors for the cell survival. Cell encapsulation devices can be broadly classified into intravascular and extravascular devices. There are different techniques available for encapsulation of these cells and the encapsulation efficiency depends on several parameters such as polymer concentration and solubility.

2.4.1 Intravascular devices

Intravascular devices are those devices which are connected as a shunt to the blood stream and this enables a rapid exchange of nutrients to regulate physiological function. The device is composed of a microporous tube which allows for blood flow through its lumen and a housing on the outside on which the tissue is implanted. Subsequently the device is implanted into the blood stream of the host by vascular anastomoses. The artificial capillary beds perfused with cell culture units provide a matrix which enables the cells to develop into tissue-like substances *in vivo*. The high surface area to volume ratio also allows large numbers of cells to be cultured with considerably less space and equipment [27]. Chick *et al*. tested the efficacy of this method by culturing beta islet cells from neonatal rats on bundles of artificial capillaries perfused with tissue culture medium. Results of this study proved that cells released insulin continually

and were responsive to glucose concentration changes internally. The quantity of insulin produced was also similar to that produced by conventional flask cultures [28].

Another widely studied intravascular device is the modified diffusion chamber. The device consists of a number of small diameter artificial capillaries composed of fibers of polyacrylonitrilie and polyvinylchloride copolymer (PAN-PVC) and has a lumen loaded with rat islets between the outside of the capillary units. This allows the islets to be in close contact with the circulation and allows the passage of only low molecular weight substances. Although this system was quite effective in regulating glucose levels, their usage was limited for longer durations due to thrombus formation within the lumen despite the administration of anticoagulants [29]. Results from a study conducted using polycarbonate diffusion chambers to encapsulate islets in the treatment of diabetes demonstrated that implantation of islets in these chambers failed to reverse diabetes over 12 weeks and that there was also a loss of viable islet tissue [30]. Diffusion chambers also posed another problem when they were used in treating diabetes as they allowed the entry of large molecules such as IgG and IgM. These devices caused the death of the xenogeneic islets due to the severe local accumulation of inflammatory cells and reduced vascularization. Due to these problems associated with diffusion chambers, their clinical application and usage has been limited [31].

Another type of intravascular device that has been used in several applications is the tubular membrane. Semi-permeable tubular membranes with larger diameter were used to replace the diffusion chambers with the hope of reducing thrombus formation. The membrane made of PAN-PVC has a single, coiled tubular membrane and has a high flow rate which prevents adhesion of the cells to the membranes. These devices have been tested in the transplantation of allogeneic and xenogeneic islets to diabetic dogs. Modifications to the device to reduce thrombus formation has limited the diffusion of essential nutrients and has limited the usage of these devices due to the reduce long term mechanical stability [32] [33].

Although intravascular devices have been tested in different applications, they are associated with several drawbacks which have greatly limited their usage. First, these devices are

highly thrombogeneic, posing danger of clot development in the host. The materials used in fabricating these devices and for vascular anastomosis are believed to be the main reason for thrombogenecity and for inducing inflammatory responses. This in turn affects the viability and long term stability of the transplanted substances such as islets. Hence, better strategies or devices with improved properties would help achieve efficient transplantation.

2.4.2 Extravascular devices

Extravascular devices are those which are implanted into the body without the need for vascular anastomosis. These devices encapsulate cells or other therapeutic agents within semi permeable membranes and hollow fibers and may be fabricated in the form of a flat, circular or tube like structures. Implantation of these devices requires minimal surgery unlike the intravascular devices. These devices are placed in close contact with the blood vessels and diffusion of essential substances through the capsules ensures the viability and function of the enclosed substances. These devices also eliminate thrombus formation and the need for immunosuppressive agents. Two major classes of extravascular devices are macrocapsules and microcapsules.

2.4.2.1 Macrocapsules

Macrocapsules are generally larger than 1000um in diameter and are composed of natural and synthetic biomaterials. They are usually implanted through peritoneal cavity, subcutaneous site or the renal capsule of the host. Although initial studies with macrocapsules were not very favorable, several modifications with respect to the materials used to fabricate them and their parameters have helped achieve quite effective devices. Macroencapsulated porcine islets have been quite effective in treating type I diabetes. In this study, a mesh capsule containing a Teflon cylinder was inserted into the abdominal cavity of the patient. This led to the formation of collagen membrane and the cylinder was removed and replaced with porcine islets into the tube. Six of the twelve patients who had received these grafts, showed some significant improvements. The functionality of the graft was prolonged and one child was insulin-independent for a year

[16]. In another study conducted by Takebe *et al*., porcine fetal liver fragments encapsulated in polypropylene membrane enclosed macrocapsules were implanted into rat models. These fragments survived for more than a year and were able to support the liver function [34].

Although macrocapsules have had some successes in treating diseases, there are still critical issues that must be addressed that must be addressed before their use can become more widespread. The main drawback with these capsules is that their spherical geometry affects the diffusion of the essential substances and gases and the encapsulated cells might not remain functional for a longer duration as they might die sooner due to the large diffusion distances between the core of the capsule and the outer portion of the capsule. Another problem associated with these capsules is the immune response provoked by the tissues due to the contact with the antibodies. Hence, microcapsules are preferred over macrocapsules mainly due to their spherical geometry.

2.4.2.2 Microcapsules

Microencapsulation is a process by which a group of cells are enclosed in substances made of various polymeric materials which are highly biocompatible and bio-degradable. As the substances within the capsules are isolated from the host systemic circulation, this method eliminates the need for the administration of immunosuppressive reagents. The semi-permeable polymeric membranes are composed of biodegradable materials which have proven to be compatible within the host without eliciting any inflammatory response (figure 4). Microcapsules are usually in the range of 3-800um in diameter can be produced efficiently by many techniques and by the use of natural and synthetic polymers for different applications. Several factors such as polymer concentration, morphology of the spheres and they type of material used to make the spheres have to be considered while fabricating these devices to ensure that they support cell integrity and viability and also for the long-term biocompatibility within the host environment [35].

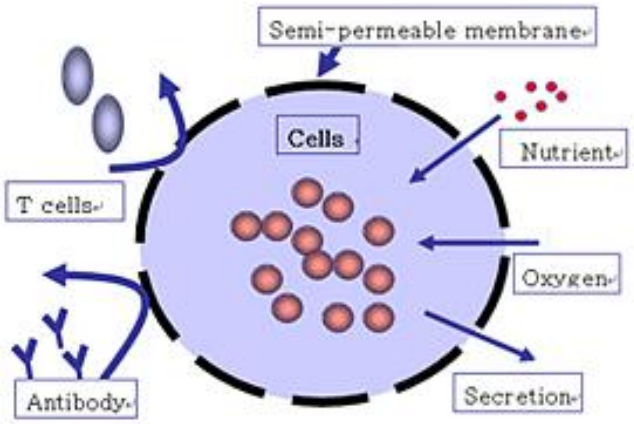

Figure 4: Micro-encapsulation [5]

In 1980, microencapsulation was tested as a way of isolating transplanted cells from the host environment in the treatment of diabetes by encapsulating pancreatic islets in alginate microcapsules by Lim *et al*. Results from the experiment showed that encapsulated islets remained functionally intact for over 15 weeks. These microcapsules were also able to effectively correct the diabetic state of the rats for approximately 2-3 weeks [36]. Since then several advances and modifications have been made in the type of materials and the techniques used to fabricate these microcapsules to expand their usability.

Microencapsulation has several advantages compared to macrocapsules and other intravascular devices. Most importantly, microcapsules permit the influx of molecules which are essential for the cellular viability (such as nutrients, oxygen, etc.) and eliminate the passage of larger molecules such as antibodies which may elicit an inflammatory response. The semi-permeable membrane also allows for the outward diffusion of waste products. Their spherical morphological structure provides a higher surface to volume ratio and also enhances the selective permeability of these microcapsules, ensures cell viability and aids in rapid secretory responses to external stimuli. Microcapsules have also been found to be more mechanically stable than

macrocapsules due to the precise parameters and the cost-effective fabrication methods that are used in manufacturing these devices.

Multiple techniques have been described to produce microcapsules. Within each of these techniques, there are steps available for optimizing sphere production. To ensure the fabrication of effective and mechanically stable microcapsules, several factors have to be taken into consideration. The semi-permeable membrane which encapsulates the cells should be selectively permeable to the essential nutrients and gases and protect the encapsulated cells from the immune system by preventing contact of the cells with the host environment. Studies have also shown that faster rates of diffusion can be achieved with thinner microsphere membranes. Another factor controlling the encapsulation efficiency of these microspheres is the polymer concentration. Increased polymer concentration enhances encapsulation as the polymer precipitates faster and prevents drug diffusion. It also delays drug diffusion due to the increased viscosity of the solution [37]. In addition to these factors, molecular weight of the polymer and the material used to fabricate these microspheres are also important parameters as they ensure long term biocompatibility and durability.

Interfacial polymerization was one of the earliest approaches developed to encapsulate biological substances where the capsule shell forms on the surface of the particle encapsulated by the polymerization of multifunctional reactive monomers. This technique helps in the generation of liquid-core capsules [37]. Another technique employed in the development of microcapsules made of a hydrogel is the extrusion method. In this method, cross linkable polymer solutions containing cells are extruded through a small needle through a syringe pump at desired speed. The droplets are allowed to fall freely and come in contact with an appropriate reagent such as calcium chloride or other divalent cations and by the principle of crosslinking this aids in the production of microbeads or microspheres encapsulated with cells. In a study conducted by Bressel *et al*. this method was effective in preserving live baby hamster kidney cells within the alginate microcapsules for a period of 4 weeks without any signs of necrosis and the cells were also able to secrete an active enzyme [38]. In a study conducted by Zhang *et al*, it was reported

that mouse mesenchymal stromal cell lines were able to attach the alginate mircocapsules and that immediate cell viability post encapsulation was 89 percent. Hence, microcapsules fabricated through this technique had no adverse effects on the reproductive activity and the viability of the cells [39].

Coating of hydrogel beads produced through the extrusion method has also been tested in the development of microcapsules. Coating of the microcapsules enhances their stability, permeability and bio-compatibility. These microcapsules are commonly coated by polyelectrolytes, covalent ions, silicates, agarose or polyethylene glycol. In one of the studies conducted by Dupuy *et al*. pancreatic islets were encapsulated in beads by the extrusion method and were subsequently coated by acrylamide/biacrylamide gel by *in situ* polymerization. Although this procedure seemed to be favorable, there were concerns regarding the toxicity of the coating material and this has limited their usage in clinical applications [40].

Several synthetic and natural polymers are utilized to fabricate these microspheres. Synthetic polymers widely used in cell encapsulation are polyacrylamide-coated agarose, cellulose sulfate, polyvinylalcohol, polymethacrylate, polyethylene glycol and its derivatives. Although the synthetic polymers can be manufactured according to specific needs with desired properties and reproducibility, they pose potential risks to the host environment as they may release toxic or carcinogenic substances. Due to the problems associated with the long-term biocompatibility of these devices, synthetic polymers have not been widely used for clinical applications.

Some of the commonly used natural polymers for cell encapsulation are polysaccharides and polyamino acids. Of these, the most widely used natural polymer is alginate. Alginates are linear unbranched block-polymers found on the cell walls of brown seaweed and are composed of guluronic acid (G) and manuronic acid (M). Due to their excellent biocompatibility and relatively low cost, alginates are commonly used in fabricating microcapsules and microspheres. Cells suspended in varying concentrations of alginate are sprayed into solutions containing divalent ions such as calcium, barium and strontium and when in contact with these solutions

they form spherical microbeads by crosslinking. Calcium is the widely used divalent ion and alginate-molecules crosslink with calcium ions by biding to consecutive blocks of G-molecules. Alginate microcapsules have shown excellent long-term biocompatibility and stability for a year when they were used to encapsulate islets in the treatment of type I diabetes [41].

Alginate-based microspheres and microcapsules have been used to encapsulate different mammalian cell lines and stem cells. Alginate microspheres have proven to be one of the most effective and long-term biocompatible devices. Microencapsulation of islets has shown quite promising results in treating diabetes. Islets encapsulated in alginate microcapsules were transplanted into streptozoticin-induced diabetic animal models and blood glucose levels were monitored during pregnancy. Results from the study indicated that blood glucose levels throughout pregnancy were similar to non-diabetic controls. Encapsulation of islets also reduced the immunogenicity of the allografts which would otherwise be involved in an organ transplantation and the results sustained for approximately one year post treatment [42].

In another study, the performance of alginate encapsulated HepG2 cells were tested in normal and liver failure human plasma for a period of 6-8 hours in a fluidized bed bioreactor. Results from the study indicated that in liver failure plasma, HepG2 cells maintained viability and detoxifying activity similar to normal liver and thus alginate-encapsulated HepG2 cells could potentially serve as a bio artificial liver [43]. In a study by M. Endres *et al*., Ca-alginate microcapsules with human mesenchymal progenitor cells obtained from the bone marrow showed the potential for chondrogenic differentiation. Alginates have clearly been widely demonstrated to be highly biocompatible natural polymers with the capability to be used in different cell encapsulation procedures.

In our project, sodium-alginate microspheres obtained through a droplet generating system have been used to encapsulate different cells mainly the liver and kidney cell lines. The potential of the cell lines to reduce phenylalanine levels in maternal phenylketonuria both *in vivo* and *in vitro* has been studied. In addition, cell viability, morphology of the microspheres and long term stability and biocompatibility have also been analyzed.

CHAPTER 3: PREVIOUS WORK

Cell encapsulation is a relatively new approach in treating phenylketonuria. Preliminary *in vitro* studies were done in Dr.Hunter's lab to test the efficacy of 293T kidney and WRL68 liver cell lines in reducing phenylalanine levels in phenylketonuria. Comparisons were also made between mock transfected and transiently transfected cell lines in reducing phenylalanine levels. The cells made to overexpress phenylalanine hydroxylase were encapsulated and their ability to reduce phenylalanine levels was compared against the unencapsulated cells.

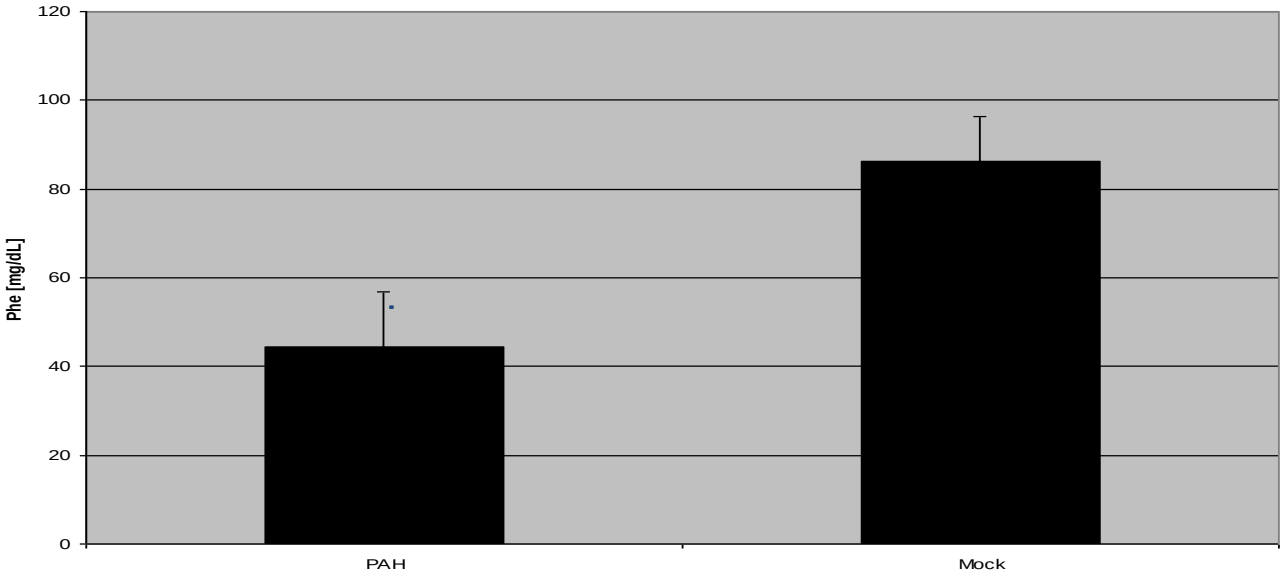

Figure 5: Effect of unencapsulated PAH transfected 293T cells on Phe levels

To ensure expression of phenylalanine hydroxylase, full-length complementary DNA (cDNA) of human PAH was cloned into human plasmids containing the strong cytomegalovirus promoter (pFLAGCMV2 and pFLAGCMV4 vectors). Transient transfections of these cells were performed to test their ability in reducing phenylalanine levels *in vitro.* Results from the study demonstrated that the 293T transiently transfected cells reduced phenylalanine levels significantly by 50% after 3 days in culture when compared to the mock transfected cells (Figure 5). Western blotting was done to confirm the PAH expression in these transfected cells 8 days

post transfection. Encapsulation of these cells demonstrated that there was no effect on cell viability and function as the encapsulated PAH-expressing cells still reduced phenylalanine levels significantly [46].

Stable clones of the liver and kidney cell lines were also created based on the favorable results obtained during the initial experiments. Clones overexpressing PAH were selected by RT-PCR and their ability to reduce phenylalanine levels was analyzed over a 10 day period. On day 10 293T PAH clone 1 reduced phenylalanine concentration by approximately 85%. Similarly the WRL68 cells also showed the ability to reduce phenylalanine concentration in cell culture media (Figure 6). The cell encapsulated microspheres were also viable during the course of the experiment and remained morphologically intact [46]. Hence, initial proof of concept studies demonstrated that cell encapsulation could be a potential non-dietary therapy in treating phenylketonuria.

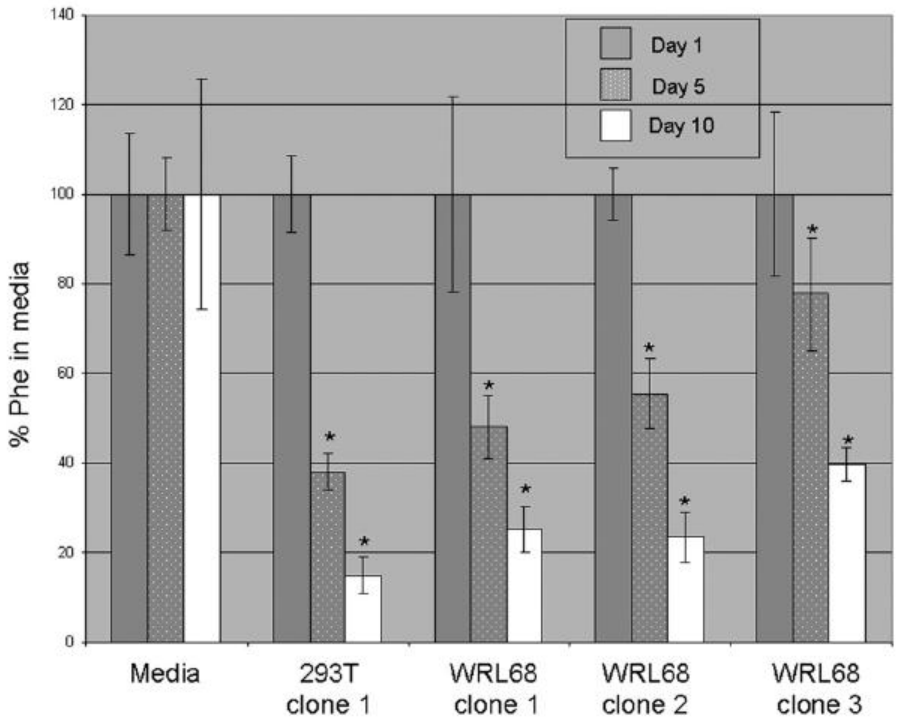

Figure 6: Comparison of cell lines in reducing phenylalanine levels over a 10 day period

CHAPTER 4: HYPOTHESIS

The hypothesis of our current study is encapsulated cells overexpressing phenylalanine hydroxylase can reduce phenylalanine levels *in vivo* and thereby prevent maternal PKU syndrome. Based on the preliminary studies done in our lab, we believe that cell encapsulation could serve as a potential non-dietary therapy in treating phenylketonuria. This approach could be more ideal for short term use such as pregnancy. Hence, our goal is to determine the ability of the cell encapsulated microspheres in reducing phenylalanine levels *in vivo* and *in vitro.*

The main aim of the *in vivo* and *in vitro* studies was to determine the ability of the kidney and the liver cells in reducing phenylalanine levels over a longer duration than previously studied. We believe that this would help us better understand the efficacy of the treatment in controlling phenylalanine levels during pregnancy by carrying out the studies for approximately 21 days *in vivo* and *in vitro* which marks the gestational period of the mice. We hypothesize that the encapsulated cells will demonstrate long term viability while still being encapsulated. The viability of the cells within the spheres would be maintained and prolonged during the course of the experiment due to the spherical geometry of the microspheres which would aid in efficient transport of essential nutrients and gases. We also hypothesize that the cells which retain the highest viability will also retain the ability to express phenylalanine hydroxylase and process phenylalanine and will be effective as an artificial organ system. To test this hypothesis, the ability of different cells in reducing phenylalanine concentrations will be analyzed and comparisons will be made between the cells as this would help us better understand the characteristic of the different types of cells.

CHAPTER 5: METHODS

Microencapsulation of cells could be an effective method in ensuring the reduction of phenylalanine levels in maternal PKU. The cells encapsulated within the microspheres would function similarly to the PAH and aid in the conversion of phenylalanine to tyrosine. Encapsulating cells within alginate microspheres also allows for the diffusion of essential nutrients and gases eliminates the passage of large substance which would otherwise affect the cell viability long time. To ensure a stable and effective encapsulation of cells, various factors to be considered are biocompatibility of the encapsulating material, stability, permeability, size and durability of the microspheres.

The cell lines that were encapsulated in the sodium-alginate microspheres to create a bioartificial organ system were 293T kidney, WRL 68 and HepG2 hepatic cell lines. 293T kidney cell lines were used as they can be easily transfected. WRL68 and HepG2 liver cell lines were used as the liver has endogenous PAH expression and is the primary site of Phe metabolism within the body. Additionally, HepG2 cells have also been shown to act as a bioartificial liver. The ability of the cell encapsulated alginate microspheres in reducing Phe levels, long-term viability of the cells and morphology of the spheres were monitored through both *in vitro* and *in vivo* experiments.

5.1 Cell culture

All cell lines were cultured under sterile conditions at 37˚C 5% CO_2 in a humidified incubator. 293T cells were cultured in a medium containing Dulbecco's modified eagle medium (DMEM) 10 percent fetal bovine serum (FBS). WRL68 liver cell lines were cultured in MEM 10 percent FBS media and HepG2 cells were cultured in DMEM 10 percent FBS containing 1 percent penicillin-streptomycin, 1 percent non-essential amino acids, 1 percent L-glutamine and encapsulated within the microspheres.

5.2 Cell encapsulation

Encapsulation of the cells was performed through a modification of the procedure described by Coromili and Chang [44]. Cells were suspended in 1.5 percent sodium alginate solution, loaded into a syringe and were allowed to flow through a 26 gauge blunt-end needle. To ensure the uniform movement of cells down the syringe and to obtain regulated spherical geometry of the spheres, an air jet system was used (Figure 5). The syringe containing the cell-alginate mixture was clamped to a syringe (Harvard) pump and the cells were subsequently allowed to pass through the syringe by optimizing the air pressure and speed. The air pressure and speed was optimized to be 2.5L/min and 250kPA respectively to aid in uniform flow of the microspheres through the syringe. The syringe was clamped onto a setup and placed at a height of 15cm from the petri dish containing 1.1 percent calcium chloride solution in which the microspheres were encapsulated.

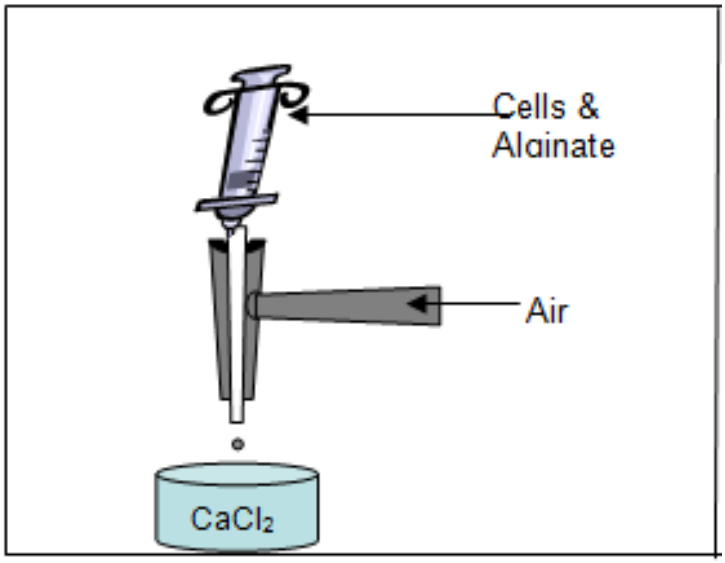

Figure 7: Air jet system to generate cell encapsulated microspheres

The calcium chloride helps form the cell encapsulated sodium alginate microspheres by cross linking with alginate globules which were captured in the solution. Variations in cell

concentrations were tested to determine the most optimum concentration necessary to produce the desired results. Cells were initially encapsulated in microspheres at a concentration of 10^5 cells/ml. Due to the variations in the phenylalanine and tyrosine concentrations in the experiments, a higher concentration of cells at higher quantities was also tested to determine the most optimal cell concentration. Finally an average of $1x10^6$ cells/ml was maintained to ensure that enough cells were transplanted without compromising the morphological structure of the microspheres.

5.3 *In vitro* experiments

In vitro studies were carried out in parallel to the *in vivo* studies to determine the ability of the cell encapsulated microspheres to reduce the concentration of Phe in cell culture media. Kidney and liver cell lines were used and their ability to reduce Phe levels was studied by two different experiments – 3 day experiment where media was changed every 3 days and 7 day experiment where media was changed every 7 days. For the *in vitro* studies, 293T cells, WRL 68 liver cells and HepG2 cells were respectively encapsulated in sodium alginate beads. The objectives of the *in vitro* studies were

1. to determine how long the cells continue to express active PAH and process phenylalanine *in vitro* by PAH conversion assay and quantitative RT-PCR assay
2. to compare different cell types and analyze their ability to process Phenylalanine
3. to determine cell viability by trypan blue staining

5.3.1 Phenylalanine concentration determination

Phenylalanine conversion assay helps analyze the ability of the cells to reduce Phe levels over a period of 28 days by comparing the Phe and tyrosine levels in media containing the encapsulated microspheres and plain media. This experiment was also used to compare the ability of different cell types in controlling phenylalanine levels. Media samples from each of the dishes were spotted on newborn screening cards and the phenylalanine concentrations were measured by tandem mass spectrometry at the University of Iowa Hygienic lab. The

microspheres encapsulated with cells were cultured in their respective media as described above. Two different experiments were setup as described below.

5.3.1.1 Experiment I – 7 day experiment

Each sample was setup in triplicates and the Phe concentrations in the cell culture media were compared between plain media, and microspheres encapsulated with cells. 293T kidney, WRL68 and HepG2 liver cells mentioned above were used for this experiment. The media was changed every 7 days. Care was taken not to remove the microspheres from cell culture dishes. The microspheres were allowed to settle down at the bottom of the dish and approximately half the media right above the cells was removed and replaced with an equal amount of fresh media. Dishes were observed under a microscope before and after media changes to ensure the presence of microspheres. There is potential that a small number of microencapsulated cells were lost at each media change.

- Batch 1: Empty Media
- Batch 2: Microspheres with cells

4mls of sodium alginate microspheres encapsulated with cells with a final concentration of $1x10^6$ cells per ml were added to the dishes in batch 2 (total of 4×10^6 cells/dish). On each day, 100ul of media from each of the 3 different dishes from each batch was taken and centrifuged. The supernatant alone was spotted on the newborn screening card leaving behind the pellet. Media samples from each of the dishes were spotted on newborn screening cards on days 0, 1, 7, 14, 21. The cards were subsequently placed in a tightly sealed desiccated bag and stored at 4°C until being batch analyzed at the conclusion of the experiment.

5.3.1.2 Experiment II – 3-day experiment

The 2nd experiment was setup similar to experiment 1, but the media was changed every 3 days to monitor its effects on the cell viability. Media samples were spotted on several days similar to the first experiment and phenylalanine concentrations were measured by tandem mass spectrometry at the University of Iowa Hygienic lab.

5.4 *In vivo* studies

A PKU mouse model was created by McDonald and colleagues in the BTBR strain of the mouse. Inbred male mice of the BTBR strain treated with a mutagen called ethylnitrosourea (ENU) were mated with female mice and their progenies were found to show symptoms of PKU such as elevated phenylketones in the urine, elevated serum phenylalanine levels (10-20X), abnormal learning/behavior and hypopigmentation as they were carriers of the mutations causing PKU at the PAH gene locus. The strain was termed PAHenu2. C57 PAHenu2 mice strains were also used for the experiments due to their improved fertility over the BTBR PAH enu2 mice (the most commonly used PAHenu2 mice for experimental studies). C57 PAHenu2 mutant mice are the BTBR PAHenu2 mice that have been backcrossed to the c57 background. The PAHenu2 mice models have a T835 missense mutation in exon 7 at the PAH gene which is found to be homologous to the gene causing PKU in humans. Thus PKU mice models deficient in PAH had a similar PKU disease as that in humans [45].

5.4.1 Mouse genotyping

Genotyping of colony mice was routinely performed by allelic discrimination using a protocol from the lab of Denise Ney at the University of Wisconsin. Homozygous PKU affected mice have grey fur and are easily distinguishable. However, wild-type mice and heterozygous mice both have black fur, making it impossible to distinguish them. Because of their difficulty in breeding, heterozygous male and female mice are bred for colony maintenance. Based on a Mendelian inheritance pattern, we would expect ¼ of the mice to be homozygous for the PAH mutation, ½ to be heterozygous, and for ¼ to be wild-type. Mice tails were snipped 21 days after their birth and were stored at -80°C. A modified version of the ABI custom Taqman assay protocol was used. 11.25ul of sample DNA (at a concentration of 10ng/ul) obtained from the mice tails using the IBI Scientific Genomic Tissue DNA isolation kit was added to each well on a 96-well plate and was mixed with 13.75ul of master mix containing TaqMan genotyping master mix and TaqMan SNP assay (40X) (Applied Biosystems). To activate the Taq enzyme

the PCR was setup at 95°C for 10 minutes initially followed by denaturation and annealing/extension for 15 s at 92°C and 1 min at 59.5°C respectively for 40 cycles. The PCR was set up using the 96-well plate and the plate was read on an ABI 7900 real time PCR system in the DNA core facility at the University of Iowa. The PKU mice models were genotyped and the homozygous male and female mice were selected to transplant the encapsulated cells.

5.4.2 Transplantation of cell encapsulated microspheres

Encapsulated cells were generated through the above protocol were injected into PKU mice models to monitor their ability to control Phe levels. The size of the microspheres was measured using digital calipers on the microscope software. In a preliminary experiment, 3mls of microspheres containing cells at a concentration of $1x10^6$ cells/ml were transplanted into the peritoneal cavity of the mice. To determine if there is a dose response, 4mls of microspheres containing HepG2 cells at the same concentration were injected peritoneally into another group of male mice (4×10^6 total cells). Comparisons were made between the two groups of mice and their phenotypic changes and phenylalanine and tyrosine levels were analyzed. A group of 5 mice were used to transplant each of the different cell lines. To determine whether gender influences our results, comparisons were also made between male and female C57/PAHenu2 mice transplanted with microspheres containing HepG2 cells (4×10^6 total cells). Female mice implanted with HepG2 cell encapsulated microspheres were bred with wild type male mice and their ability to produce pups was analyzed. Wild type male and wild type female mice were also set as breeding pairs to serve as controls for the experiment. Following implantation of the microspheres, blood samples obtained from the sub-mandibular space of the mice were spotted on newborn screening Guthrie cards for several days: day 0, day 1, day 7, day 14 and day 28. Subsequently the phenylalanine concentrations were measured by tandem mass spectrometry by the University of Iowa Hygienic lab.

Similar to the *in vitro* studies, statistical analysis was also done on normalized phenylalanine concentrations from the blood samples obtained from each of the mice. Student t-

test was also performed to determine any statistical significance. *P* less than 0.05 was considered significant.

5.5 Cell viability studies

Cell viability was done to monitor the ability of the cells to survive over the period of the experiment. Trypan blue staining was performed on day 21 for the *in vitro* experiment which marks the end of the gestational period of the mice. Trypan blue is a stain which selectively stains dead cells due to their permeability and leaves viable cells refractile. It is widely used in cell counting. Sodium alginate encapsulated microspheres were dissolved in sodium citrate buffer to degrade the alginate beads. The spheres were dissolved 3ml of sodium citrate buffer. The cells were allowed to sit on ice for 10-20 minutes until the spheres completely dissolved. The mixture was pipetted well up and down and the cells were stained using trypan blue in 1:2 ratio and live and dead cells were counted using a hemocytometer. Similarly cell viability studies were carried out as part of the *in vivo* studies to analyze the viability of the cells. Student t-test was carried out to determine the statistical significance in cell viability data.

5.6 Quantitative real time PCR

Quantitative real time (qRT)PCR was used to measure PAH mRNA expression. RNA was isolated from frozen cell pellets using the mirVANA RNA isolation kit as per the manufacturer's protocol. cDNA was generated from RNA samples from 293T kidney, WRL68 liver and HepG2 cell lines using the RT First Strand Kit by Qiagen and run on a 96-well plate on ABI 7900 located in the DNA core facility at the University of Iowa. The gene expression levels of different cell lines were analyzed and compared.

CHAPTER 6: RESULTS

6.1 Cell encapsulation

The morphology of the sodium alginate microspheres which encapsulated the cells were monitored during the 28 days period. The size of the cell encapsulated spheres was maintained at an average of 98 ± 8 µm (n=36) (Figure 6).

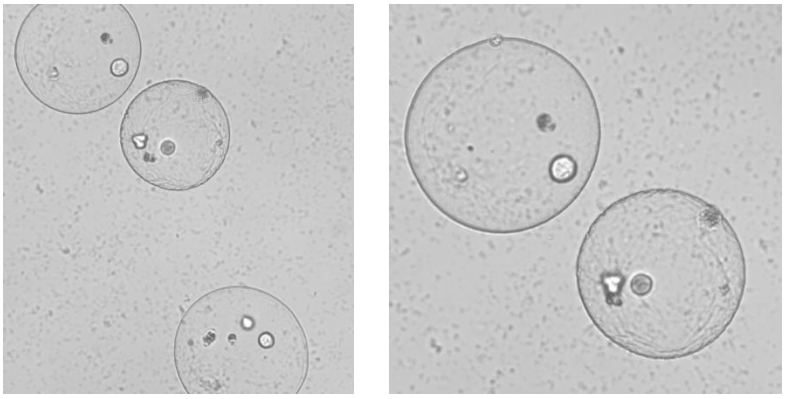

Figure 8: Cell encapsulated sodium alginate microspheres

6.2 *In vitro* results

293T, WRL68 and HepG2 cells encapsulated microspheres were cultured in their respective media and the phenylalanine concentrations were measured. The following results were obtained for the *in vitro* experiments with media changes done every 3 and 7 days respectively.

Figure 9 shows the comparison of normalized phenylalanine concentrations in media containing 293T cell encapsulated microspheres and plain media. In this 7 day experiment, reduction in phenyalanine levels was observed throughout the 21 day period. Phenylalanine concentrations in media with cell encpasulated microspheres were lower in comparison to the phenylalanine in control media at each of the different measured time points. Significant reduction ($P < 0.05$) in media containing cell encapsulated microspheres was observed on days

14 and 21 when compared to the control media. On day 21, a reduction of approximately over 60% Phe in media containing the encapsulated microspheres was observed when compared to the control media. These results suggest that 293T cell encpasulated spheres do possess the ability to reduce phenylalanine levels.

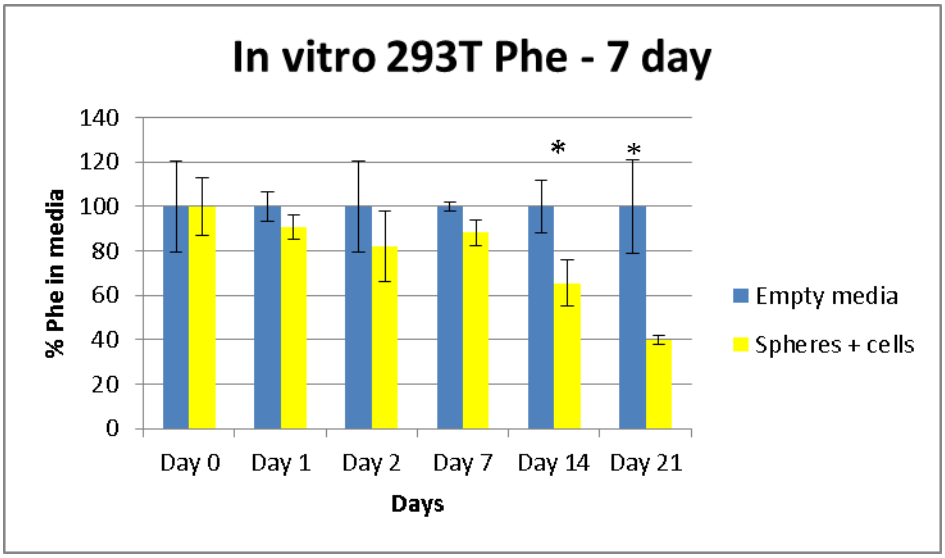

Figure 9: Percent Phenylalanine in media with 293T cell encapsulated microspheres

Similarly 3-day and 7-day experiments were carried out with WRL68 cell encapsulated microspheres and their phenylalanine concentrations were normalized and measured against the phenylalanine concentrations in control media. Gradual decline in phenylalanine concentrations over the 21 day period was observed when the media containing WRL68 cell encapsulated microspheres was changed once every 7 days (Figure 10). Significant reduction in phenylalanine concentrations in media containing WRL68 cell encapsulated microspheres was osberved on days 14 and 21 (Figure 10). Reduction in phenylalanine levels was also observed with the 3 day experiment with these WRL68 cell encapsulated microspheres. A significant reductions in phenylalanine concentration was observed on day 14 in media containing the WRL68 cell encapsulated microspheres when compared to the control media (Figure 11). Media change once

every 3 days could affect the ability of the sodium alginate microspheres encapsulated with cells in reducing phenylalanine in media as this could lead to the over replinishment of phenylalanine through the non-essential amino acids present in culture media. Hence, results from these two experiments indicate that a change in media once every 7 days is a system which better reflects the changes in Phe in media.

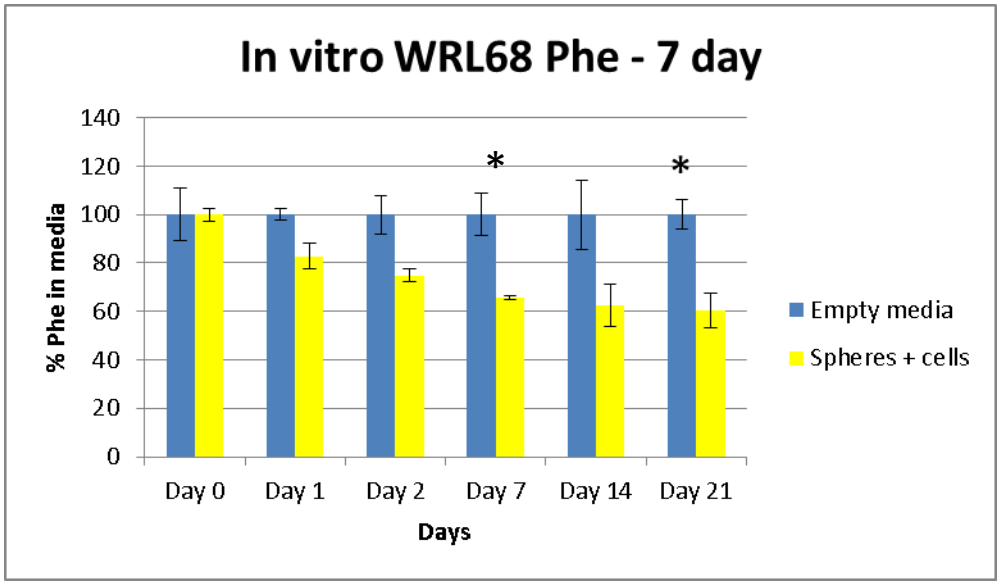

Figure 10: Percent Phenylalanine in media with WRL68 cell encapsulated microspheres

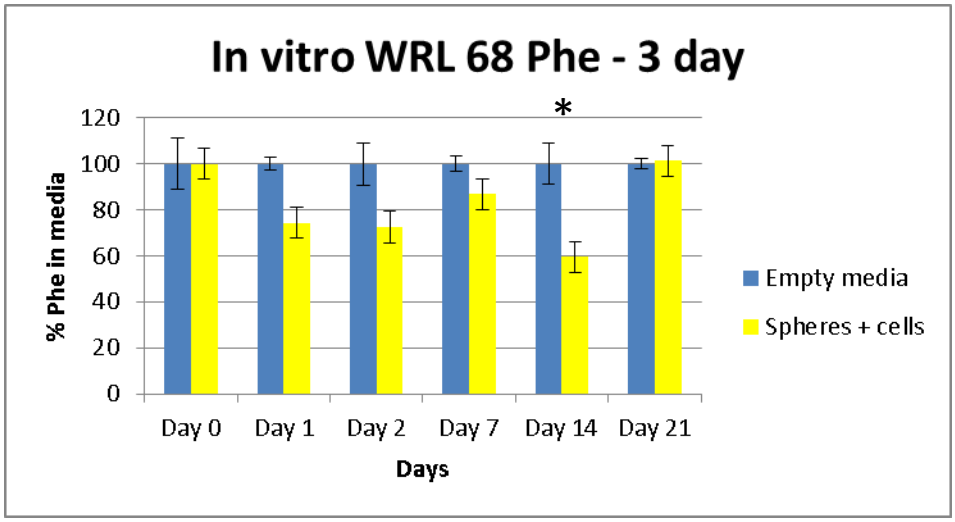

Figure 11: Percent Phenylalanine in media with WRL68 cell encapsulated microspheres (3-day)

Figures 12 and 13 show the comparison of normalized phenylalanine concentrations in media containing HepG2 cell encapsulated microspheres and plain media. Variations in phenylalanine concentrations were osberved throughout the 28day period with a significant reduction in phenylalanine levels ($P<0.05$) observed in media conatining the HepG2 cell encpasulated microspheres on day 28 (Figure 12). Inconsistent trends were observed in phenylalanine concentrations when there was a media change every 3 days (Figure 13). This could indicate that frequently replenishing the media would not prove to be fruitful for the cell encapsulated microspheres as this would not only lead to the over accumulation of phenylalanine but would also not provide sufficient time for these PAH expressing cells to function effectively and reduce the phenylalanine levels. The variations could also be attributed to other factors involved in the *in vitro* experiments such as the evaporation of media in certain dishes thereby altering the phenyalanine concentration in some of the dishes. These results were commonly observed with all the three different cell types. In addition, these *in vitro* experiments were conducted in parallel to the *in vivo* experiment as it ensures that the cell encapsulated microspheres were generated uniformly under same conditions. As the *in vitro* and *in vivo*

experiments were conducted in parallel we did not have the benefit of analyzing the *in vitro* results which would have helped in optimizing the *in vivo* assay. Thus analysis of the *in vivo* data would help in arriving at a better conclusion regarding the efficacy of these cells.

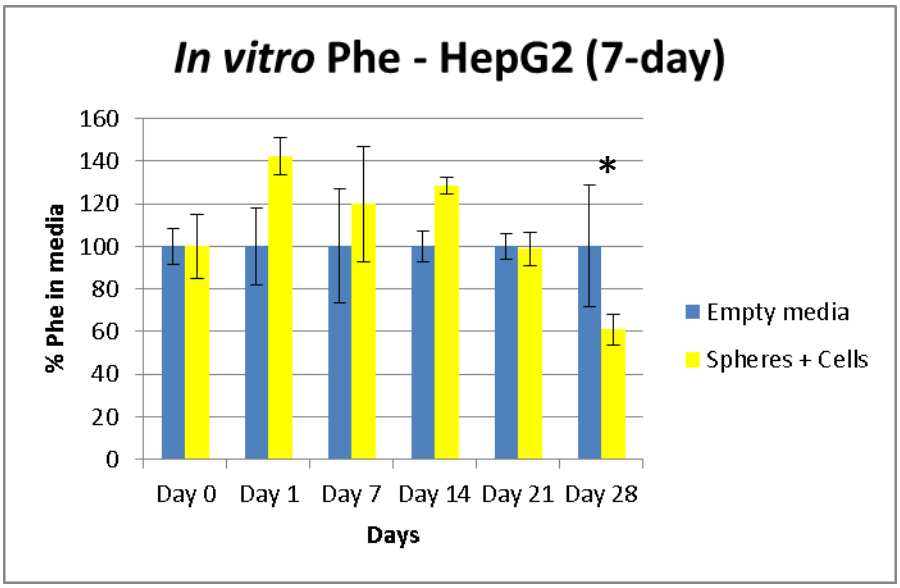

Figure 12: Percent Phenylalanine in media with HepG2 cell encapsulated microspheres

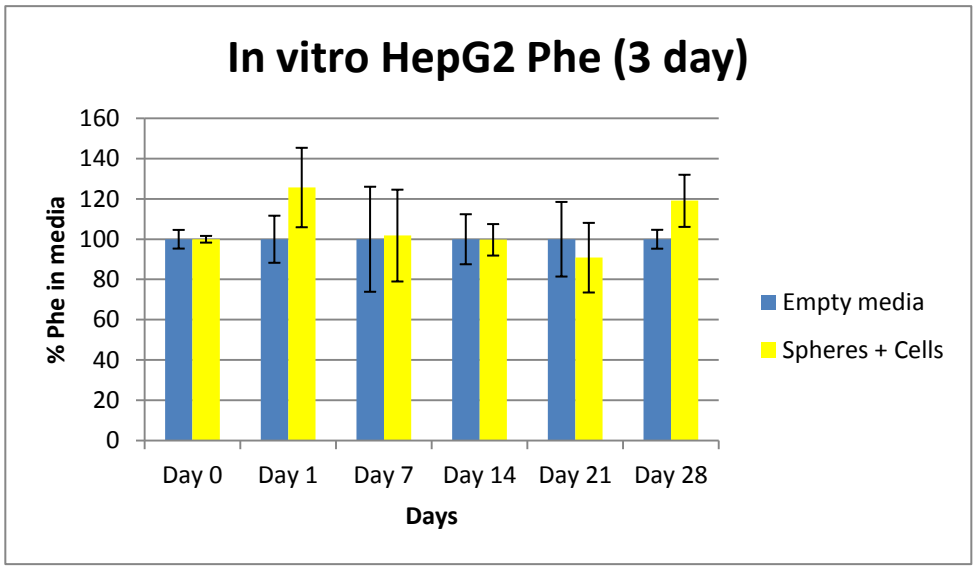

Figure 13: Percent Phenylalanine in media with HepG2 cell encapsulated microspheres (3-day)

6.3 *In vivo* results

Prior to injection of these cell encapsulated sodium alginate microspheres into the mice, genotyping of C57 and BTBR PAH enu2 mice was performed using a modification of the Applied Biosystems Custom TaqMan assay protocol. Genotyping helped us identify the homozygous PKU mice which had to be treated for the disease, wild type and heterozygous mice. Figure 14 shows the allelic discrimination plot where the samples are clustered based on their genotyping. Allele X represents the wild type mice, allele Y represents homozygous mice and the green color (both) represents the heterozygous mice.

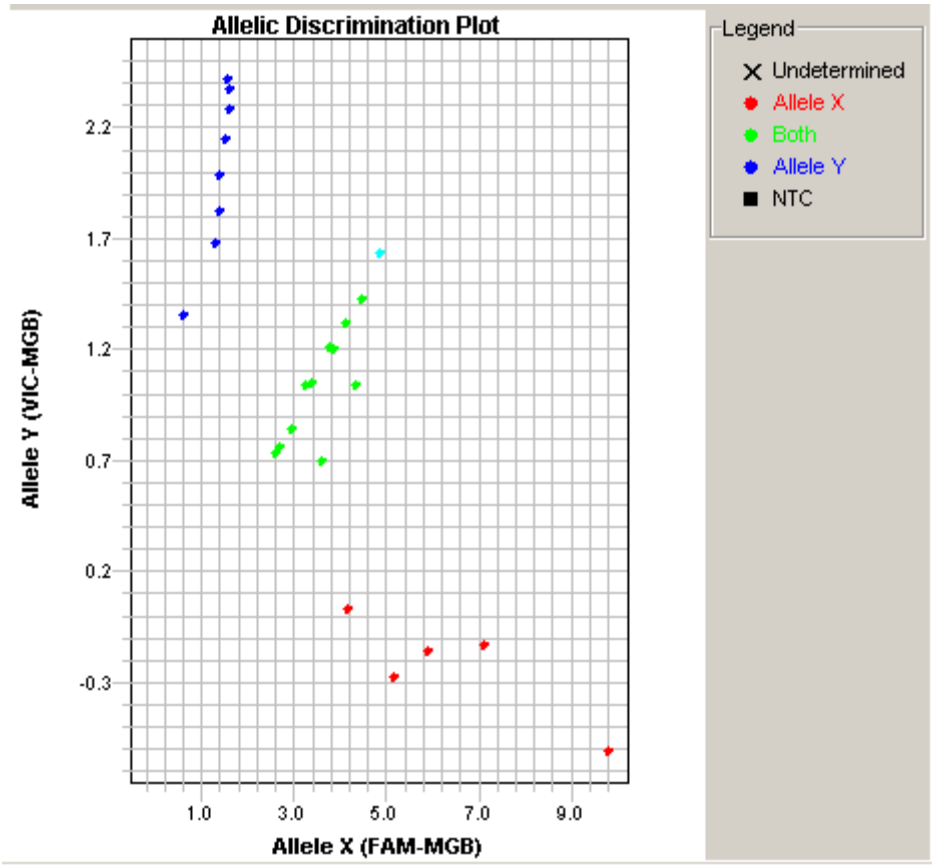

Figure 14: Allelic discrimination plot – genotyping PKU mice

Once the mice were genotyped, groups of homozygous PKU mice were randomly selected for the injection of the sodium alginate microspheres. 293T cell encapsulated microspheres and HepG2 cell encapsulated microspheres were transplanted into 2 respective groups of mice. Whole blood samples obtained from the sub-mandibular region of the mice were spotted on newborn screening cards on days 0, 1, 2, 7, 14, 21 and 28 post-transplantation and phenylalanine concentrations were measured by tandem mass spectrometry. Comparisons were made between normalized phenylalanine concentrations in mice containing cell encapsulated microspheres and mice without any spheres.

Phenylalanine concentrations was reduced by 28 days in mice injected with 293T cell encapsulated microspheres compared to the control mice (which had no spheres). On day 28

significant reduction in phenylalanine concentrations were observed in mice with the cell encapsulated microspheres compared to those which did not have any microspheres (Figure 15). Comparisons were also made between day 0 and day 28 in the phenylalanine concentrations *in vivo* with the 293T cell encapsulated microspheres in each of the mice. An average of 20% reduction in phenylalanine was observed on day 28 compared to day 9 particularly with three of the mice injected with the 293T cell encapsulated microspheres. The *in vivo* results with the 293T cell encapsulated microspheres correspond with the results seen in the *in vitro* experiment thereby suggesting that PAH expressing 293T cell encapsulated microspheres would be effective in controlling elevated phenylalanine levels in PKU.

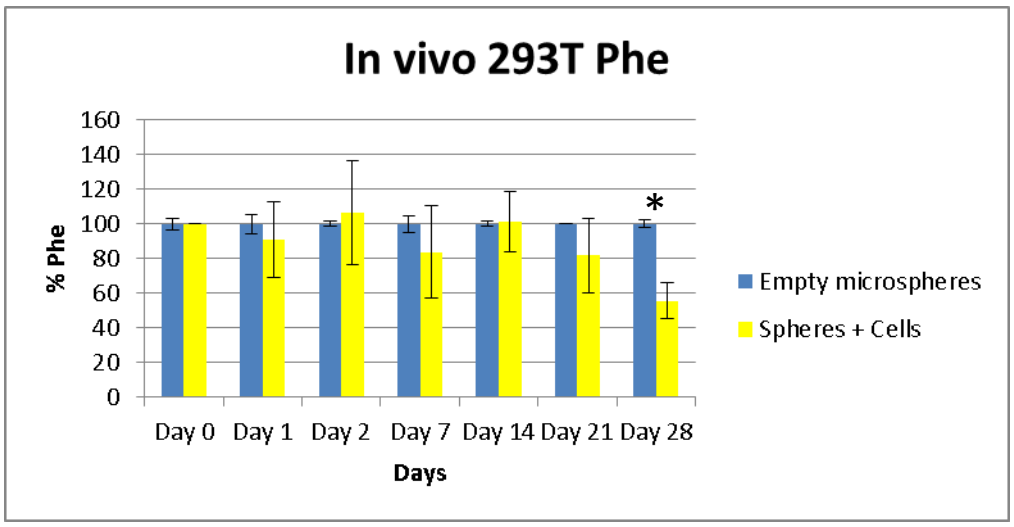

Figure 15: Percent phenylalanine in mice injected with 293T cell encapsulated microspheres

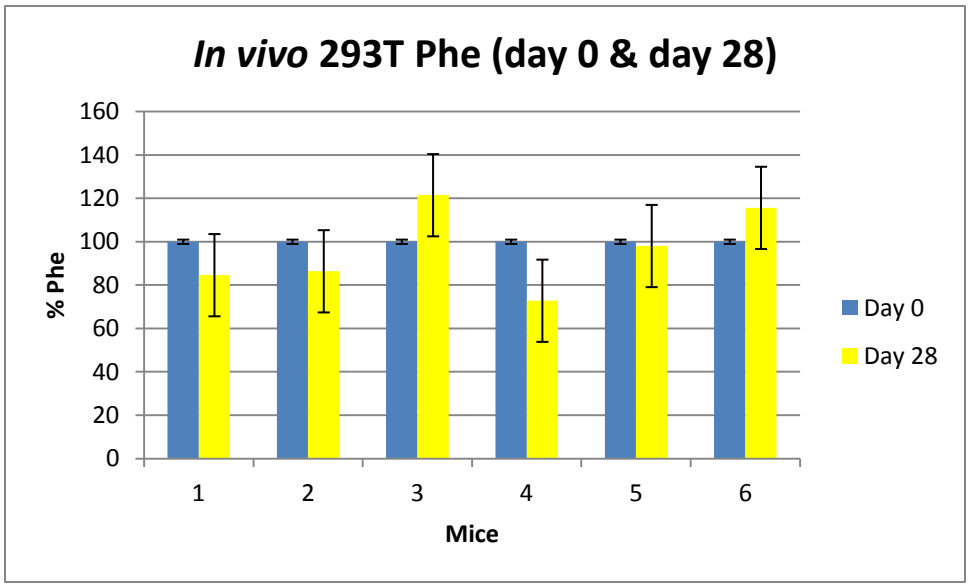

Figure 16: Percent phenylalanine in mice injected with 293T cell encapsulated microspheres (day 0 & day 28)

Similarly, *in vivo* experiments with HepG2 cell encapsulated microspheres were conducted in parallel to the *in vitro* experiment. Two different dosages of microspheres were tried in two different groups of mice. In one of the experiments, $3x10^6$ ml/mouse was tested. Results from the experiment showed that there was a significant reduction ($P<0.05$) in phenylalanine concentrations in mice containing the cell encapsulated microspheres on days 21 and 28 (Figure 16). When $4x10^6$ ml/mouse was used overall reduction in phenylalanine concentrations was observed on days 7, 14, 21, and 28 (Figure 17). Although significant reduction in phenylalanine concentrations was observed on days 21 and 28 with cell encapsulated microspheres at a concentration of $3x10^6$ ml/mouse compared to $4x10^6$ ml/mouse, a more consistent and sustained reduction in phenylalanine concentrations was observed with $4x10^6$ ml/mouse.

Figures 19 & 20 show the comparison of phenylalanine concentrations in each of the mice with cell encapsulated microspheres on days 0 & 28. The results demonstrate that the mice

encapsulated with $3x10^6$ ml/mouse show a gradual reduction in phenylalanine in each of the mice compared to the $4x10^6$ ml/mouse. Although these results demonstrate that $3x10^6$ ml/mouse are effective in reducing phenylalanine levels, it has to be taken into consideration that the $4x10^6$ ml/mouse were injected in both the male and the female homozygous PKU mice unlike the $3x10^6$ ml/mouse which was injected only in the male mice. As mentioned earlier the presence of estrogen in female mice could influence the results observed and could contribute to the trends observed in phenylalanine concentrations with $4x10^6$ ml/mouse. Hence, these results suggest that HepG2 cell encapsulated microspheres at a higher concentration would also help in controlling phenylalanine concentrations.

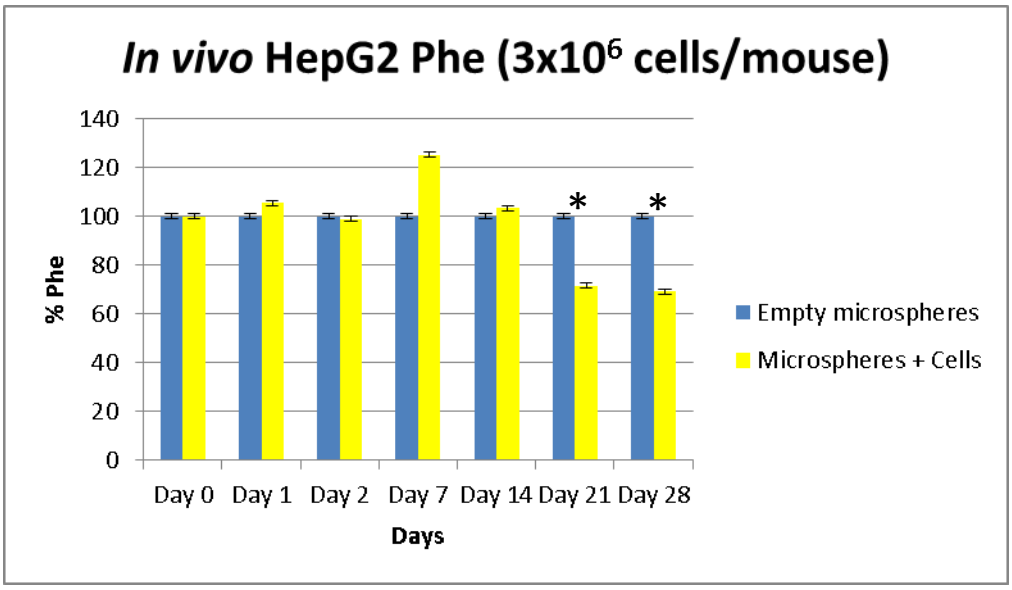

Figure 17: Percent phenylalanine in mice injected with HepG2 cell encapsulated microspheres (3ml/mice)

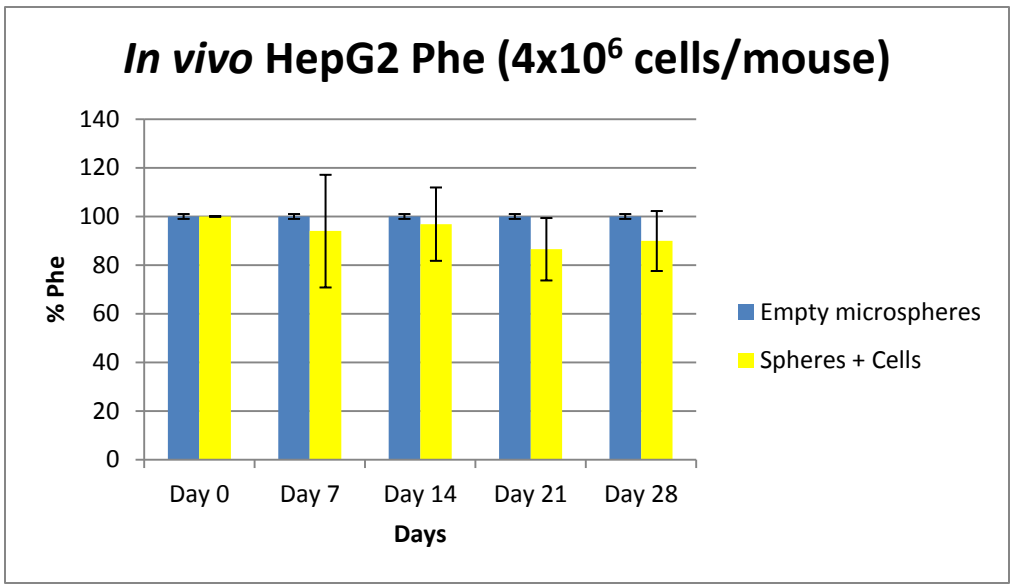

Figure 18: Percent phenylalanine in mice injected with HepG2 cell encapsulated microspheres (4ml/mice)

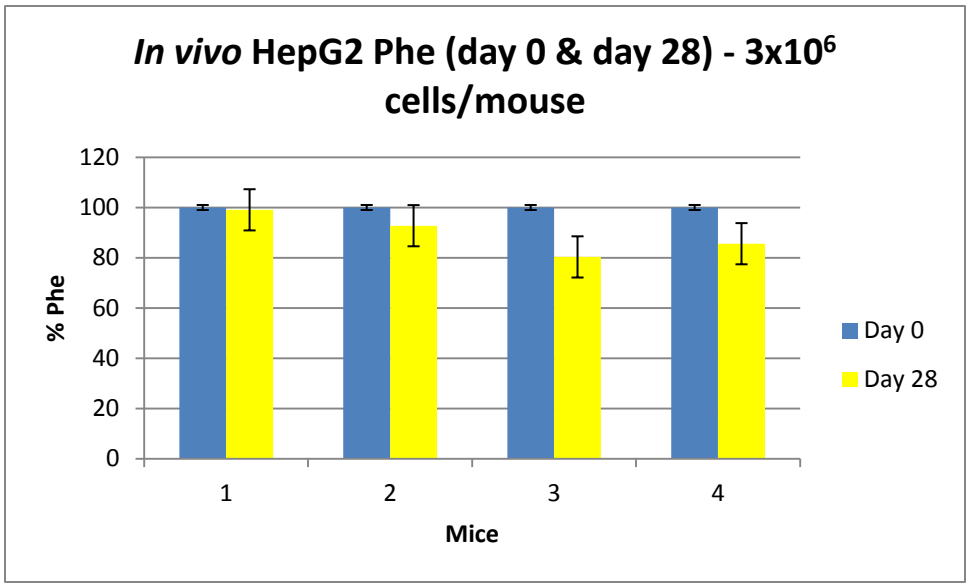

Figure 19: Percent phenylalanine in mice injected with HepG2 cell encapsulated microspheres (day 0 & day 28 - 3ml/mice)

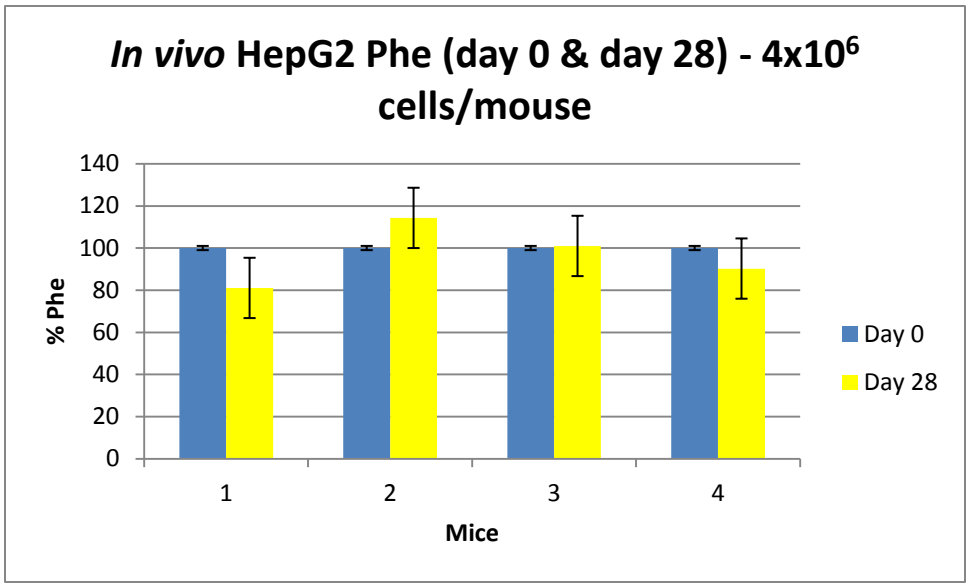

Figure 20: Percent phenylalanine in mice injected with HepG2 cell encapsulated microspheres (day 0 & day 28 - 4ml/mice)

6.3.1 Phenotypic changes in mice

HepG2 cells encapsulated microspheres were transplanted into 3 male and 3 female homozygous PKU mice. Phenylalanine concentrations were monitored during the 28 day period. Phenotypic changes were also observed during the same time period. On day 0 the male and female untreated homozygous mice displayed a fur color which was similar and much lighter than the wild type control mouse (Figure 21).

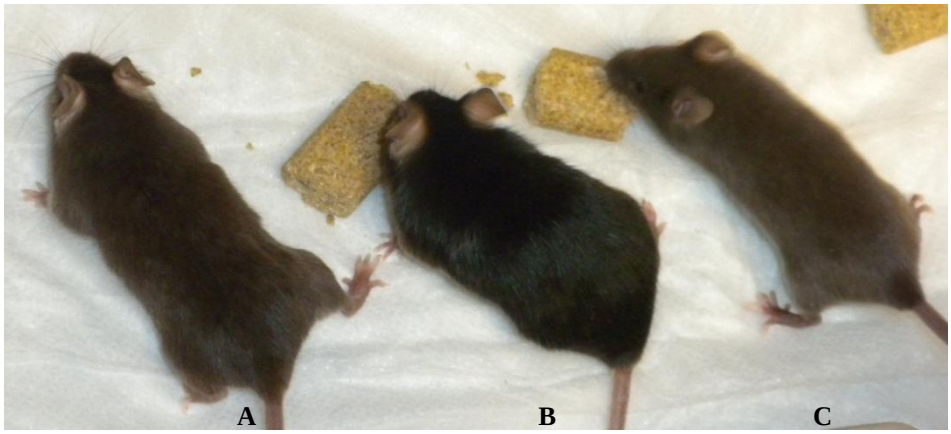

Figure 21: C57 mice strains transplanted with HepG2 cells on day 0: Mice A – male homozygous PKU mice transplanted with microspheres, Mouse B – Untreated wild type mouse (control), Mouse C – female homozygous PKU mouse transplanted with microspheres

28 days post injection of the HepG2 cell encapsulated microspheres, darkening of the fur color of both the male and the female mice was observed suggesting that there could be an increase in tyrosine levels (which is an important factor in the production of the skin pigment melanin) responsible for the phenotypic changes observed in these mice (Figure 22). One particular male mouse treated with HepG2 cells displayed the greatest darkening of fur color which was almost exactly the same (phenotypically) as the wild type male mouse (Figure 23).

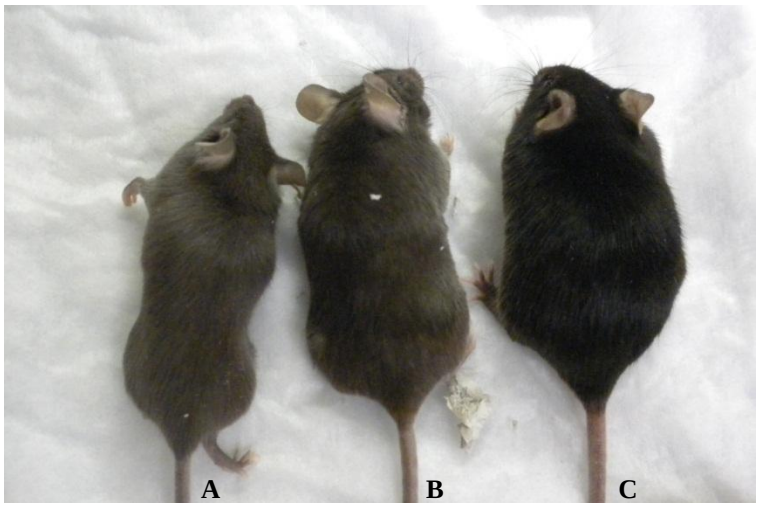

Figure 22: C57 mice strains transplanted with HepG2 cells on day 28: Mouse A – female homozygous PKU mouse transplanted with microspheres, Mice B & C – male homozygous PKU mice transplanted with microspheres

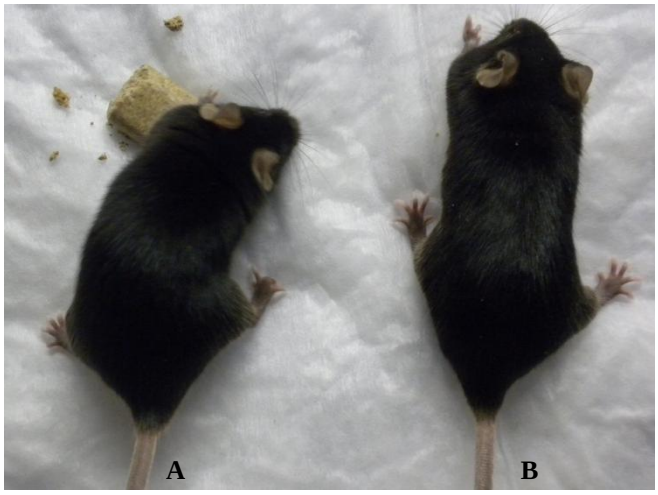

Figure 23: C57 mice strains on day 28: Mouse A – male wild type mouse, Mouse B – male homozygous PKU mouse transplanted with HepG2 cell encapsulate microspheres

Comparisons between the male and female treated homozygous mice on day 28 were also done. Results from our study demonstrated that the phenylalanine concentration in the female mice (17mg/dL) was approximately 47% higher than the phenylalanine concentrations in the male mice (15mg/dL). Results also indicated that male mice had a greater tendency to have a darkened fur color compared to the female mice. This could be due to the presence of the estrogen hormone in females conflicting with their ability to better respond to the therapy. This reasoning was also speculated in another research conducted with the adeno-associated vectors and PEGylated PAL in treating PKU [21].

In the final study performed with the HepG2 cell encapsulated microspheres, treated female homozygous mouse which was set to breed with the wild type male mouse produced five pups which weighed an average of 1.1 ± 0.06g on day 1. The results from this study was very different and unexpected as it has been particularly very difficult for homozygous female mice and the wild type male mice (which were set as breeding pairs and controls) to produce pups. Hence, these results suggest that HepG2 cell encapsulated microspheres could probably hold the greatest potential in reducing phenylalanine levels and could also play a significant role in the tyrosine production. HepG2 cells were injected in the mice over the other two cell encapsulated microspheres as previous studies have shown their ability to function as a bio-artificial liver. These encapsulated microspheres could potentially help or improve the reproducing capability in homozygous female mice by reducing their phenylalanine levels and thereby could serve as effective form of a treatment strategy for maternal PKU.

6.4 Cell Viability

Cell viability was measured by trypan blue staining. Cell viability data helps us further analyze the ability of the cells to remain functional during the course of the experiment thereby helping us determine the ability of these cells to function effectively in reducing phenylalanine. For the *in vitro* experiment, cell viability was performed by trypan blue staining with different cell lines: 293T, WRL 68 and HepG2 cells. Cells were counted on days 0, 4, 7, 14, 21 and 28. As

show in table 2 the cell viability remains high throughout the 28 days of cell encapsulation. Average percent cell viability remained substantially high amongst the HepG2 cells and there was no statistical significance (*P>0.05)* observed in cell viability amongst any of the cells between day 0 and day 28 for each of the respective cell lines.

Average percent cell viability ± SEM			
Days	**293T cells**	**WRL68 cells**	**HepG2 cells**
0	92.33 ± 1.45	89.33 ± 1.15	91.53 ± 1.34
4	89 ± 1.56	87.67 ± 1.53	90.2 ± 0.75
7	88.43 ± 1.54	85.33 ± 1.17	88.45 ± 1.24
14	86.2 ± 1.51	84.2 ± 1.63	86.86 ± 1.43
21	83.67 ± 1.83	81.76 ± 1.74	84.52 ± 1.78
28	79.32 ± 1.92	78.42 ± 2.12	82.79 ± 1.49

Table 2: Average percent cell viability (*in vitro)*

The mice transplanted with the cell encapsulated microspheres were sacrificed on day 28 and the microspheres were removed from their peritoneal cavity. The spheres were subsequently dissolved in sodium citrate and the cells were counted. A Student t test was performed to compare the cell viability on day 0 and day 28 and a *P*-value of 0.07 was obtained indicating no difference in cell viability prior to and post transplantation of the cell encapsulated microspheres (Table 3).

Cell viability data for microspheres encapsulated with HepG2 cells show that an average of 78.7% remained viable on day 28 (Table 4). Initial cell viability was 88% day 0 prior to encapsulation. This result demonstrates that cell viability remained similar to that initially

observed and that long-term encapsulation of cells did not cause significant harm to the cells. Additionally, percent cell viability corresponded with the darkening of fur color as the darkest fur color (mouse 4 – Table 4) was observed in the mouse with the highest percentage cell viability (85 percent). The phenylalanine concentration of this mouse was 15mg/dL which was on average approximately 75% lower than the other mice. Therefore, the cell viability data further support our analysis and finding that the HepG2 cell encapsulated microspheres are particularly more effective in reducing phenylalanine levels. The spherical geometry of the sodium alginate microspheres encapsulated with the three different cells were analyzed and to ensure that they retained their spherical shape the spheres were analyzed under a microscope every time the media was changed. The cell viability data also demonstrated that sodium alginate microspheres were quite morphologically intact and that the spherical geometry allowed the diffusion of essential substances thereby aiding the cell viability.

293T	
Mouse No.	**Percent cell viability**
1	78.4
2	79.8
3	82.1
4	76.45
5	77.3
6	74.3
7	75.7

Table 3: Percent cell viability (293T cells - *in vivo)*

HepG2	
Mouse No.	**Percent cell viability**
1	83.3
2	80
3	72.9
4	85
5	79.4
6	74.21
7	84.7

Table 4: Percent cell viability (HepG2 cells - *in vivo)*

6.5 Quantitative real time PCR

RT-PCR was done to quantitatively determine mRNA expression of phenylalanine hydroxylase by different cell lines. Figure 21 shows the comparison of mRNA expression levels of 293T, WRL68 and HepG2 cells. mRNA expression levels of the different cell lines are represented as a measure of their cycle threshold (ΔCt). ΔCt represents the number of cycles necessary for the fluorescent signal generated by dyes to cross the threshold level and is inversely proportional to the amount of target nucleic acid present in the sample. RNA expression was observed among the three different lines with the greatest levels of mRNA expression observed with the HepG2 cells (Figure 24).

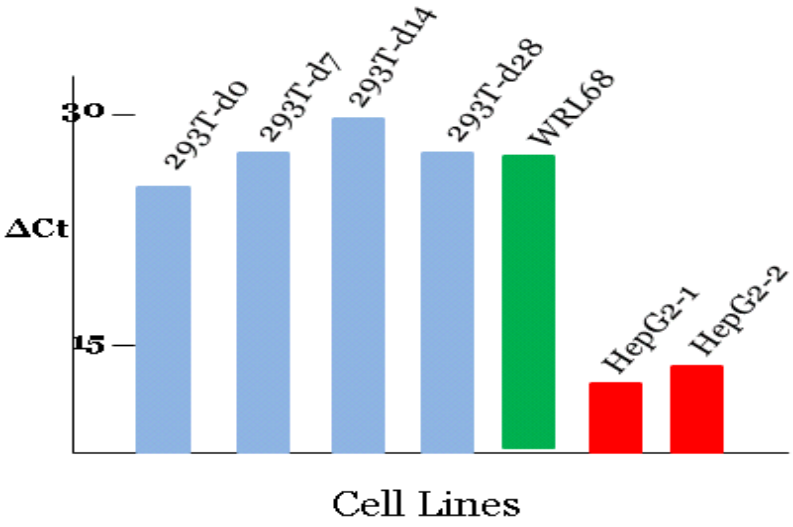

Figure 24: RNA expression levels of different cell lines

CHAPTER 7: DISCUSSION

The main objective of our study was to analyze the ability of cell encapsulation technology, a potential artificial organ system treatment strategy for maternal PKU. Cell encapsulation is relatively a new treatment strategy that aims to reduce phenylalanine levels by immunoisolating PAH expressing cells. In our study, several cell lines have been tested in reducing phenylalanine levels. Initial studies done by Dr.Hunter's lab showed that transfected cells possessed the ability to reduce phenylalanine levels *in vitro* transplanted with 293T and WRL68 cells for days 0 – 10 compared to mock cell lines [46]. Further studies were performed using these non-transfected cell lines to test their efficacy in controlling PKU over a longer duration.

Because previous studies were all performed *in vitro*, our goal was to evaluate the ability of encapsulated cells both *in vitro* and *in vivo*. Results from the *in vivo* and *in vitro* experiments demonstrated that 293T, WRL 68 and HepG2 cells encapsulated within polymeric microspheres possess the ability to reduce phenylalanine levels. 293T cell encapsulated microspheres helped attain significant reduction ($P<0.05$) in phenylalanine concentrations on day 21 during the 7 day experiment. Overall, these cells proved to be quite efficient in reducing phenylalanine levels during the 21 day period *in vitro*. These results were further validated by the *in vivo* results where a significant reduction in phenylalanine concentrations was observed on day 28. Although an inconsistent trend was observed in the reduction of phenylalanine levels during the experimental period both *in vivo* and *in vitro*, the results from the experiments utilizing these cells trended toward a reduction in phenylalanine concentrations on each of the different days of the experiment. This supports our hypothesis that the PAH expressing cells, as an artificial organ system aid in phenylalanine metabolism. The variations in phenylalanine concentrations could be attributed to the increase in phenylalanine concentrations in mice through the diet and the presence of phenylalanine in the media used for the *in vitro* experiments. The efficacy of these 293T cells in reducing phenylalanine levels was further supported by the substantially high cell

viability data observed during the 28 day period both *in vivo* and *in vitro.* The mRNA expression levels also helped confirm that these cells express PAH which is vital to obtain sustained reduction in phenylalanine levels both *in vivo* and *in vitro*. The results from these experiments demonstrated that 293T cell encapsulated microspheres remained viable during the 28 day and possess the ability to reduce phenylalanine levels *in vivo* and *in vitro*.

Similarly results from the experiments with WRL68 cell encapsulated microspheres showed a gradual reduction in phenylalanine levels during the 21 day period (in the 7-day experiment) and a significant reduction of more than 60% in phenylalanine concentrations was observed particularly on day 21 in the same experiment. Results from the 3-day experiment also demonstrate that there was a reduction in phenylalanine concentrations in media containing the cell encapsulated microspheres compared to the media containing empty microspheres. But comparison of the results from the 7-day and 3-day experiments suggest that media change every 7-days was more favorable. This could possibly be due to the fact that media change every 3 days might be too frequent to allow us to detect a reduction in phenylalanine in the media. The efficacy of the WRL68 cell encapsulated microspheres in reducing phenylalanine effectively was further validated by the substantially high cell viability data where no significant difference ($P>0.05$) in the average percent cell viability between day 0 and day 28 was observed. The mRNA expression levels of the WRL68 cells also help further confirm that these cells do possess the ability in reducing phenylalanine levels when compared to the phenylalanine levels in empty media and could be potentially useful in treating maternal PKU.

Encapsulated HepG2 cells reduced phenylalanine levels *in vitro* by approximately 60% compared to the phenylalanine levels in empty media with the 7-day experiment. In addition, cell viability data from the mice encapsulated with these cells show that HepG2 cells remain highly functional (82.79% on day 28) over a longer duration compared to the other cells. Their higher PAH mRNA expression levels as evidenced by the low cycle threshold values obtained through quantitative RT-PCR analysis also suggest that HepG2 cells would be more efficient in controlling phenylalanine levels in PKU mice. *In vivo* studies with the HepG2 cell encapsulated

microspheres also showed favorable results in reducing phenylalanine levels in homozygous PKU mice. There is a statistically significant reduction in the phenylalanine concentrations as measured from the *in vivo* experiment which had 4 mls of microspheres containing encapsulated cells injected into the mice (4×10^6 total cells). Comparison of the results from the experiments having two different volumes (3×10^6 total cells and 4×10^6 total cells) of cells injected in the mice demonstrate that 4×10^6 total cells/mouse was optimal as it helped attain a more sustained and gradual reduction in phenylalanine concentrations in media.

Results also demonstrated that HepG2 cells are more efficient in controlling phenylalanine levels in comparison to WRL68 and 293T cells *in vivo*. When 4×10^6 total cells were injected into each of the mice, darkening of the fur color of the treated homozygous PKU mice was observed on day 28. One particular homozygous male mouse showed an increased darkening of fur color which was exactly the same phenotype as the wild type male mouse. Reversal of hypopigmentation observed in some of the mice treated with these microspheres suggests that the cell encapsulated microspheres could also possess the ability to convert phenylalanine to tyrosine. Further investigations in understanding the role played by tyrosine in influencing the fur color of the mice and the pathway by which phenylalanine is converted to tyrosine could be useful in not only reducing phenylalanine levels but also in increasing the tyrosine production in these mice.

Comparisons made between the male and female mice transplanted with HepG2 cell encapsulated microspheres showed that male mice respond better to the treatment than the female mice as it was evidenced by the phenylalanine concentrations and also the fur color of the treated homozygous PKU mice. Previous genome-targeted PAH gene therapy studies and preclinical studies using PEGylated PAL have indicated that the presence of estrogen hormone in females could affect the reduction in phenylalanine levels. Hence, in order to obtain sustained and more efficient control of phenylalanine levels in females higher volumes of spheres could be tested in the PKU mice. Finally, the pups produced on breeding the treated female homozygous PKU mouse with the wild type male mouse also suggests that cell encapsulation could

potentially control phenylalanine levels in homozygous female mice thereby aiding them in becoming pregnant. This was not observed among the control breeding pairs in which the untreated female homozygous PKU mice were bred with wild type male mice. Previous studies with HepG2 cells have also shown their innate ability to act as a potential bio artificial liver [43]. Although results from the *in vitro* experiments did not show a reduction in phenylalanine throughout the 21 day period, results from the *in vivo* experiment were favorable with these cells and are generally more replicative of the human system. Thus we conclude that encapsulated HepG2 cells do possess the ability to reduce phenylalanine levels.

In conclusion, results from our study indicate that cell encapsulation technology could aid in the reduction of phenylalanine levels. In order to obtain sustained and increased darkening of the fur color, higher quantity of the cell encapsulated microspheres could be tested. This could even help achieve sustained reduction in phenylalanine levels and increase in tyrosine levels. Results from the *in vivo* and the *in vitro* experiments indicate that 293T, WRL68 and HepG2 cells possess the ability to reduce phenylalanine concentration. In particular, HepG2 cells possessed the greatest ability in reducing phenylalanine levels. They display high expression levels of the PAH mRNA in the encapsulated microspheres which could possibly be due to their endogenous activity to function as a bio artificial liver and also remain highly viable throughout the experiment. Further investigations could help us better understand their role played in the phenylalanine metabolism and also their ability to produce tyrosine.

CHAPTER 8: CONCLUSION

Results from the current study prove the concept that cell encapsulation could serve as a potential non-dietary therapy in treating phenylketonuria. Cell encapsulated microspheres do possess the ability to reduce phenylalanine levels both *in vitro* and *in vivo* as evidenced by the 293T kidney, WRL68 and HepG2 hepatic cells. Significant reduction in phenylalanine levels observed with these cells suggest that future work by increasing the volume of cell encapsulated microspheres injected into the mice could further reduce phenylalanine levels and help attain a statistically significant increase in tyrosine levels. Comparison of the 293T kidney, WRL68 and HepG2 hepatic cells suggest that HepG2 cells possess higher potential in treating PKU. Although WRL68 cells helped achieved a statistically significant reduction in phenylalanine levels *in vitro,* HepG2 cells show favorable results in the *in vivo* studies which are better study models as they more closely mimic the human condition.

Cell encapsulation could serve as an alternative therapeutic strategy to the dietary therapy in treating PKU as it eliminates the need for the administration of immunosuppressants. This suggests that it could be used safely and more economically in PKU patients. This is particularly favorable for pregnant women as they fail to adhere to the diet prior to and throughout pregnancy and the administration of immunosuppressants could lead to other problems during pregnancy. Further investigations on the use of cell encapsulation as a potential non-dietary therapy in treating PKU as well as maternal PKU would be necessary to achieve sustained results.

CHAPTER 9: FUTURE WORK

In the current study, HepG2 cells showed the greatest potential in treating PKU compared to the 293T kidney and WRL68 liver cells. In the future, stable clones of these cells overexpressing PAH could be developed and tested for their ability to significantly reduce phenylalanine levels and increase tyrosine levels. As 4ml (4×10^6 total cells) of encapsulated cells transplanted into each of the mice showed favorable results this amount could be tested in a larger group of mice to determine if more mice reversed their phenotype in response to our non-dietary therapy. HepG2 cell encapsulated microspheres could also be injected into more female mice. Their response to the treatment could be observed by breeding them with wild type male mice and the pups produced on breeding could be further tested to determine the ability of this treatment to prevent maternal PKU syndrome. Specifically, the phenylalanine levels of the dams throughout gestation and the pups outcomes could be analyzed. In the pups, we could look for changes in cognitive abilities using standard cognitive function tests such as spatial novelty test and object recognition test which tests for their memory [46]. In the spatial novelty test, mice are expected to recognize objects that have been moved from previous positions to determine their ability to encode spatial relationships. Object recognition test helps determine the ability of the mice to recognize a new object which is similar to a sample that they were previously exposed to. Additionally, cell viability studies and gene expression analysis help us confirm the viability of the cells and their ability to express PAH mRNA, protein expression studies can be performed through western blotting to confirm the expression of PAH protein in the encapsulated cells.

In conclusion, our results have demonstrated that the generation of an artificial organ system through cell encapsulation holds great potential as a novel non-dietary therapy for PKU and could be used to prevent the deleterious effects of maternal PKU syndrome.

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