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EFFECTS OF A SYSTEMIC HIGH UREA CONCENTRATION ON THE ENDOMETRIAL AND EMBRYONIC TRANSCRIPTOMES OF THE MARE

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EFFECTS OF A SYSTEMIC HIGH UREA CONCENTRATION ON THE ENDOMETRIAL AND EMBRYONIC TRANSCRIPTOMES OF THE MARE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

By
Yatta Linhares Boakari
Lexington, Kentucky
Director: Dr. Barry Ball, Professor of Equine Reproduction
Lexington, Kentucky
2019

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ABSTRACT OF DISSERTATION

EFFECTS OF A SYSTEMIC HIGH UREA CONCENTRATION ON THE ENDOMETRIAL AND EMBRYONIC TRANSCRIPTOMES OF THE MARE

Pregnancy loss remains a major source of economic cost to the equine industry. Frequently, the exact causes of pregnancy loss remain unknown. It has been shown, in other species, that increased dietary protein leading to elevated blood urea nitrogen concentrations (BUN) can be a factor in decreased survival of the early embryo. Our studies provided novel information regarding the effects of elevated BUN on endometrium and embryos from mares as well as insights on changes in their gene expression. Our first objective was to develop an experimental model to elevate BUN during diestrus using intravenous urea infusion. We analyzed the effects of an acute elevation in BUN on uterine and vaginal pH along with changes in the endometrial transcriptome of mares with RNA sequencing. There was a significant increase in BUN and a decrease in uterine pH in the urea group compared to the control group. A total of 193 genes were differentially expressed (DEG) between the urea and control groups. The DEG were predicted to be related to cell pH, ion homeostasis, changes in epithelial tissue, fatty acid metabolism, and solute carriers. Our second objective was to evaluate the effects of elevated BUN in the endometrium of mares using a chronic oral urea administration to elevate BUN in mares. Uterine and vaginal pH were evaluated and RNA sequencing of the endometrium was again performed. There was an increase in BUN in the urea-fed mares, but no significant change in uterine or vaginal pH between the groups. A total of 60 DEG were characterized, with prediction of transcriptomic changes in the endometrium of mares related to cell death (necrosis) and cellular movement (invasion of cells). Our third objective was to determine the effects of a high BUN on the transcriptome of day-14 embryos. There was a positive correlation between plasma BUN and blastocoele fluid urea nitrogen concentration. Changes in embryo transcriptome were related to survival of organism, angiogenesis, adhesion, and quantity of cells. Our final objective was to evaluate the correlation between BUN and follicular fluid urea nitrogen and evaluate the survival of embryos collected from donor mares with high BUN

concentrations. Urea nitrogen concentration was positively correlated between the plasma and follicular fluid of mares. Additionally, there was a higher pregnancy rate when embryos were collected from mares with lower BUN. Overall, these results further elucidate the mechanisms through which urea affects endometrial and embryonic transcriptome of mares with high BUN, serving to identify effects of a high BUN in the reproductive tract of mares that might lead to decreased fertility.

KEYWORDS: High protein diet, uterus, embryo, horse, RNA sequencing, fertility.

Yatta Linhares Boakari

06/14/2019

Date

EFFECTS OF A SYSTEMIC HIGH UREA CONCENTRATION ON THE ENDOMETRIAL AND EMBRYONIC TRANSCRIPTOMES OF THE MARE

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06/14/2019

Date

DEDICATION

I dedicate my dissertation to my families: Boakari, Linhares, and Silva. You truly are my strength! “Do the best you can until you know better. Then when you know better, do better.” – Maya Angelou

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CHAPTER 1. Literature review

1.1. Introduction

In the United States, early embryonic (prior to 40 days) and early fetal (prior to 90 days) losses remain important causes of infertility in mares, with approximately 8% of early embryos lost and a further 5% rate of early fetal loss. The rate of pregnancy loss can present considerable variation across season and year (B. A. Ball, 2011). Pregnancy loss remains a major source of economic cost to the equine industry. However, many times, the exact causes of pregnancy losses remain unknown.

Composition of maternal diet, such as dietary polyunsaturated fatty acids, high caloric diets, and starch-rich diets may have negative impacts on oocyte, embryo, follicular fluid and uterine environment in mice, cows, and ewes (Coyne, Kenny, & Waters, 2011; Fouladi-Nashta et al., 2009; Hughes et al., 2011; Igosheva et al., 2010; Mattos, Staples, & Thatcher, 2000; van Knegsel et al., 2007; Zachut et al., 2010). More specifically, the role of forage and elevated crude protein (CP) intake leading to high BUN has been proposed as an important factor in oocyte and embryo quality and overall pregnancy failure in cows and ewes (Butler, 2000a; Elrod & Butler, 1993; C. Elrod, M. Van Amburgh, & W. Butler, 1993; T. McEvoy, J. Robinson, R. Aitken, P. Findlay, & I. Robertson, 1997; Rhoads, Gilbert, Lucy, & Butler, 2004; Rhoads, Rhoads, Gilbert, Toole, & Butler, 2006). However, the effect of elevated BUN on fertility has never been reported in mares. Therefore, the research proposed here will provide novel information regarding the effects of high BUN on reproductive function in mares. Additionally, these projects will provide insights on alterations in gene expression in the endometrium and early embryos to elucidate possible mechanisms of action.

This literature review will summarize crude protein digestion, urea cycle, and effects of high blood urea nitrogen *in vitro* and *in vivo*. Although the focus of this work is horses, protein digestion in ruminants was reviewed here because most studies done to identify the effects of high protein diet or BUN were performed with ruminants. Subsequently, this dissertation describes the first studies with mares evaluating the effects of elevated BUN on the endometrial and embryonic transcriptome as evaluated with RNA sequencing.

1.2. Protein digestion in horses

Horses are monogastric, more specifically, they are classified as non-ruminating roughage grazers. The horse is a hindgut fermenter, with a very well developed cecum and ascending colon adapted for fermentation of plant cellulose and hemicellulose by cellulolytic bacteria (Reece, Erickson, Goff, & Uemura, 2015). Consumed proteins are composed of hundreds of amino acids linked together by peptide bonds. To move across the intestinal absorptive cells, enterocytes, proteins need to be digested into dipeptides or tripeptides. The main sites of enzymatic protein digestion are the stomach and small intestine (NRC, 2007; Reece et al., 2015). Protein digestion starts in the stomach where hydrochloric acid (HCl) denatures dietary protein by hydrolyzing part of the peptide bonds. The gastric glands, chief cells located in the fundic stomach, secrete pepsinogen, a precursor of proteolytic enzymes, that is inactive so as to prevent autodigestion of gastric cells. Subsequently, pepsinogen will be cleaved and transformed into its active form- pepsin. Pepsin is responsible for cleaving peptide bonds from the molecule, which will then be composed of 25 to 100 amino acids and ready to enter the first portion of the small

intestine, the duodenum. These proteins activate receptors of the enteroendocrine cells in the duodenum making them secrete cholecystokinin, which enters systemic circulation and triggers the secretion of pancreatic proteolytic enzymes into the duodenum. The pancreatic enzymes including trypsin, chymotrypsin, elastase, and the carboxypeptidases cleave peptide bonds converting protein to peptides with six or less amino acid residues as well as free amino acids. Subsequently, these smaller compounds move to the brush border of the enterocytes, the microvilli-covered surface of the intestine, where peptidases will split oligopeptides (six or less amino acids in length) into di- and tripeptides. These are transported into the enterocyte by a H^+-coupled transporter and hydrolyzed to free amino acids by peptidases or transported across the basolateral membrane (Krehbiel & Matthews, 2003). Additionally, the free amino acids will be absorbed by transporters and will either be transformed into protein or transported across the basolateral membrane to systemic circulation (Krehbiel & Matthews, 2003; Reece et al., 2015). Single amino acids accumulate in a large concentration above the apical membrane of the villous cells in the duodenum and jejunum after ingestion of a meal. There are facilitated carriers in the apical membrane of the villous cells, such as amino acid transporters, that bind the amino acid and a sodium atom. When the amino acid moves down its concentration gradient and the sodium ion moves down its electrical and concentration gradient, it helps move the amino acid across the apical membrane. Dipeptides and tripeptides can be absorbed at the brush border by tertiary transporters, as they are large and need an ATP molecule and a transporter protein to pump them across the apical membrane. They then arrive in the cytosol of the villous enterocytes and are hydrolyzed to single amino acids by intracellular peptidases (Reece et al., 2015; Woodward et al., 2010).

Amino acids will either be used for protein synthesis in the body or undergo catabolism and excretion as carbon dioxide and urea, for example. In the latter case, amino acids enter the extracellular fluid and are transported in the portal circulation to the liver for deamination and urea formation (Reece et al., 2015; Reitnour, Baker, Mitchell, Little, & Kratzer, 1970; Woodward et al., 2010). A large amount of the urea that is produced in the liver is secreted into the ileum and moved to the large intestine for bacterial degradation to ammonia through the bacterial enzyme, urease. Intestinal bacteria are found in the duodenum, jejunum, ileum, cecum, and colon of mature horses. Different from ruminants, most of the equine microbial bacteria are found in the hindgut (Kern, Slyter, Leffel, Weaver, & Oltjen, 1974). The proteolytic bacteria reutilize most of the resulting ammonia or urea to synthetize protein and the remaining ammonia and urea diffuse into the blood (Frape, 2008; Mackie & Wilkins, 1988). Consequently, after the deamination of amino acids in the liver and synthesis to urea, the concentration of blood urea nitrogen (BUN) increases (Frape, 2008; Lewis, 1995). When there is an excess protein intake, there is an associated increase in BUN; consequently, BUN levels are used to assess whole-body metabolism, protein metabolism, and levels of systemic urea nitrogen in horses (Kohn, Dinneen, & Russek-Cohen, 2005; Latham, Wagner, & Urschel, 2019; Mok, Levesque, & Urschel, 2018).

1.3. Protein digestion in ruminants

Although our experiments used horses, studies in ruminants are a major source of information for the following projects. Therefore, it is important to understand the basic physiology in protein digestion in ruminants and differences with equids. Firstly,

ruminants have forestomaches composed of the rumen, reticulum, and omasum. In this foregut, bacterial fermentation digests cellulose and hemicellulose that are not digestible by enzymes produced by the animals (Kern et al., 1974; Reece et al., 2015).

Additionally, for ruminants, the ingested protein can be either rumen degradable or rumen undegradable. The rumen degradable protein is deaminated by proteolytic enzymes coming from rumen bacteria and protozoa to produce ammonia. The microbes will use this ammonia to synthetize microbial protein. When there is an excess ingestion of rumen degradable protein, it is degraded and forms ammonia in the rumen, which is transported out of the rumen into the portal circulation and travels to the liver to be converted into urea. Part of the rumen degradable protein is converted to microbial protein, as the microbes use nitrogen from ammonia or urea to form the amino acids that compose their structure. Subsequently, when these microbes die or go into the intestine, the proteins from their structures are digested by proteolytic enzymes and the ruminant utilizes the resulting amino acids. The resulting protein from the digestion of bacteria is very high quality and serves as a source of essential and nonessential amino acids. Conversely, the protein that is not degraded by the rumen microbes is known as rumen undegradable protein and passes through the rumen unchanged. An amount of rumen undegradable protein is digested in the small intestine of ruminants serving as another source of essential and nonessential amino acids. The rumen undegradable protein can also contain non-protein nitrogen (nitrogen that is not in the form of amino acids and protein). Urea is one of the most commonly used non-protein nitrogen source, as it supplies nitrogen to the microbes in the rumen (Reece et al., 2015; Tamminga, 2006). Urea is hydrolyzed into ammonia and carbon dioxide by bacterial urease, then it is available for rumen microbes to make microbial

protein. When ammonia is absorbed, it is converted to urea by the liver and can either be recycled back to the rumen or be excreted by the kidneys (Jin, Zhao, Zheng, Beckers, & Wang, 2018).

1.4. Ammonia and urea metabolism and excretion in the horse

Urea is a small-uncharged particle, with a molecular weight of 60 g/mol, it is very soluble and has low toxicity compared to other catabolic metabolites. There are also urea transporters, membrane transport proteins responsible for transporting urea molecules across membranes. The importance of transporters is that a large amount of urea molecules can be moved to different portions of the body in a timely fashion. Two main types of urea transporters genes are *SLC14A1* and *SLC14A2.* These genes produce multiple protein isoforms, such as UT-A1 to -A5 and UT-B. These transporters were first cloned and isolated from erythrocytes and kidney. Currently, it is known that these urea transporters have a widespread distribution in tissues from mammals. These transporters are responsible for the rapid transport of urea across cell membranes which is important for urea equilibrium in tissues. This urea translocation is done by facilitated transport, independent of Na^+ and Cl^-. (Sands, 1999, 2003; Shayakul & Hediger, 2004). Additionally, there are aquaporins (AQPs), a family of membrane water channels, composed of diverse isoforms, including transporters that allow the passage of water, glycerol and urea (AQP3, 7, 8 and 10). AQPs are distributed in multiple organs and tissues, forming pores across the membranes to facilitate the transport of water and other small molecules (Li & Wang, 2014). Of interest, expression of AQPs was seen in the endometrium of mares and these

were predicted to be related to changes in the endometrium during the estrous cycle and pregnancy (C. Klein, Troedsson, & Rutllant, 2013).

Urea is absorbed by the horse from the small intestine with subsequent excretion through the kidneys in the urine. It will be transported into the lumen of the large intestine, cecum, and colon, where it will be used by bacteria to produce ammonia, then incorporated into their protein (Reece et al., 2015). If an excess of protein is ingested, the amino group is removed from the amino acids and converted to urea in the liver, thus increasing the blood urea nitrogen (BUN) concentration (Frape, 2008; Lewis, 1995).

An experiment to understand how horses utilize urea (Martin, McMeniman, Norton, & Dowsett, 1996) used isotope labeled urea intragastrically or intravenously. When a single intragastric dose was given, urea was rapidly absorbed into the blood, with an average of 90% of the dietary urea entering the plasma. Over a 5-day period, 26% of the dietary urea was retained. With a single intravenous injection of urea, there was a significant increase in BUN concentrations with a percentage of urea retained after 5 days varying between 15 to 38% depending on the diet the animals received. This study showed that urea is not an ideal supplement to substitute protein for horses in most situations, because most of it is absorbed from the small intestine and excreted in the urine before it can reach the large intestine and be used by bacteria for protein synthesis. A small amount of urea will reach the cecum and colon to be used for bacterial protein synthesis. Thus, supplementation with a nonprotein nitrogen source, such as urea, seems to be beneficial only when the horse is receiving a protein deficient diet. Urea-supplementation, in a dose of 0.14 g urea/kg of body weight to horses daily, resulted in elevated BUN concentrations,

probably because urea was rapidly absorbed from the small intestine by transporters (H. F. Hintz & Schryver, 1972; Reitnour, 1978).

Furthermore, if urea is given in a large amount, it is converted to ammonia by bacterial urease in the large intestine, and can be toxic. Horses that ingest a large amount of urea will show incoordination, press their heads against objects, become comatose, convulse, and even die. A dose of 450 g of urea fed orally was fatal for ponies weighing 125 to 136 kg, which would equal 1.65 kg of urea for a 500 kg horse (H. Hintz, Lowe, Clifford, & Visek, 1970; Lewis, 1995).

1.5. Protein requirements in horses

Crude protein (CP) is a nutrient required for growth and maintenance and is a major component of most tissues, enzymes, hormones, and antibodies in the body; it can also be used for energy (Lewis, 1995; NRC, 2007). The 22 amino acids that compose proteins have been classified as: nonessential (dispensable), those that can be synthesized *de novo* in animal cells, or essential (indispensable), which have to be supplied in feed. For the horse, there are 10 essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Proteins are composed of amino acids bonded together and contain nitrogen. The CP content of feed is estimated by calculating its nitrogen content and dividing this amount by 0.16 (because most protein contains 16 ± 2% of nitrogen), for example, a feed containing 1.6% of nitrogen would be equivalent to 10% CP (Lewis, 1995; NRC, 2007).

Horses have different daily protein requirements according to their stage of development. The following formula is used to calculate daily protein requirements for a

mare during early pregnancy: body weight x 1.26 g CP/kg of body weight. A 500 kg mare from conception to early pregnancy needs approximately 630 g of CP daily (NRC, 2007). Similarly, pregnant mares from 9 months to 11 months of gestation require the same amount of CP as nonpregnant and sedentary horses, which is 656 g. Pregnant mares from 9 to 11 months of gestation require a higher amount, 798 to 893 g of CP daily. Finally, mares that are in the first to sixth month of lactation require from 1535 to 1265 g of CP daily (Lewis, 1995; NRC, 2007). A more individualized daily requirement of CP was proposed, by suggesting three different levels of protein intake according to individual characteristics of the horse. Using a minimum, average or elevated daily nutritional requirement, depending on how easy it is for the horse to maintain their normal weight. A 500 kg mare during early pregnancy should consume a minimum of 540g, an average of 630 g or an elevated amount of 720 g CP/daily. A 500 kg pregnant mare during the last month of gestation, should consume a minimum of 803, an average of 893 or an elevated amount of 983 g of CP/daily (Lawrence, 2011).

Even though high protein diets might have deleterious effects on reproduction in ruminants, diets supplying excessive amounts of CP to horses are common. Surveys conducted with horse owners helped to better elucidate feeding practices, more specifically, type of grain, hay and supplements that these animals received. Results showed that in 70% of cases the CP offered exceeded the NRC recommendations. Additionally, horses received diets with 157 ± 21.6% (range 79-263%) of the CP recommended by the NRC (Harper, Swinker, Staniar, & Welker, 2009; Honoré & Uhlinger, 1994). This excessive amount of CP will result in BUN concentrations above the normal range for horses that might result in negative consequences to the reproductive

tract and consequently to pregnancy rates. However, no reports to date have addressed the effects of a high protein diet on fertility in the mare.

1.6. Effect of high blood urea nitrogen *in vitro* and *in vivo*

Elevated CP consumption or urea-supplementation elevates BUN concentrations systemically, thus increasing the amount of urea in the body (NRC, 2007). In general, urea concentrations in the female reproductive tract of cows and ewes have been positively correlated with urea concentrations in plasma, thus elevations in BUN would be expected to result in elevated tissue concentrations. For example, studies showed a high correlation between follicular fluid and plasma concentrations of urea in women, cows, ewes, female buffaloes, and mares (Baki Acar, Birdane, Dogan, & Gurler, 2013; Collins et al., 1997; D. S. Hammon, Holyoak, & Dhiman, 2005; Jozwik, Teng, & Battaglia, 2006; Leroy et al., 2004; Nandi, Kumar, Manjunatha, & Gupta, 2007). Follicular fluid is composed of water and solutes from plasma and metabolites from follicular cells, and its composition is an important factor because it is in direct contact with the oocyte-cumulus complexes before ovulation (Jozwik et al., 2006; Nandi et al., 2007). The follicular fluid composition changed as follicles grew (Leroy et al., 2004), possibly due to an increase in follicular vascularity (Acosta, Hayashi, Matsui, & Miyamoto, 2005; Gastal et al., 2007), dilutions caused by an increase in follicular fluid volume (Nandi et al., 2007), and an increase in the permeability of the blood-follicle barrier (Bagavandoss, Midgley, & Wicha, 1983).

Similarly, an increase in BUN during the luteal phase caused an increase in urea nitrogen concentrations in the uterus of cows and ewes and a decrease in intrauterine pH (Elrod & Butler, 1993; C. Elrod et al., 1993; D. S. Hammon et al., 2005; Ellen R Jordan,

Thomas E Chapman, Donald W Holtan, & Lloyd V Swanson, 1983; T. McEvoy et al., 1997; Slade, Robinson, & Casey, 1970). However, an absence of changes in the uterine pH related to an increase in BUN after a high protein diet was also reported (Amundson et al., 2016). These changes in the intrauterine environment will affect early embryos when they arrive to the uterus, as they are dependent on the uterine histotroph for nutrition (Allen & Wilsher, 2009; C Klein & Troedsson, 2011). On a molecular level, high urea concentrations changed the mRNA expression evaluated with targeted real-time PCR *in vitro* of bovine endometrial explants with an altered endometrial gene expression related to cell growth, proliferation, differentiation, and immune function (Gunaretnam, Pretheeban, & Rajamahendran, 2013).

Ewes that received either urea or a diet with CP higher than daily recommendations that increased BUN, did not have a difference in the number of embryos recovered at days two, three, four or five (Berardinelli, Weng, Burfening, & Adair, 2001; Fahey, Boland, & O'Callaghan, 2001). Interestingly, the duration of urea-treatment influenced the number of embryos recovered at seven days after artificial insemination (AI) in cows. Cows that received urea-treatment for a short period (from the day of AI until the day of embryo collection) had a lower number of embryos recovered per cow, when compared to those that received a long urea-treatment (ten days before AI), or that received no urea (3.8 ± 0.3, 6.7 ± 0.4, and 6.1 ± 0.3 embryos, respectively) (Dawuda et al., 2002). However, urea-treatment reduced the mean number of cells per embryo recovered from ewes at day 4 (10.27% ± 0.27 and 8.17% ± 0.29 for untreated and urea-treated donors, respectively) (Fahey et al., 2001), and also reduced the percentage of recovered day-4 embryos with more than 16 cells (33% and 86% for urea-treated and control ewes, respectively) (T.

McEvoy et al., 1997). Similarly, cows that received urea-treatment for a short time had a lower percentage of embryos (53.3%) morphologically graded as 'very good' and 'good' when compared to animals that received a control diet or urea-treatment for a longer time (67.4% and 67.5%, respectively) (Dawuda et al., 2002). These results suggest that although the total number of embryos is not affected, treatments that resulted in an increase of BUN did have negative effects on the early embryo development up to 4 days after estrus. Additionally, the negative effects of urea-treatment on the embryo development was influenced by the duration of treatment, with a shorter treatment having more negative effects, perhaps because the animal did not have time to adapt and create compensatory mechanisms.

Furthermore, when embryos with good morphological quality and at least eight cells, collected at day 4 from control or urea-treated donor ewes, were transferred to recipients (in a donor to recipient combination of: [1] urea-treated donor to untreated or urea-treated recipient, or [2] untreated donor to untreated or urea-treated recipient), the pregnancy rate until days 34 - 36 after estrus was not reduced (70% for untreated and 75% for urea-treated recipients) (Fahey et al., 2001). Embryos were collected from ewes that received a urea-supplementation, at day 4 or day 11, and transferred to ewes also receiving treatment, with different pregnancy rates at day 18 (75% and 33%, respectively, for control and urea-treated animals) (T. McEvoy et al., 1997). Thus, the changes in uterine environment due to a BUN increase is not the only factor related to the reduction of embryo survival and pregnancy maintenance in ewes (Berardinelli et al., 2001). Conversely, embryos collected at day 3 or 7 from cows that received urea-treatment or diets with high CP had similar percentages of recovery, stage of development, and morphological quality

when compared to animals that received a control treatment (Amundson et al., 2016; Gath et al., 2012; Rhoads et al., 2006). Additionally, embryos from cows with high BUN, transferred to recipients, receiving diets resulting in low or high BUN, had lower pregnancy rates when compared with embryos collected from cows with low BUN. The recipient diets did not affect pregnancy rates (Rhoads et al., 2006). These results suggest detrimental effects caused by elevated urea and/or other metabolites *in vivo* to embryos during early development.

In vitro maturation and fertilization were performed with cumulus oocyte complexes from animals with high or low BUN or with medium supplemented with different concentrations of urea. Embryos collected from ewes at day 4 after urea-treatment, oocytes collected from cows with high BUN and cultured *in vitro*, and oocytes cultured in medium with high urea (from 0 to 10 mM urea concentration) had reduced fertilization rates, lower cleavage rates, higher total apoptotic cell rate, lower rate of blastocyst hatching, and a smaller proportion of embryos which developed into blastocysts (De Wit, Cesar, & Kruip, 2001; Ferreira et al., 2011; Kowsar et al., 2018; T. McEvoy et al., 1997; Ocon & Hansen, 2003; Santos et al., 2009). After maturation of bovine oocytes in the medium supplemented with urea (18.7 mg/dL) for 24 hours, oocytes had a shrunken morphology (Kowsar et al., 2018). Conversely, other studies found no difference between rates of recovery or quality of oocytes collected from cows that received a control or high CP diet, or cows that received urea-treatment after fertilization and culture *in vitro* (Amundson et al., 2016; Ferreira et al., 2011), and no difference in oocyte cleavage or blastocyst hatching (Amundson et al., 2016). Therefore, several *in vitro* studies show a

sensitivity of oocytes and embryos to urea with a number of adverse effects during their early stages related to their morphology as well as their developmental potential.

Oocytes matured *in vitro* with different media pH containing acid dimethadione for 8 days (resulting in media pH of 7.4, 7.1, 7.0, and 6.8 pH), resulted in a consequent lower cleavage rate and development to the blastocyst stage and higher oocyte degeneration (Ocon & Hansen, 2003). These detrimental effects of a low pH environment on the development of oocytes support the hypothesis that a lower uterine pH associated with higher BUN would have a negative effect on resulting pregnancy rates.

Concentrations of BUN greater than 16 mg/dL or 19 mg/dL resulted in an approximate 20% reduction in pregnancy rates in heifers and lactating dairy cattle (W. R. Butler, J. J. Calaman, & S. W. Beam, 1996; Elrod & Butler, 1993; Ferguson, Galligan, Blanchard, & Reeves, 1993). Similarly, ewes with an elevated BUN had lower pregnancy rates at day 18 after embryo transfer, 33% and 75%, respectively, when compared to controls (T. McEvoy et al., 1997). Conversely, lactating cows and beef heifers with normal or high BUN had no differences in pregnancy rates evaluated 30 days after the period of natural breeding (cows were housed with bulls for a 21-day natural breeding period) (46 ± 8.4% and 47 ± 8.5%, control and high protein diet groups, respectively) (Amundson et al., 2016; Carroll, Barton, Anderson, & Smith, 1988). The differences in effects of BUN on pregnancy rates among these studies have not been explained; however, treatments used to increase BUN were different, the time of exposure to elevated BUN were different and the time of pregnancy diagnosis were different among these studies.

Based on results from studies with cows and ewes, there was also a relationship between BUN and systemic progesterone concentrations, although the reported results

have been inconsistent. For instance, progesterone concentrations were lower when cows had a higher BUN concentration (Jordan & Swanson, 1979; Sonderman & Larson, 1989). However, other studies with cows reported no variation in the concentrations of progesterone according to BUN concentrations (Amundson et al., 2016; W. R. Butler et al., 1996; Elrod & Butler, 1993; Rhoads et al., 2004). Similarly, when ewes received different diets that resulted in high or low BUN concentrations, there was no difference in progesterone concentrations (Berardinelli et al., 2001; T. McEvoy et al., 1997). The differences in the pregnancy rates between these studies might be due to the different treatments used to elevate BUN, the different time of exposure to elevated BUN and the different time of pregnancy diagnosis.

Although, high CP diets or urea-treatment in horses resulted in an increase in BUN, studies have focused on the relationship between BUN and exercise performance in horses and not on reproductive function, as was done with ruminants. For example, when horses trained for three-day-eventing received diets containing 7.5%, 9.0%, 11.0% or 13.0% CP during 140 days of training, there was an increase in BUN proportional to CP levels of 34.2, 39.8, 46.8, and 51.2 mg/dL, respectively (Oliveira et al., 2014). Similarly, racing Standardbred geldings were fed a forage-only diet (timothy and meadow fescue fertilized with different levels of nitrogen) for 23 days, with high CP (16.6%) or recommended intake of CP (12.5%). The higher CP diet resulted in an elevated BUN when compared to the other diet, 17.65 mg/dL and 15.69, respectively (Connysson et al., 2006). Horses that received either a basal diet (5.9% CP) or a diet with fishmeal (12.7% CP) for 14 days had different BUN values, 9.5 mg/dL and 15.0 mg/dL (Reitnour & Treece, 1971). Horses that received a 13.5% CP content over 63 days of training had a significantly higher BUN when

compared to horses that received 7.6% CP content, 27.44±1.0 and 25.72±1.1, respectively (P. Graham-Thiers, Kronfeld, Kline, Sklan, & Harris, 2000). Additionally, sedentary horses that received a high protein diet (12% CP) compared to a low protein diet (7.5% CP) had lower venous blood pH (7.449 pH or 7.395 pH) which might be due to the oxidation of amino acids resulting in an acid load that lowered the blood pH(P. M. Graham-Thiers & Kronfeld, 2005). Overall, these studies show that BUN has a strong positive relationship with dietary CP and blood pH, providing evidence of the importance of CP levels in the diet of broodmares because of the possible impact on their reproductive tract and fertility.

Even though high protein diets caused an increase in equine BUN, and an elevated BUN has deleterious effects on reproduction in cows and ewes, it is part of normal equine management practices to feed diets exceeding CP requirements (Harper et al., 2009; Honoré & Uhlinger, 1994). The effects of a high BUN on the reproductive tract urea concentrations and on embryonic development in mares are unknown. Thus, it is paramount that studies be done in order to fill the gaps in knowledge regarding the influence of elevated BUN on reproductive functions in mares.

CHAPTER 2. A systemic urea-infusion to model a high protein diet alters the endometrial transcriptome of mares

2.1. Abstract

Fertility of ruminants is decreased with high blood urea nitrogen (BUN); however, the mechanisms are unknown and have not been investigated in mares. We developed an experimental model to elevate BUN during diestrus. There were both urea and control treatments (7 mares/treatment), done in a crossover design. Urea-treatment consisted of a loading dose of urea (0.03 g/kg of BW) and urea injections over 6 hours (0.03 g/kg of BW/hr). Control mares received the same volume of saline solution. Blood samples were collected to measure BUN. Uterine and vaginal pH were evaluated after the last intravenous infusion, then endometrial biopsies were collected for RNA-sequencing done with a HiSeq 4000. Cuffdiff(2.2.1) was used to calculate differentially expressed genes (DEG) between urea and control groups (FDR-adjusted p-value < 0.1). There was a significant increase in BUN and a decrease of uterine pH in the urea group compared to the control group. A total of 193 genes were DEG between the urea and control groups, with five genes identified as upstream regulators (*ETV4*, *EGF*, *EHF*, *IRS2* and *SGK1*). The DEG were predicted to be related to cell pH, ion homeostasis, changes in epithelial tissue, fatty acid metabolism, and solute carriers. Changes in gene expression reveal alterations in endometrial function that could be associated with adverse effects on fertility of mares.

KEYWORDS: High protein diet, high blood urea nitrogen concentrations, uterus.

2.2. Introduction

Early embryonic and fetal losses affect reproductive efficiency and productivity in farm animals (B. A. Ball, 1988). Nutritional imbalance and/or disorders are some of the factors that could lead to these losses. Several studies have shown that a high protein diet results in an increase in blood urea nitrogen (BUN), associated with lower fertility in cows and ewes (Butler, 2000b; W. R. Butler et al., 1996; C. C. Elrod, M. Van Amburgh, & W. R. Butler, 1993; Ferguson et al., 1993; T. G. McEvoy, J. J. Robinson, R. P. Aitken, P. A. Findlay, & I. S. Robertson, 1997).

Cows receiving a high protein diet exhibited an increase in systemic BUN and a decrease in uterine luminal pH at day 7 of the estrous cycle (diestrus) (C. Elrod et al., 1993; D. S. Hammon et al., 2005). This alteration in the uterine environment is critical, as by this time the embryo would have reached the uterus and the endometrium would be undergoing remodeling to prepare for the early conceptus (C. Klein, Scoggin, Ealy, & Troedsson, 2010). When urea, a metabolite of protein digestion, was given intravenously or orally to cows and ewes during the luteal phase, conditions mimicked those of a high protein diet, including an acute elevation of BUN, an increase in uterine urea, and a decrease in uterine pH (Elrod & Butler, 1993; C. Elrod et al., 1993; C. C. Elrod et al., 1993; D. S. Hammon et al., 2005; E. R. Jordan, T. E. Chapman, D. W. Holtan, & L. V. Swanson, 1983; T. G. McEvoy et al., 1997; Rhoads et al., 2004; Smith et al., 2000). Additionally, the *in vitro* effects of high urea concentrations on bovine endometrial explant from diestrus resulted in altered endometrial gene expression related to cell growth, proliferation, and differentiation (Gunaretnam et al., 2013). It has been suggested that there might be deleterious effects of an altered endometrial tissue and uterine environment on early

embryonic development due to a high BUN in animals (De Wit et al., 2001; Gunaretnam et al., 2013). For instance, a lower media pH resulted in detrimental consequences to bovine embryos cultured *in vitro* (Ocon & Hansen, 2003). Overall, these studies showed that a high protein diet caused an increase in systemic BUN, an increase in intrauterine urea, and a decrease in uterine pH.

Even though high protein diets might have deleterious effects on reproduction in cows and ewes, it is part of normal nutrient management practices to feed protein in excess of requirements to horses (Harper et al., 2009; Honoré & Uhlinger, 1994). This excessive amount of protein might result in BUN concentrations above the normal range for horses that may result in negative consequences to the uterine environment. However, no studies to date have addressed the effects of a high protein diet on fertility in the mare.

It is important to study the effects of a high BUN in the endometrium of mares, as broodmares might be routinely receiving a high protein diet. To the best of our knowledge, there are no published studies regarding the influence of elevated BUN on the endometrial transcriptome of mares. Therefore, we hypothesized that an acute intravenous infusion of urea would elevate BUN with a concomitant decrease in uterine pH resulting in transcriptomic changes in the endometrium of mares. The objectives of the present study were to 1) develop a model to elevate BUN, 2) correlate changes in BUN to changes in uterine pH, and 3) evaluate how changes in circulating BUN affected the endometrial transcriptome of mares.

2.3. Material and methods

All animal procedures were completed in accordance to the Institutional Animal Care and Use Committee of the University of Kentucky (Protocol #2011-0876). Clinically healthy mares of mixed breeds, ranging from 5 to 15 years of age were used in this study. All mares underwent a reproductive examination and transrectal ultrasonography for reproductive tract evaluation (vulva, cervix, uterus, and ovaries) before the experiment. The researchers only used mares with no detectable abnormalities of the reproductive system.

Mares received a treatment or control infusion (n = 7 mares/group) in a crossover design. The intervening estrous cycle was skipped and served as a washout cycle. Mares received 2,500 IU of human chorionic gonadotropin (hCG) (Chorulon; Intervet, Millsboro, DE) intravenously when they had a follicle of at least 35 mm in diameter and pronounced uterine edema as determined by transrectal ultrasonography (ExaGo; ECM Co., Angouleme, France). Animals were scanned daily by ultrasound for ovulation detection (Day 0 = ovulation) and infusions were initiated at Day 7 of diestrus (D7). On the day of infusion, both jugular veins were catheterized using a 14Gx2" gauge indwelling catheter (NIPRO medical corporation, Miami, FL). One jugular catheter was used for blood collection, and the opposite jugular catheter was used for infusion of urea (treatment) or saline (control). Treatment consisted of a loading dose of 0.03 g/kg of body weight of urea (Sigma-Aldrich Company, St. Louis, MO) diluted in 100 mL of saline solution (Hospira, Inc, Lake Forest, IL) (15 g of urea for a 500 kg horse) to achieve a rapid increase in urea concentrations, and control mares received 100 mL of saline solution. Subsequently, mares received a bolus injection of urea (0.03 g/kg of body weight/hr) diluted in 15 mL of saline

solution every 30 minutes over 6 hours (90 g of urea over 6 hours for a 500 kg horse). The control group received the same amount of saline solution as the urea group, 100 mL of saline solution as a loading dose and 15 mL of saline solution every 30 minutes over 6 hours.

2.3.1. Blood urea nitrogen concentration

Blood samples were collected hourly in 10 mL vacutainer tubes with sodium heparin (BD Vacutainer, Franklin Lakes, NJ). Blood samples were promptly centrifuged at 1500 x *g* for 10 minutes at 4°C, and plasma was stored at -20°C. BUN was measured with a colorimetric spectrophotometric assay following an adapted protocol previously described (Mok et al., 2018). All reagents were purchased from Sigma-Aldrich. The standard curve ranged from 5.6 mg/dL to 56.0 mg/dL. Urea was diluted (8M after constitution with 16 mL high purity water) to 5.6 mg/dL and 56.0 mg/dL to be used as low and high controls. The reaction consisted of analyzing urea by enzymatic hydrolysis to ammonia at room temperature. The reaction was done in microcentrifuge tubes (2 mL) with 10 µL of each plasma sample in duplicate and 125 µL urease buffer was added, and the samples were incubated for 20 minutes. The urease enzyme hydrolyzes urea to produce carbon dioxide and ammonia (CH_4N_2O + Urease buffer → $CO_2 + 2NH_3$). Then, 250 µL of phenol nitroprusside solution, 250 µL of alkaline hypochlorite solution (0.2%), and 1000 µL of distilled water were added (NH_3 + phenol nitroprusside + alkaline hypochlorite + H_2O → Indophenol blue) (Tabacco & Meiattini, 1985). After a 25-minute incubation, a 200 µL aliquot was transferred to a 96-well plate and read in an Epoch microplate spectrophotometer plate reader (Biotek, San Francisco, CA) at 570 nm. The intra- and

interassay coefficients of variation for BUN concentrations were 0.5% and 9.8%, respectively. The lower limit of detection of the assay was 0.11 mg/dL.

2.3.2. Uterine and vaginal pH

Immediately after the last intravenous infusion with urea or saline solution, uterine and vaginal pH were measured. The mares were restrained in palpation stocks, and their tails were wrapped and tied. Feces were removed from the rectum manually. The perineal region was washed three times with povidone-iodine scrub, rinsed with clean water, and dried with clean paper towels. An adapted epoxy pH probe (model number 911600, Thermo Fisher Scientific, Waltham, MA) attached to a portable pH meter (Accumet AP115, Thermo Fisher Scientific) was used for pH measurements. Immediately before pH measurements, the probe was calibrated with calibration solution buffers at pH 4, 7 and 10 (Thermo Fisher Scientific). The pH probe was introduced into the vagina with the tip protected by a sterile gloved hand and passed through the cervix. The pH probe was advanced into the uterus until it reached the uterine body and was held in place by the examiner. The examiner introduced the other hand into the rectum to increase the contact surface between the uterine wall and the pH device. When the uterine pH measurement was completed, the pH probe was removed from the uterus and placed into the vagina, in contact with the vaginal mucosa next to the cervix. The pH meter probe was maintained in the same position until two stable reads were completed. This procedure was done two times, in the uterus and vagina, and the mean of the readings was calculated.

2.3.3. Endometrial biopsies

After uterine pH measurements, the perineal region was washed again three times with povidone-iodine scrub, rinsed with clean water and dried with clean paper towels. A Jackson uterine biopsy forceps designed for horses (60-cm length, 4 mm × 28 mm cut-off area, Jorgensen Laboratories, Inc., Loveland, CO) was guarded in a sterile gloved hand and passed through the cervix into the uterus. A uterine biopsy was collected from the base of the uterine horn. The sample was removed from the instrument with a sterile needle (NIPRO medical corporation), and preserved in RNAlater (Thermo Fisher Scientific), kept at 4°C overnight and then kept at -80°C until RNA isolation (Herrera et al., 2018). Mares received dinoprost tromethamine (5 mg, im; Lutalyse; Pfizer, New York, NY) to help with uterine clearance after intrauterine procedures.

2.3.4. RNA extraction

Total cellular RNA was extracted from endometrial samples using TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer's recommendations. After extraction, RNA concentration and quality were analyzed using a NanoDrop DP-1000 spectrophotometer (Agilent Technologies, Palo Alto, CA) and a Bioanalyzer® (Agilent, Santa Clara, CA). All samples had a 260/280 ratio > 2.0, a 28S:18S rRNA ratio > 2.0 and RNA integrity number (RIN) > 8 (8.95 ± 0.4, mean ± SEM). A total of 1 µg of RNA was treated with DNase I (Ambion Inc., Austin, TX) for 30 minutes at 37°C to remove genomic DNA according to the manufacturer's instructions. The extracted RNA was kept at -20°C until further analyses.

2.3.5. mRNA library preparation and Next Generation Sequencing

The extracted RNA (1µg), as described above, was sent to the University of Illinois at Urbana-Champaign for library preparation and RNA Sequencing. Paired-end reads with 150 nucleotides in length were produced. The RNAseq libraries were prepared with Illumina's TruSeq Stranded mRNAseq Sample Prep kit (Illumina, San Diego, CA). Read 1 aligns to the antisense strand and Read 2 aligns to the sense strand. The libraries were quantitated by qPCR and sequenced on one lane for 101 cycles from each end of the fragments on a HiSeq 4000 using a HiSeq 4000 sequencing kit version 1. The lane produced a total of 700 million reads. Fastq files were generated and demultiplexed with the bcl2fastq v2.17.1.14 Conversion Software (Illumina).

2.3.6. Next Generation Sequencing data analysis

The Fastq files were evaluated for read quality using FastQC 0.11.4 (Andrews, 2010b). Subsequently, Trim Galore 0.4.1 (Krueger, 2012) was used for adapter and read quality trimming (Phred score threshold of 30). Reads were mapped to the *Equus caballus* reference genome (EquCab 3.0) using the software STAR 2.5.3a (Dobin et al., 2013), then annotated with the equine reference annotation from NCBI using Cufflinks 2.2.1 (Trapnell et al., 2012). Fragments per kilobase per million (FPKM) were used to determine the expression level of genes. Lastly, we used Cuffdiff 2.2.1 (Trapnell et al., 2012) to calculate differentially expressed genes (DEG) between samples from the control and urea groups. Significance level was set at FDR-adjusted p-value of the test statistic < 0.1 using a Benjamini-Hochberg correction.

2.3.7. Functional annotation and pathway analysis

The Database for Annotation, Visualization, and Integrated Discovery Bioinformatics Resources (DAVID, version 6.8, https://david.ncifcrf.gov/home.jsp) was used to annotate DEG in relation to biological process, molecular function, and cellular component (Huang da, Sherman, & Lempicki, 2009). A functional classification was performed based on the Official Gene Symbol of *Equus caballus* genes in DAVID. GOplot (http://wencke.github.io/) was used to illustrate the results. Additionally, PANTHER (version 13.1, http://www.pantherdb.org/) statistical overrepresentation test with a Fisher's Exact test with no correction was used to show protein class and pathways (Mi & Thomas, 2009). GOplot was used to illustrate the results. DAVID and PANTHER were used to describe the functions of DEG based on public genomic resources through gene-set enrichment.

A core analysis of the DEG was conducted using Ingenuity Pathway Analysis (IPA, QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) as this commercial software package uses networks based on cause and effect relationships reported in previous studies. A diseases and biological function analysis was done to show networks of biological interest. Additionally, an upstream regulator analysis was conducted to identify molecules that are upstream of the genes in this study that affect and help to explain the changes in expression observed (Kramer, Green, Pollard, & Tugendreich, 2014).

The R-based Weighted Correlation Network Analysis (WGCNA) package was used to evaluate the correlation patterns among the genes in this RNA-sequencing experiment (Langfelder & Horvath, 2008). The FPKM data was transformed to log2(x+1)

prior to the analysis to normalize the data. WGCNA was used to generate clusters of highly interconnected genes identified by different colors, called modules. A power of 10 was chosen because it was the lowest possible power term that topology fitted a scale free network. Additionally, genes that were highly connected in the modules were identified as hub genes.

2.3.8. Protein-protein interactions

STRING consists of a protein network of genome-wide functional connectivity from published and predicted protein-protein interactions (PPI), allowing the prediction of the protein-protein interactions of the proteins coded by our DEG (Szklarczyk et al., 2017). The protein-protein interactions related to the DEG between the urea and control treated animals were predicted through a correlation analysis of expression level using the STRING database (version 10.5, http://www.string-db.org/). Additionally, a PPI analysis using the genes from each of the IPA disease and biological functions was done to further characterize the protein interactions between genes that have a similar function.

2.3.9. Quantitative Real-Time PCR

Expression levels of a subset of DEG determined by RNA sequencing between the control and urea groups were confirmed with RT-qPCR. The extracted RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit and random hexamers (Thermo Fisher Scientific). The cDNA was kept frozen at -20°C until quantitative real-time PCR (RT-qPCR) was done. Primers for the selected transcripts were designed using the Primer-BLAST (National Center for Biotechnology Information, NCBI) function

(Table 2.1). The RT-qPCR was done using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) with the program: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and 55–95 °C for dissociation cycling conditions. Each reaction was done in duplicate.

Table 2.1. Forward and reverse primers used for quantitative Real-Time PCR analysis.

Gene symbol	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Accession number	Product size
B2M	GTGTTCCGAAGG TTCAGGTT	ATTTCAATCTCA GGCGGATG	NM_001082502.3	103
EEF1A1	CAACATCGTCGT CATTGGGC	CAGCAGCCTCCT TCTCGAAT	NM_001081781.1	119
ANGPTL4	GGCTCCGTGGAC TTTAACCA	GGTCCCCCATGA TGAGATGC	XM_023644667.1	107
AQP5	CCTGCTCTTCCC CAACTCG	GGCTCATACGTG CCCTTGAC	AJ514427	66
CA2	TACTGGACCTAC CCAGGCTC	TGCCCTCCGCGT TGAAATTA	XM_001488490.4	136
EGF	CCCCAGGCAAT GGAGTGTAG	AGCTCCATTTAG AGCGGTGG	XM_014737940.2	143
ENPP1	GATCCAGACCA GGCTCCCTC	TCCGAGCTCTGT GTAACCTCA	XM_023651100.1	148
ERRFI1	AAGACAGGCCT CCGAAAGTG	CAGGCTTTTAGG ACTGGGGG	XM_023635858.1	77
ETV1	GGGGAAGTGCT GGGCAATAA	GCAATGGCGATC AACGAGAC	XM_005609238.3	122
FADS1	CCACGTCTTCTT CCTGCTGT	CCCCCTGAACTG TGCTGAG	XM_023654188.1	134
IGFBP3	GGAAACAGCAG TGAGTCGGA	CTTGGTGTGGAT CGTGTGGA	XM_023639032.1	111
INSR	GTGAGTACGAG GAGTCTGCC	GAGACGGTCTGG GGACAAAA	XM_023644608.1	141
ITGB8	CATCGTGGTGCC AAATGACG	GGCCTAGTGAGG GATGTTCC	XM_001497221.5	86
KCNA3	AGTTTGATGGAC CCGTCAGC	TTCCAGGAGGGG AGTTTCCA	XM_023641433.1	148
LAMC2	CTGGAGAGCGC TGTGATAGG	GGTACAGCCCTG AGGGTTTC	NM_001081768.1	71
PIGR	GTTTTGGCAGCA GCATCCAG	ACTCCTTGCGAG GGATGTTT	XM_014739411.2	134
PRLR	GTCACTGCTCCC GAAACAGA	GTCACCTGGGAC ACCTTAGC	XM_001500104.4	96
SERPINA14	CTGACAGATGC AAAGAGCAGC	AAAAGTCCGCAG AGGGTGAG	XM_014735597.1	131

Table 2.1(continued). Forward and reverse primers used for quantitative Real-Time PCR analysis.

SGK1	GGGTGTGAAGT GAAAGAGCCA	AAAGTCGTTCAG GCCCATCC	XM_023651133.1	123
SPINK7	TTTTCCCTCGTG TTGGCTGA	AGGGCACAACAA CCTTCTCC	XM_003362870.2	88

Primers were generated using the National Center for Biotechnology Information (NCBI) primer-BLAST tool. Key: beta-2-Microglobulin (B2M), eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A1), angiopoietin like 4 (ANGPTL4), aquaporin 5 (AQP5), carbonic anhydrase 2 (CA2), epidermal growth factor (EGF), ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), ERBB receptor feedback inhibitor 1 (ERRFI1), ETS variant 1 (ETV1), fatty acid desaturase 1 (FADS1), insulin like growth factor binding protein 3 (IGFBP3), insulin receptor (INSR), integrin subunit beta 8 (ITGB8), potassium voltage-gated channel subfamily A member 3 (KCNA3), laminin subunit gamma 2 (LAMC2), polymeric immunoglobulin receptor (PIGR), prolactin receptor (PRLR), serine peptidase inhibitor clade A (alpha-1 antiproteinase, antitrypsin) member 14 (SERPINA14), serum/glucocorticoid regulated kinase 1 (SGK1), serine peptidase inhibitor, Kazal type 7 (putative) (SPINK7).

The RT-qPCR efficiency was determined using LinRegPCR (version 2012.0) to ensure that it was between 1.8 and 2.2 (Ruijter et al., 2009). Mean threshold cycles (CT) were used to show changes in the mRNA expression and then normalized to the housekeeping genes Beta-2-Microglobulin (*B2M*) and Eukaryotic Translation Elongation Factor 1 Alpha 1 (*EEF1A1*) to calculate delta CT values (ΔCT) (Livak & Schmittgen, 2001). The two housekeeping genes were chosen with GeNORM (De Spiegelaere et al., 2015) as the most stably expressed genes in the endometrial samples.

2.3.10. Statistical analyses

The BUN, uterine and vaginal pH and RT-qPCR were tested for normality with a Shapiro-Wilk test. The BUN concentration was not normally distributed and a normal quantile transformation was done. A Fit Least Squares model using hour, treatment and interaction between hour and treatment with mare as a random effect was used, followed by a Student's t-test. The uterine and vaginal pH data had a normal distribution and were analyzed with a one-tailed paired t-test. Pearson's correlation coefficients were done

between the BUN concentration at the end of the treatment (H6) and the uterine pH and also between the uterine and vaginal pH.

A Pearson's correlation coefficient was used to determine the correlation between the -ΔCT (negative delta CT) from RT-qPCR results and the FPKM from RNA-sequencing results. Data was reported as mean ± SEM. Significance was set at $P \leq 0.05$ and trend at $0.1 > P > 0.05$. JMP Pro (version 14; SAS Institute, Cary, NC, USA) was used for all statistical tests.

2.4. Results

2.4.1. Blood urea nitrogen concentrations

There was an effect of time of sampling ($P < 0.0001$) and of the interaction between time of sampling and treatment ($P = 0.0008$). There was no treatment effect ($P = 0.90$). Immediately before the start of the treatment (H0), the urea and control groups had BUN concentrations of 14.26 ± 0.69 and 14.12 ± 0.99 mg/dL in the control and treated mares, respectively, with no statistical difference ($P > 0.05$). The BUN at H6 was 14.33 ± 0.66 and 20.36 ± 0.75 mg/dL in the control and treated mares, respectively, showing a statistical difference ($P < 0.05$) (Figure 2.1).

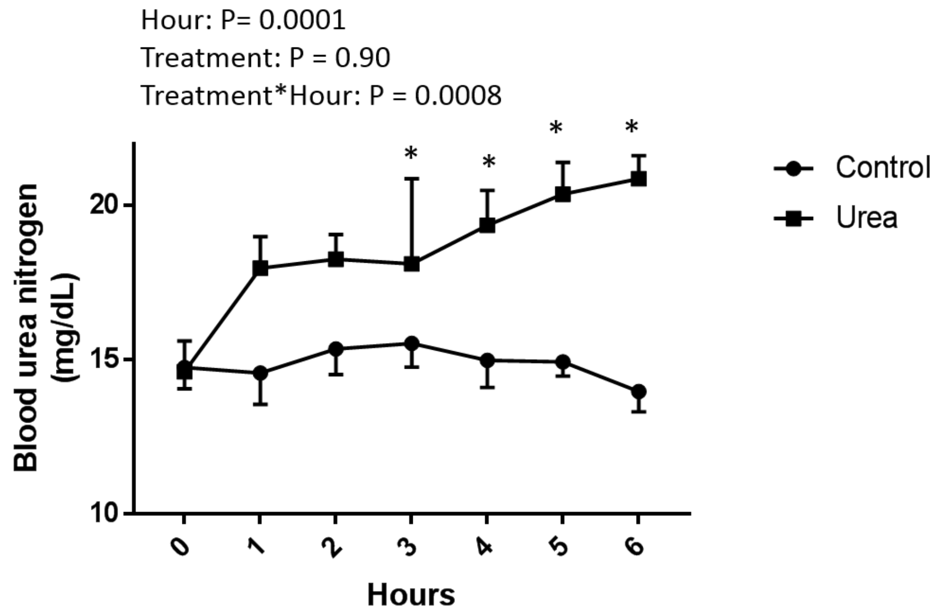


Figure 2.1. Blood urea nitrogen (mg/dL) analyzed in diestrus mares receiving an intravenous control or urea treatment over 6 hours done in a crossover design. Results are presented as mean and SEM. The main effect of hour, treatment, and interaction are shown. * $P \leq 0.05$.

2.4.2. Uterine pH

Based upon a one-tailed paired t-test there was a lower uterine pH in the urea group ($P = 0.05$). Uterine pH was 7.02 ± 0.06 and 6.83 ± 0.05 pH in the control and treated group at H6, respectively (Figure 2.2). There was a negative correlation ($R = -0.56$, $P = 0.04$) between the BUN at H6 and uterine pH (Figure 2.2).

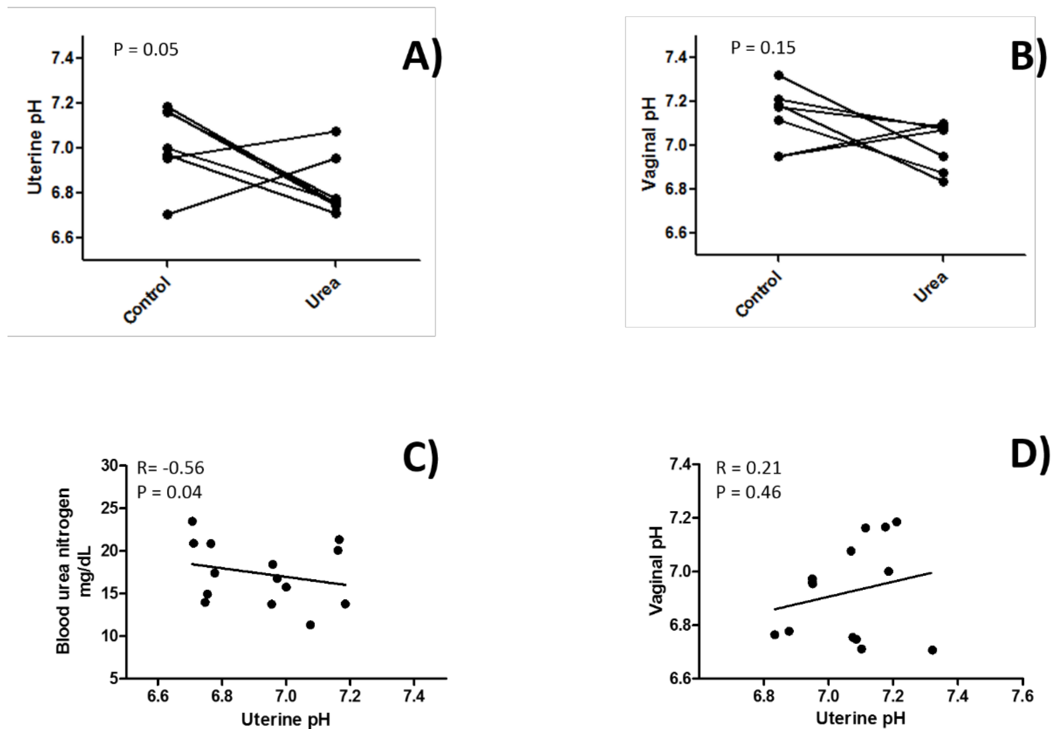


Figure 2.2. Uterine pH (A) and vaginal pH (B) analyzed in diestrus mares receiving an intravenous control or urea treatment over 6 hours done in a crossover design, lines connect the values for each mare after respective treatment C) Correlation between blood urea nitrogen (BUN, mg/dL) concentrations at hour 6 and uterine pH D) Correlation between uterine and vaginal pH at hour 6. Results are shown as mean.

2.4.3. Vaginal pH

Based upon a one-tailed paired t-test the vaginal pH was not different between the treated and control group (P = 0.15). Vaginal pH was 7.13 ± 0.05 and 6.99 ± 0.11 in the control and treated group at H6, respectively (Figure 2.2). The correlation between uterine and vaginal pH was not significant (R = 0.21, P = 0.46, Figure 2.2).

2.4.4. RNA sequencing

The RNA-sequencing analysis performed on 14 endometrial samples resulted in 18,950 genes. The average of input reads per sample was 28,045,588; the input read length for paired end reads was 150 and 95.5% of uniquely mapped reads were obtained for the samples sequenced (Table 2.2).

Table 2.2 Summary of RNA sequencing data for 14 endometrial samples.

Number	Group	Number of input reads	Input read length for paired end reads	Uniquely mapped reads	Uniquely mapped reads %
1	CONTROL	21,609,396	150	20,577,813	95.23
2	UREA	38,190,014	150	36,639,458	95.94
3	CONTROL	29,143,588	150	27,860,949	95.6
4	UREA	20,992,262	150	19,975,343	95.16
5	CONTROL	40,534,917	150	38,931,171	96.04
6	UREA	23,391,217	150	22,171,251	94.78
7	CONTROL	34,523,977	150	33,077,984	95.81
8	UREA	30,571,360	150	29,211,317	95.55
9	CONTROL	20,954,527	150	19,920,283	95.06
10	UREA	25,449,880	150	24,400,353	95.88
11	CONTROL	33,555,929	150	32,008,513	95.39
12	UREA	28,445,829	150	27,138,814	95.41
13	CONTROL	21,115,137	150	20,130,465	95.34
14	UREA	24,160,197	150	23,093,575	95.59

2.4.5. Differentially expressed genes

A total of 193 genes were differentially expressed between the urea and control groups. A total of 29 were upregulated and 162 were downregulated in the urea group in comparison to the control group (Figure 2.3, Appendix 1). Additionally, 2 DEG, aldo-keto

reductase family 1 member C23 (*AKR1C23*) and alpha-fetoprotein (*AFP*) were of particular interest, as they had no expression in the urea group, only in the control group.

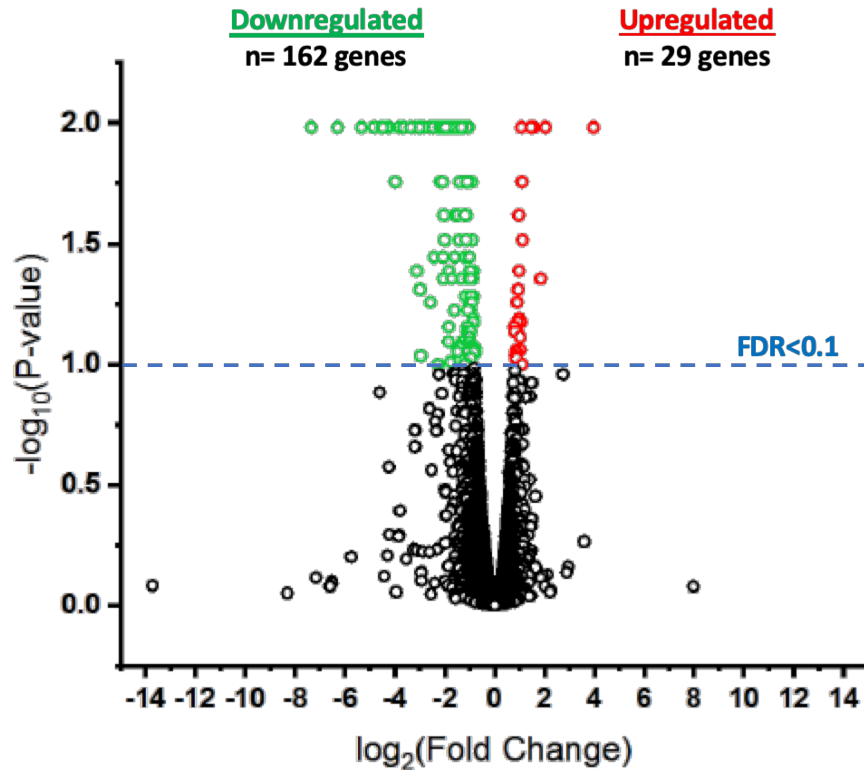


Figure 2.3.Volcano plot showing differentially expressed genes between mares from the urea treatment and control treatment using a FDR cutoff value < 0.1. Red dots show genes that were not differentially expressed, blue dots show genes that were up- or down-regulated.

Genes that were uncharacterized in the NCBI database for *Equus caballus* had their nucleotide sequence (FASTA format) identified in the NCBI database (http://www.ncbi.nlm.nih.gov/), then the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST) (Camacho et al., 2009) was used to identify their orthologs in other species (*Canis lupus dingo*, *Equus asinus*, *Equus przewalskii*, *Homo sapiens*).

2.4.6. Functional analyses

Functional characterization of the DEG between the urea and control treatments was done with GO analysis using the DAVID software for biological processes, cellular components and molecular functions (Figure 2.4).

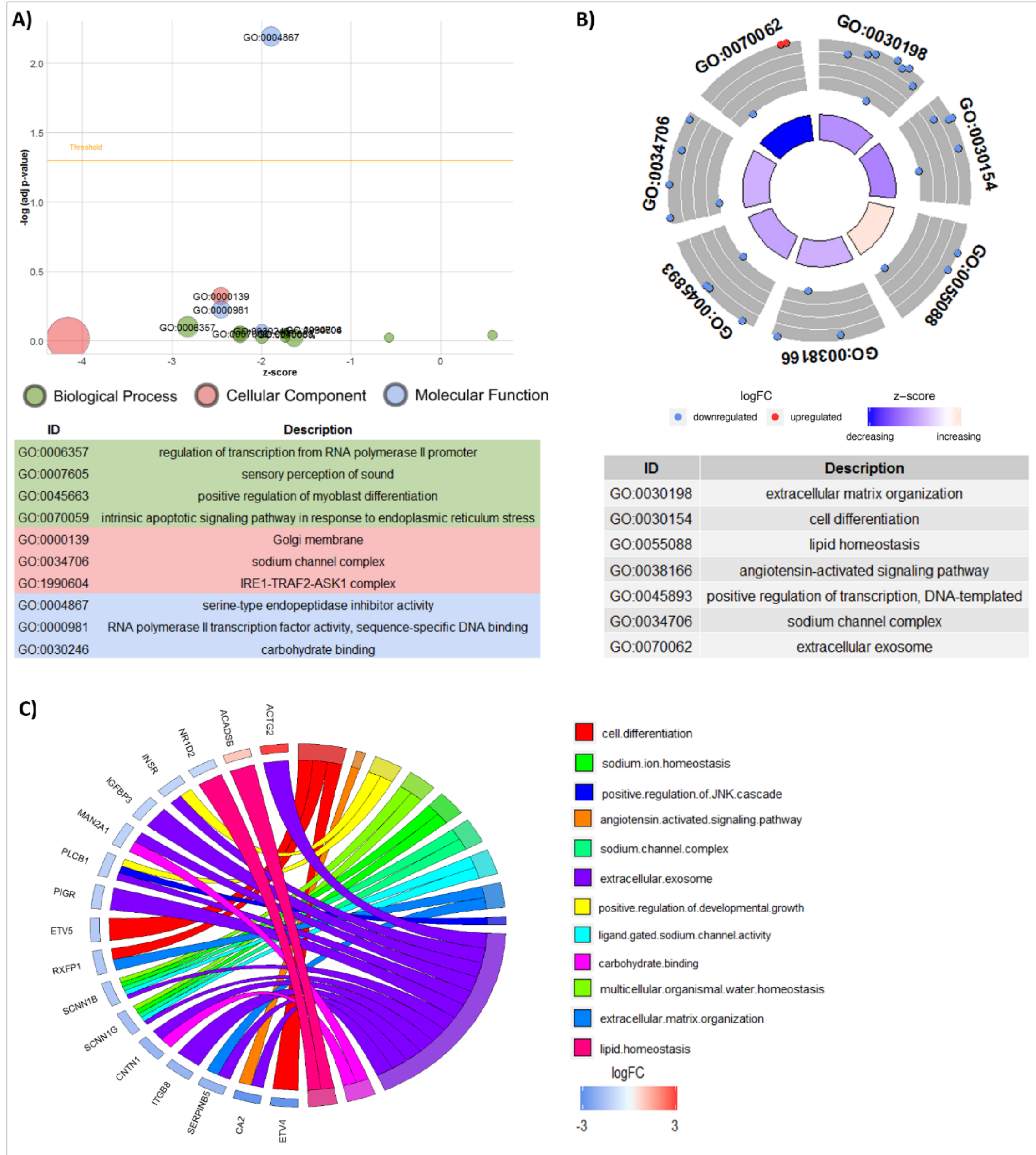
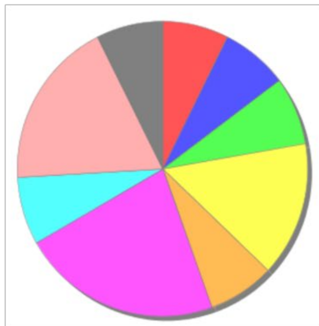


Figure 2.4. Gene ontology of differentially expressed genes analyzed by DAVID and the GOplot package. A) GOBubble plot of differentially expressed genes analyzed by DAVID. The y-axis shows the –log10(adjusted P-value) and the x-axis shows the z-score. The area of the circles is proportional to the number of genes related to the term. A threshold for the labeling is set based on the negative logarithm of the adjusted p-value. The Biological Process, Cellular Component and Molecular Function are represented by green, pink and blue, respectively. The identification (ID) and description of each term is given. B) GOCircle plot showing the number of genes in each GO term. The inside rings are a bar plot, with the height representing the significance of the term (-log10 adjusted P-value). Different colors of the inside rings represent the z-score, with blue showing a decrease and red an increase. The outside rings show the expression levels (logFC) for each gene in the GO term. Each dot in the outside rings represents one gene from the GO term, red dots show upregulated genes and blue dots show downregulated genes. C) GOChord plot show genes linked by

ribbons to their respective GO terms and the logFC of each gene. The colored squares next to each gene represent the logFC, with red showing positive logFC, blue showing negative logFC and white showing no change. Each GO Term is assigned a color corresponding to the color of the ribbon indicating the relationship with the genes.

The PANTHER pathway analysis showed nine pathways related to the urea-treatment. Among them were EGF receptor signaling pathway, integrin signaling pathway, and gonadotropin-releasing hormone receptor pathway (Figure 2.5). The functional annotation analysis of differentially expressed genes done with PANTHER, based on protein class, showed a prevalence of serine protease inhibitor-enzyme modulator, signaling molecule, and transporters (Figure 2.5).

A) Pathways



B) Protein classes

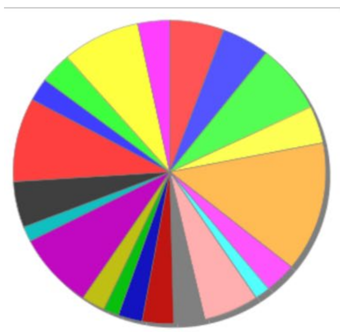


Figure 2.5. Functional annotation analysis of the differentially expressed genes using PANTHER (version 13.1) statistical overrepresentation test with a Fisher's Exact test. A) Pathways overrepresented analysis shown in pie chart identified by the GO terms in different colors, B) Protein classes overrepresented analysis shown in pie chart identified by the GO terms in different colors.

The core analysis of the DEG using IPA showed 501 categories of diseases and biological functions. The categories that had biological interest for this study and a $P < 0.05$ were ion homeostasis of cells (12 genes related, $P = 0.007$), fatty acid metabolism (13 genes related, $P = 0.000$), pH of cells (3 genes related, $P = 0.002$), growth of epithelial tissue (11 genes related, $P = 0.002$), and development of epithelial tissue (8 genes related, $P = 0.002$) (Figure 2.6).

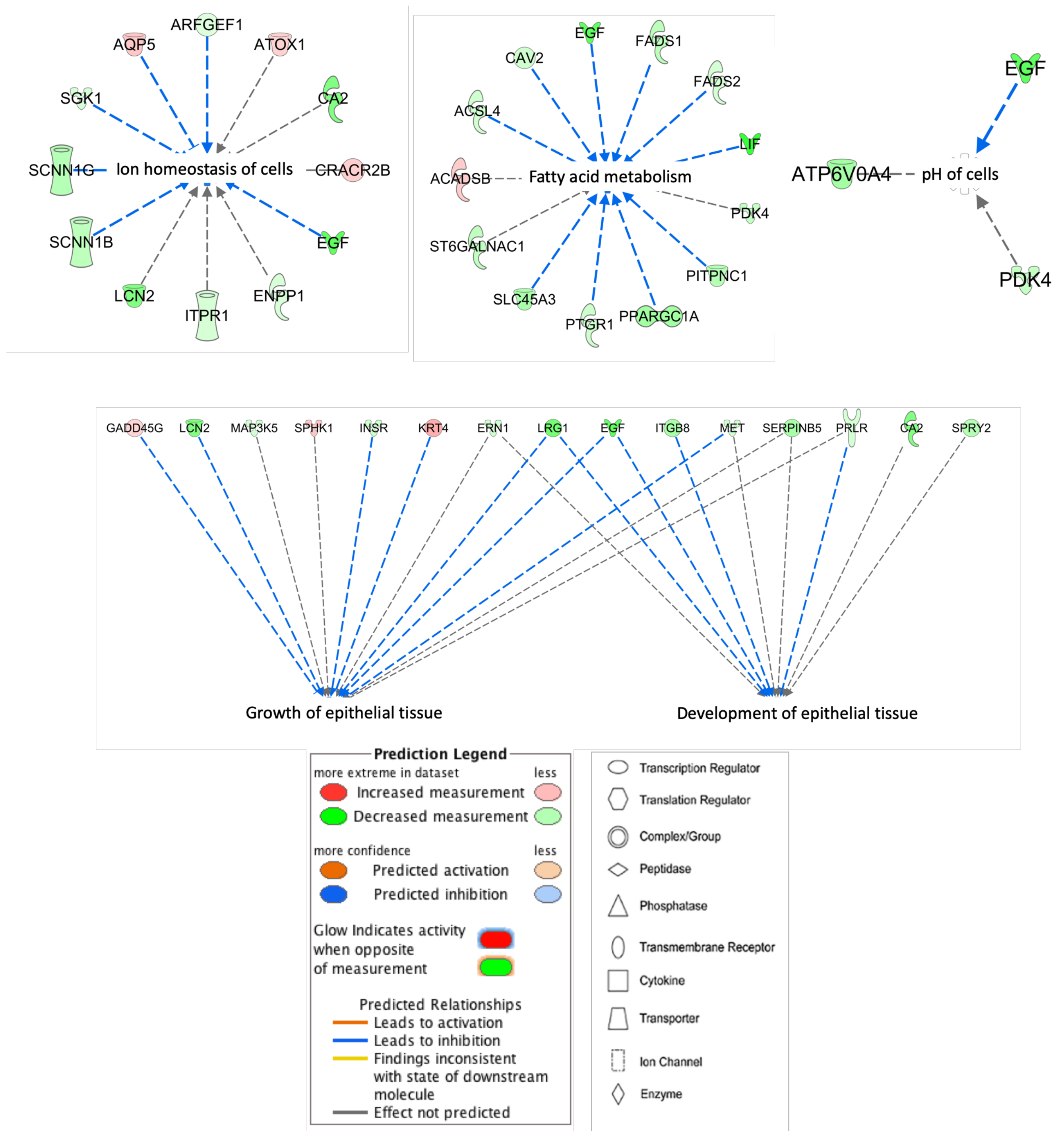


Figure 2.6. Ingenuity Pathway Analysis (IPA) of diseases and biological functions of the differentially expressed genes displayed as nodes (genes) and edges (biological relationship between nodes). The color intensity of each node represents fold change expression, red (upregulated) and green (downregulated). The edges connecting the genes to the respective functions represent the predicted relationships, blue representing inhibition and grey representing effect not predicted based on the IPA activation z-scores, combination of directional information encoded by the gene expression with information curated from the literature.

2.4.7. Upstream regulators

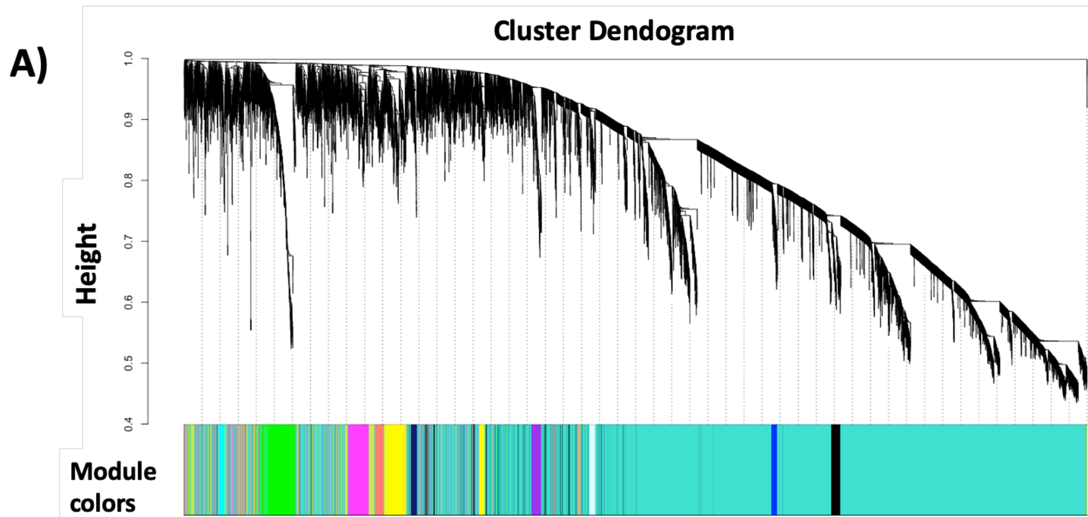
ETS variant 4 (*ETV4*), epidermal growth factor (*EGF*), ETS homologous factor (*EHF*), insulin receptor substrate 2 (*IRS2*), and serum/glucocorticoid regulated kinase 1 (*SGK1*) were predicted as upstream regulators and differentially expressed in our dataset. Their respective target molecules in the dataset are shown in Table 2.3.

Table 2.3 Upstream regulators from the IPA analysis when comparing the urea and control groups.

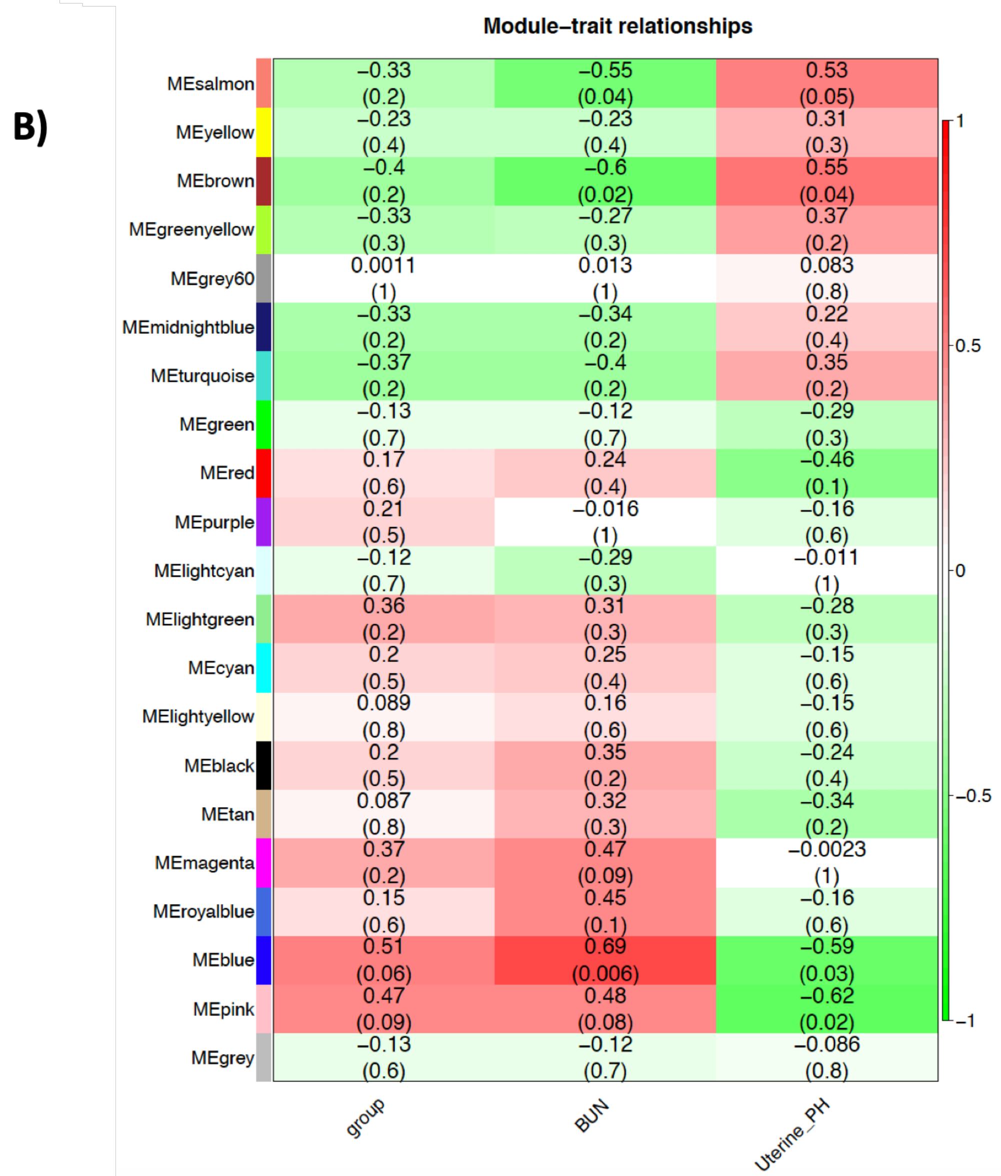
Upstream Regulator	Expr Log Ratio	Molecule Type	p-value of overlap	Target molecules in dataset
ETV4	-5.332	transcription regulator	0.024	ETV4, MET
EGF	-3.834	growth factor	0.000	ACSL4, IDS, IGFBP3, LCN2, MAP3K5, PDK4, SLC37A1, SP4, SPHK1, SPRY2
EHF	-1.688	transcription regulator	0.022	ANGPTL4, EHF, MET
IRS2	-1.253	enzyme	0.028	PPARGC1A, THRSP
SGK1	-0.939	kinase	0.000	KCNA3, LIF, SCNN1B, SCNN1G

2.4.8. Weighted correlation network analysis (WGCNA)

The WGCNA analysis identified hub genes, pointing out genes that have an important role in the genetic interaction network, by regulating other genes in their module showing that they might be markers for urea-treatment in the endometrium of mares. WGCNA identified 21 modules that were highly correlated to the traits of interest. The Brown Module is of interest, as it had genes with a high membership related to the IPA diseases and biological functions; it had a positive correlation with BUN and a negative correlation to uterine pH (Figure 2.7).



A)
Cluster Dendogram
Height
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Module colors



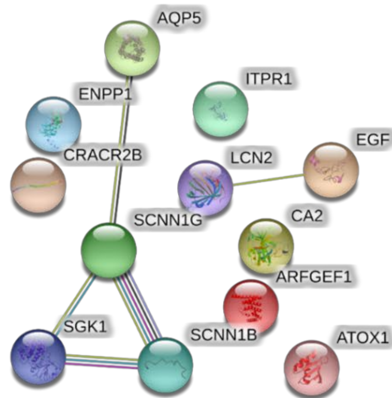
B)
Module–trait relationships
MEsalmon −0.33 (0.2) −0.55 (0.04) 0.53 (0.05)
MEyellow −0.23 (0.4) −0.23 (0.4) 0.31 (0.3)
MEbrown −0.4 (0.2) −0.6 (0.02) 0.55 (0.04)
MEgreenyellow −0.33 (0.3) −0.27 (0.3) 0.37 (0.2)
MEgrey60 0.0011 (1) 0.013 (1) 0.083 (0.8)
MEmidnightblue −0.33 (0.2) −0.34 (0.2) 0.22 (0.4)
MEturquoise −0.37 (0.2) −0.4 (0.2) 0.35 (0.2)
MEgreen −0.13 (0.7) −0.12 (0.7) −0.29 (0.3)
MEred 0.17 (0.6) 0.24 (0.4) −0.46 (0.1)
MEpurple 0.21 (0.5) −0.016 (1) −0.16 (0.6)
MElightcyan −0.12 (0.7) −0.29 (0.3) −0.011 (1)
MElightgreen 0.36 (0.2) 0.31 (0.3) −0.28 (0.3)
MEcyan 0.2 (0.5) 0.25 (0.4) −0.15 (0.6)
MElightyellow 0.089 (0.8) 0.16 (0.6) −0.15 (0.6)
MEblack 0.2 (0.5) 0.35 (0.2) −0.24 (0.4)
MEtan 0.087 (0.8) 0.32 (0.3) −0.34 (0.2)
MEmagenta 0.37 (0.2) 0.47 (0.09) −0.0023 (1)
MEroyalblue 0.15 (0.6) 0.45 (0.1) −0.16 (0.6)
MEblue 0.51 (0.06) 0.69 (0.006) −0.59 (0.03)
MEpink 0.47 (0.09) 0.48 (0.08) −0.62 (0.02)
MEgrey −0.13 (0.6) −0.12 (0.7) −0.086 (0.8)
group
BUN
Uterine_PH
1
0.5
0
−0.5
−1

Figure 2.7. Gene expression modules with Weighted Correlation Network Analysis (WGCNA). A) Gene clustering tree (dendogram) obtained by hierarchical clustering. The co-expression modules defined by the WGCNA are indicated by the color row below the dendogram indicating module membership. Genes were assigned to each module using the static tree cutting method. B) Matrix with the module-trait relationships and corresponding p-values of the modules on the y-axis and selected traits related with treatment on the x-axis. The y-axis is colored according to the correlation, with red representing a strong positive correlation and green representing a strong negative correlation. Traits are: group (control and urea groups), BUN (blood urea nitrogen concentrations at hour 6 of treatment) and uterine pH (uterine pH measured at hour 6 of treatment).

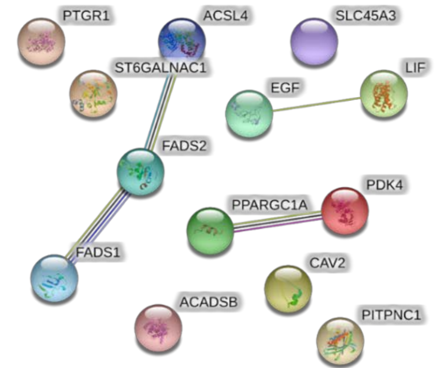
2.4.9. Protein-protein interactions (PPI)

The PPI analysis done with STRING, showed a significant PPI enrichment score (P = 0.002) with a total of 171 nodes and 59 edges. The average node degree was 0.69 and the average local clustering coefficient was of 0.297. The constructed PPI networks showed that the DEG encode a group of proteins with several interactions, and are biologically connected. This further characterizes the mechanisms of action of urea-supplementation on the endometrium of mares (Figure 2.8).

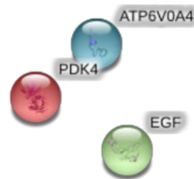
A) Ion homeostasis of cells



B) Fatty acid metabolism



C) pH of cells



D) Growth and development of epithelial tissue

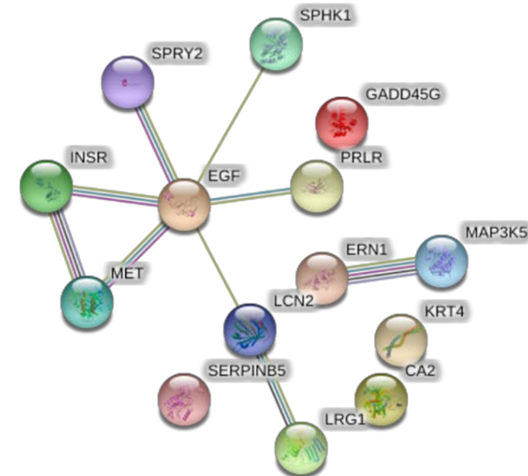


Figure 2.8. Protein-protein interaction analysis with the STRING algorithm showing nodes (proteins) and edges (protein-protein association) of proteins in the differentially expressed genes. The functional interaction network represents how the proteins coded by the differentially expressed genes between the urea and control groups are related. Thicker edges indicate stronger protein-protein interactions, each node represents a protein produced by a single, protein-coding gene locus, node colors represent proteins that are first interactors, nodes that have shapes inside indicate that the protein has a known or predicted 3D structure. Protein-protein interaction of the Ingenuity Pathway Analysis (IPA) of diseases and biological functions: ion homeostasis of cells (A), fatty acid metabolism (B), pH of cells, (C) and growth and development of epithelial tissue (D).

2.4.10. Quantitative Real-Time PCR

Analysis of the correlation between genes with RT-qPCR (-ΔCT) and the RNA sequencing results (FPKM) showed that 13 (72.23%) genes had a significant correlation

between the two methods. There was a similar pattern of regulation with a significant correlation between –ΔCT and FPKM (Table 2.4).

Table 2.4 Pearson's correlation of RNA Sequencing (FPKM) and Quantitative Real-Time PCR (-ΔCT) to confirm RNA Sequencing results.

Gene	Correlation (R)	P-value
ANGPTL4	0.538	0.047
AQP5	0.840	0.000
CA2	0.538	0.046
EGF	0.611	0.020
ENPP1	0.746	0.002
ERRFI1	0.836	0.000
ETV1	0.382	0.177
FADS1	0.710	0.004
IGFBP3	0.723	0.003
INSR	0.419	0.136
ITGB8	0.677	0.008
KCNA3	0.393	0.165
LAMC2	0.402	0.154
PIGR	0.601	0.023
PRLR	0.864	0.000
SERPINA14	0.688	0.007
SGK1	0.174	0.551
SPINK7	0.858	0.000

2.5. Discussion

To the best of our knowledge, this is the first study to elucidate global changes in mRNA expression profile in the endometrium of mares with an elevated BUN. In this study, diestrus mares received an acute infusion of urea intravenously to elevate BUN and allow evaluation of changes in the endometrial transcriptome. The main findings of the current study were that intravenous infusion of urea resulted in an increase in blood urea nitrogen and a decrease in uterine pH. The functional analyses of changes in the transcriptome in the urea and control groups identified alterations in genes related to pH homeostasis; fatty acid metabolism; and sodium, potassium, and glucose channels. These results serve to illustrate possible effects of a high BUN on the endometrium of mares.

2.5.1. pH of cells

As expected, the intravenous urea infusion resulted in a significant increase in BUN that was inversely related to uterine pH, similar to studies in cows and ewes (C. C. Elrod et al., 1993; D. S. Hammon et al., 2005; E. R. Jordan et al., 1983; T. G. McEvoy et al., 1997; Rhoads et al., 2004; Smith et al., 2000). The IPA disease and functions network associated with the current study indicated that epidermal growth factor (*EGF*), downregulated in the urea group, was related to cell pH. The growth factor *EGF* has an important role in pH changes, inducing an elevation of cytoplasmic pH by modifying the pH sensitivity of the Na+/H+ exchanger (Moolenaar, Tsien, van der Saag, & de Laat, 1983). Additionally, the exposure of renal medullary cells to urea stress was similar to *EGF* exposure, corroborating with results relative to urea supplementation changing *EGF* expression (Tian & Cohen, 2002). Interestingly, in our dataset *EGF* was identified as an upstream regulator for the DEG and a hub gene for the Brown Module (membership 0.77, P = 0.001). Additionally, the *EGF* receptor signaling pathway was identified by PANTHER as a significantly enriched term from the DEG list.

It is worth noting that *in vitro* studies have shown that a decline in pH of culture media is associated with a decrease in embryo development rate in hamsters (Squirrell, Lane, & Bavister, 2001), mice (Edwards, Williams, & Gardner, 1998), and cows (De Wit et al., 2001; Ocon & Hansen, 2003). There was also a lower blastocyst quality *in vitro* when donor cows received 75 g of urea orally (Ferreira et al., 2011). These changes could explain the decrease in pregnancy rate and embryo development, which have been reported

in other species receiving urea or a high protein diet (Butler, 2000b; W. R. Butler et al., 1996; Fahey et al., 2001).

2.5.2. Solute carriers and ion homeostasis of cells

Among the DEG, there were five solute carriers: solute carrier family 25 member 36 (*SLC25A36*), solute carrier family 37 member 1 (*SLC37A1*), solute carrier family 45 member 3 (*SLC45A3*), solute carrier family 52 member 3 (*SLC52A3*), and solute carrier family 6 member 20 (*SLC6A20*). There were also two potassium voltage-gated channels: potassium voltage-gated channel subfamily A member 3 (*KCNA3*) and potassium voltage-gated channel subfamily C member 4 (*KCNC4*). Solute carriers, such as the ones found in the present study, have been suggested to modify the uterine environment by altering the composition of the uterine fluid and receptivity in implantation (Groebner et al., 2011). Additionally, amino acid transporters have been shown to have an increased expression in the bovine endometrium during pregnancy (Groebner et al., 2011).

Homeostasis of the uterine environment is crucial for uterine functionality; therefore, maintenance of the intracellular ionic environment and consequent regulation of fluid is essential and is achieved by water channels, ion channels, and transporters (Liu, Zhang, Wang, Sheng, & Huang, 2014; Ruan, Chen, & Chan, 2014; Samborski, Graf, Krebs, Kessler, & Bauersachs, 2013; Zhu et al., 2015). Previous studies have identified genes that control the uterine fluid environment; for instance, sodium channel epithelial 1 beta subunit (*SCNN1B*) and sodium channel epithelial 1 gamma subunit (*SCNN1G*) encode the epithelial sodium channel (ENaC) which regulates sodium reabsorption in epithelial cells (Canessa et al., 1994). Both genes were downregulated in our dataset, similar to a

reduction in expression of ENaC channels reported in the endometrium of infertile women (Boggula, Hanukoglu, Sagiv, Enuka, & Hanukoglu, 2018). These two genes were also hub genes in the Brown Module, *SCNN1B* (membership= 0.76, P = 0.002) and *SCNN1G* (membership= 0.81, P = 0.000), suggesting that the urea-treatment might have altered the normal ion homeostasis in the endometrium. Additionally, the ion channel ENaC and sodium-potassium ATPases are stimulated by the serum and glucocorticoid regulated kinase 1 (*SGK1*), which regulates ion balance and extracellular fluid volume (Lang, Artunc, & Vallon, 2009). A lower expression of *SGK1*, as seen in our study, was reported to alter the local fluid environment leading to reproductive failure in women, possible by a dysregulation of uterine fluid and ion imbalance leading to failure in embryonic implantation (Salker et al., 2011). Interestingly, *SGK1* is also an upstream regulator in our dataset.

Water channels also maintain ion homeostasis, such as aquaporin channels which are permeable to water and ions, regulating reabsorption of endometrial glandular fluid to maintain the luminal fluid volume (Zhu et al., 2015). The expression of aquaporin 5 (*AQP5*) is progesterone-dependent, showing higher concentrations during high endogenous progesterone stages of the estrous cycle in rats and mares. Additionally, *AQP5* had a high expression in cyclic mares at D8 of diestrus and was upregulated in the endometrium during the time of implantation in rats (C. Klein et al., 2013; Lindsay & Murphy, 2006, 2007). In our dataset, *AQP5* was upregulated in the urea group and was a hub gene in the Brown Module (membership = -0.88, P = 0.000). Altogether, we suggest that the urea infusions disrupt the normal function of this channel, resulting in an alteration of the composition and volume of uterine luminal fluid.

Enzymes are another important mediator of ion homeostasis, as carbonic anhydrase 2 (*CA2*) which was downregulated and also a hub gene in the Turquoise Module (membership= 0.95, P = 0.000) in our dataset. *CA2* catalyzes the reversible conversion of carbon dioxide and water to bicarbonate, regulating the acid-base balance and transporting carbon dioxide (Supuran, Scozzafava, & Casini, 2003). The downregulation of *CA2* by the urea-treatment is one of the mechanisms that resulted in an imbalanced uterine environment, supported also by the role that *CA2* has on endometrial gland development in mice and sheep (Hu & Spencer, 2005).

As shown in the preceding paragraphs, ion channels are crucial for ion homeostasis and can be regulated by hormones, including progesterone and estradiol (Ruan et al., 2014). Aldo-keto reductase family 1 member C23 (*AKR1C23*), which is only expressed in the control animals, has a well-known activity of converting progesterone to 20α-hydroxy-4-pregnen-3-one (20α-DHP) and 3α-dihydroprogesterone (3α-DHP) (Brown et al., 2006; El-Sheikh Ali et al., 2019; Kozai et al., 2014; Ogle & Beyer, 1982). Therefore, we postulate that the urea-treatment might disrupt the physiological progesterone metabolism that occurs in the endometrium. Consequently, progesterone is not converted into 20α-DHP which in turn affects the hormonal regulation of ion channels in the endometrium and disrupts the normal ion and fluid balance in the uterus of mares. Further studies need to be done to verify this effect of the progesterone metabolism in the endometrium of nonpregnant mares.

2.5.3. Growth and development of epithelial tissue

The endometrium goes through morphological changes during the estrous cycle and early pregnancy (Stefan Bauersachs, Mitko, Ulbrich, Blum, & Wolf, 2008; C. Klein et al., 2010). The current urea-treatment was responsible for downregulating genes related to the growth and development of epithelial tissue: *EGF*, Serpin B5 (*SERPINB5*), dickkopf WNT signaling pathway inhibitor 1 (*DKK1*), MET proto-oncogene, receptor tyrosine kinase (*MET*), insulin receptor (*INSR*), leucine rich alpha-2-glycoprotein 1 (*LRG1*), and prolactin receptor (*PRLR*). The treatment also upregulated angiopoietin like 4 (*ANGPTL4*), keratin 4 (*KRT4*), and growth arrest and DNA damage inducible gamma (*GADD45G*). For example, *EGF* mediates endometrial proliferation (Haining et al., 1991), while *DKK1* is responsible for initiating endometrial cellular proliferation and differentiation (Macdonald et al., 2011). Additionally, *SERPINB5*, also known as Maspin, plays an important role in embryonic implantation and had a higher expression in the endometrium of pregnant mice when compared to nonpregnant mice (Huang, Cai, & Yang, 2012). Of interest, *ANGPTL4*, a gene related to angiogenesis and rearrangement of blood vessels (Le Jan et al., 2003), had a higher mRNA expression in the endometrial tissue of multiparous pregnant sows when compared to nulliparous animals at day 15 and 25 of pregnancy (Lord et al., 2006; Merkl et al., 2010). In mares, there was an upregulation of *ANGPTL4* in the endometrium of day 12 pregnant animals. In the same study, there was a downregulation in *KRT4* in day 12 pregnant mares compared to age-matched nonpregnant mares (Merkl et al., 2010), opposite of what we saw in our dataset with mares during diestrus. Overall, the urea-treatment resulted in a change in expression of genes related to normal endometrial changes, which might result in a disruption of the physiological growth and development

of endometrial tissue at this stage of diestrus. However, this hypothesis needs to be tested with further studies.

2.5.4. Fatty acid metabolism

An interesting finding was a change in fatty acid metabolism, as was predicted by the disease and function analysis. Fatty acids function as precursors for steroid and eicosanoid synthesis, and are associated with phospholipids in cell membranes being able to affect uterine function and disturb pregnancy rates (Chapman & Quinn, 1976; Mattos et al., 2000). Mainly, a significant downregulation of *EGF*, caveolin 2 (*CAV2*), acyl-CoA synthetase long chain family member 4 (*ACSL4*), *SLC45A3*, prostaglandin reductase 1 (*PTGR1*), fatty acid desaturase 1 (*FADS1*), fatty acid desaturase 2 (*FADS2*), and leukemia inhibitory factor (*LIF*) in the urea group reflected this after the current treatment.

As eicosanoids are synthetized from fatty acids, the downregulation of the gene *PTGR1,* responsible for encoding catabolic enzymes that degrade eicosanoids, such as prostaglandins, might have a negative effect on the endometrium of animals (Erkenbrack et al., 2018), as an upregulation of it might be paramount for pregnancy, suggested by the upregulation of *PTGR1* in the endometrium of pregnant sows (Samborski et al., 2013) and pregnant mares (Merkl et al., 2010). Also related to reproduction hormone production in the endometrium, *SLC45A3* enhances long-chain fatty acids and neutral lipid accumulation with a consequent incorporation into cholesterol esters and phospholipids used for steroidogenesis and energy production (Shin, Howng, Ptacek, & Fu, 2012).

Furthermore, working through a fatty acid metabolism route, the cytokine *LIF* inhibits lipoprotein lipase activity *in vitro* (Marshall, Doerrler, Feingold, & Grunfeld,

1994) which might be related to the fact that *LIF* knockout mice were infertile suggesting that *LIF* codes a protein essential for blastocyst implantation (Stewart et al., 1992). Additionally, *LIF* was a hub gene in the Brown Module (membership= 0.94, P = 0.000) in our dataset.

As previously mentioned, fatty acids are associated with phospholipids of cell membranes (Chapman & Quinn, 1976). For example, the *CAV2* gene which encodes a protein that is present in the membrane of caveolin, invaginations of the plasma membrane, responsible for transport of glycolipids (Parton, 1994) and cholesterol across endothelial cells (Rothberg et al., 1992). The resulting downregulation of *CAV2* after the urea-treatment might be due to a deficit in the transport of macromolecules in the endometrium, disrupting the normal fatty acid metabolism in the tissue. Although this work sheds light into how urea-treatment might affect fatty acid metabolism, a clear mechanism of how urea-treatment can affect fatty acid metabolism in the endometrium has not been established and remains to be elucidated.

2.6. Conclusion

Our findings suggest that mares with a high BUN exhibit a decreased uterine pH and changes in gene expression in endometrial tissue are associated with pH regulation, ion channels, changes in epithelial tissue, and fatty acid metabolism. Specifically, effects on *EGF* could play a central role by driving the effects of urea on the endometrial transcriptome. Although this study did not address the effects of elevated BUN on fertility in mares, the changes in gene expression described herein reveal alterations in endometrial function that could have adverse effects on fertility.

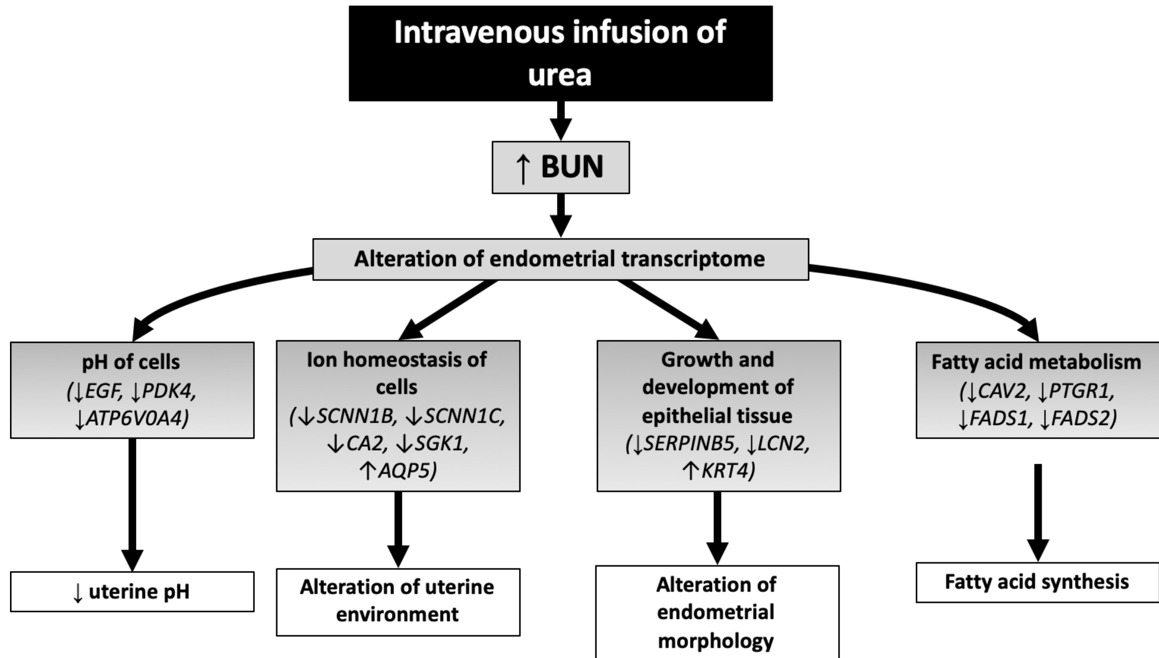


Figure 2.9. Schematic representation of proposed mechanisms of action of intravenous infusion of urea at day 7 of estrous cycle on the endometrium of mares over 6 hours. BUN = blood urea nitrogen.

CHAPTER 3. Effect of oral urea supplementation on the endometrial transcriptome of mares

3.1. Abstract

Elevated blood urea nitrogen (BUN) associated with increased crude protein consumption results in altered uterine environment, affecting early embryonic development in cows and in ewes. The objective of the present study was to evaluate the effects of increased BUN on the endometrium of mares. At ovulation detection (D0), oral treatment with urea was initiated and continued until D7. Mares received a treatment or control diet (n= 11 mares/group) in a crossover design. The treated group received urea (0.4 g/kg body weight) mixed with sweet feed and molasses, the control group received sweet feed and molasses alone. Blood samples were collected daily, one hour after feeding, for BUN determination. Uterine and vaginal pH were evaluated with an epoxy pH probe. Endometrial biopsies were taken transcervically one hour after the last feeding on D7. RNA sequencing of the endometrium of a subset of mares (n=6/group) was conducted. Reads were mapped to EquCab 3.0. Cuffdiff(2.2.1) was used to calculate differentially expressed genes (DEG) between urea and control groups (FDR-adjusted p-value < 0.1). There was an increase in BUN in the urea-treated mares, with no differences in uterine and vaginal pH between the groups. A total of 60 DEG were characterized, those with largest fold change were *SIK1*, *ATF3*, *SPINK7*, *NR4A1* and *EGR3*. Diseases and biological functions (Ingenuity Pathway Analysis, 2.2.1) resulted in processes related to cell death (necrosis) and cellular movement (invasion of cells). In conclusion, oral urea administration resulted in transcriptomic changes in the endometrium of mares related to necrosis, tissue remodeling and concentration of lipids. The observed changes in gene expression observed with an increased BUN might result in disruption to the endometrium that would influence the establishment of pregnancies and early embryo loss in mares.

KEYWORDS: High protein diet, high blood urea nitrogen, uterus, urea-treatment.

3.2. Introduction

Equine embryos reach the uterus at approximately 6.25 days after ovulation (Battut, Colchen, Fieni, Tainturier, & Bruyas, 1997; Freeman, Weber, Geary, & Woods, 1991) and are dependent on histotroph (uterine milk) to grow and develop (Allen & Wilsher, 2009). Therefore, an optimal intrauterine environment, to guarantee survival of the conceptus, is critical to pregnancy outcome. Consequently, physiological changes to the endometrium occur during diestrus to ensure that the uterine environment is prepared for a developing embryo (C. Klein et al., 2010; C. Klein et al., 2013; Sharp, 2000). Moreover, nutritional factors in the dam such as type and amount of polyunsaturated fatty acids (Coyne et al., 2011), have been implicated in causing changes in the uterine luminal milieu that might result in an unsuitable environment for embryonic development in cows and sheep. Additionally, high protein diets had detrimental effects on fertility of cows and sheep (Butler, 2000a; W R Butler, J J Calaman, & S W Beam, 1996; T. McEvoy et al., 1997; Rhoads et al., 2006). No studies to date have addressed the relationship between high protein concentration in maternal diets and fertility in mares.

A high protein diet or urea supplementation can elevate BUN in horses (Connysson et al., 2006; Martin et al., 1996), cows (Elrod & Butler, 1993; C. Elrod et al., 1993; Rhoads et al., 2006), and ewes (Fahey et al., 2001; T. McEvoy et al., 1997). A recent study from our lab used a short-term (six hours) intravenous infusion of urea as a model for high dietary protein in mares. This study revealed that urea affects endometrial transcripts related to cell pH, solute carriers, and ion homeostasis (Chapter 2). However, to better mimic the effects of a high protein diet, it is necessary to develop a diet model fed to mares over a longer period.

Currently, no studies elucidate the effects of an oral high protein diet model on the uterine transcriptome of mares; high throughput sequencing might be an excellent tool to clarify this process as it provides a global transcriptomic evaluation of tissues. As urea was shown to affect the endometrial environment, endometrial transcriptome, and pregnancy rates in cows, sheep and mares as previously stated, our hypothesis was that oral ingestion of urea would elevate blood urea nitrogen (BUN) and alter the endometrial transcriptome of mares. The objectives of the present study were: 1) to develop an oral supplementation model to elevate BUN, 2) to study the effects on the uterine and vaginal pH, and 3) to use RNA sequencing to characterize the endometrial transcriptome of mares fed oral urea compared to control mares at D7 of diestrus.

3.3. Material and methods

All animal procedures were completed in accordance with the Institutional Animal Care and Use Committee of the University of Kentucky (Protocol #2011-0876). Clinically healthy mares of different breeds, ranging from 5 to 15 years of age were used in this study. All mares underwent a reproductive examination and transrectal ultrasonography for reproductive tract evaluation.

To induce ovulation, mares received 2,500 IU of human chorionic gonadotropin (hCG) (Chorulon; Intervet, Millsboro, DE) intravenously when they had a follicle of approximately 35-mm in diameter and evident uterine edema. The animals were examined by transrectal ultrasonography (ExaGo ultrasound; ECM Co., Angouleme, France) for ovulation detection (D0). At D0, oral treatment started and continued until D7. Mares received the treatment or control diet in a random order (n= 11 mares/group) in a crossover

design, and the intervening estrous cycle served as a washout cycle between two experimental cycles. The treatment group was fed 0.4 g of feed-grade urea (Hallway Feeds, Lexington, KY) per kg of body weight daily, mixed with 2.4 kg of sweet feed (Poize 10% crude protein, Hallway Feeds), molasses and mixed grass hay (8.4% crude protein). The control group received identical sweet feed, molasses and hay. More specifically, the oral treatment supplemented the mares with grain (90% dry matter) and hay (89% dry matter). Considering a daily 2% dry matter total feed intake of body weight for a 500 kg mare, each animal received 2.4 kg of grain and 8.8 kg of hay. The amount of nitrogen supplied by the grain was of 34.56 g and by hay was of 107 g (considering a 16% percentage of nitrogen in the crude protein). The feed grade urea supplied a total of 84 g of nitrogen (considering a 42% of nitrogen). Therefore, in the urea-treatment the mares received a total of 225.56 g of nitrogen and in the control treatment received a total of 141.56 g of nitrogen. Daily meals were divided in two equal amounts given in the morning and afternoon using individual feeding pens. Mares had *ad libitum* access to water.

Blood samples were collected daily, one hour after the afternoon feeding in heparinized vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ). Blood samples were centrifuged at 1500 x *g* during 10 minutes at 4ºC, and plasma was stored at -20ºC. BUN was measured with a colorimetric spectrophotometric assay following an adapted protocol previously described (Mok et al., 2018). All reagents for this assay were purchased from Sigma-Aldrich (St. Louis, MO). The standard curve ranged from 5.6 mg/dL to 56.01 mg/dL. The researcher diluted urea (8M after constitution with 16 mL high purity water) to 5.6 mg/dL and 56.01 mg/dL to be used as low and high controls. The reaction consisted of analyzing urea by enzymatic hydrolysis to ammonia at room temperature. The reaction

(in duplicate) was done in microcentrifuge tubes (2 mL) with 10 μL of each plasma sample, and 125 μL urease buffer was added with incubation of the samples for 20 minutes. The urease enzyme hydrolyzes urea to produce carbon dioxide and ammonia (CH_4N_2O + Urease buffer $\rightarrow$ CO_2 + $2NH_3$). Then, 250 μL of phenol nitroprusside solution, 250 μL of alkaline hypochlorite solution (0.2%), and 1000 μL of distilled water were added (NH_3 + phenol nitroprusside + alkaline hypochlorite + H_2O $\rightarrow$ Indophenol blue) (Tabacco & Meiattini, 1985). After a 25-min incubation, a 200-μL aliquot was transferred to a 96-well plate and absorbance (570 nm) was determined with an Epoch microplate spectrophotometer (Biotek, San Francisco, CA). The intra- and interassay coefficients of variation for BUN concentrations were 0.5% and 9.8%, respectively. The lower limit of detection of the assay was 0.11 mg/dL.

Uterine and vaginal pH were measured one hour after the last feeding at D7 in both groups. The mares were restrained in palpation stocks and their tails were wrapped and tied. Feces were removed from the rectum manually. The perineal region was washed three times with povidone-iodine scrub, rinsed with clean water, and dried with clean paper towels. An adapted epoxy pH probe (model number 911600, Thermo Fisher Scientific, Waltham, MA) attached to a portable pH meter (Accumet AP115, Thermo Fisher Scientific) were used for pH measurements. Immediately before pH measurements, the probe was calibrated with calibration solutions buffers at pH 4, 7 and 10 (Thermo Fisher Scientific). The pH probe was introduced into the vagina with the tip protected by a sterile gloved hand and passed through the cervix. The pH probe was advanced into the uterus until it reached the uterine body and was held in place by the examiner. The examiner introduced the other hand into the rectum to increase the contact between the uterine wall

and the pH probe. When the uterine pH measurements were completed, the pH device was removed from the uterus and placed into the vagina, in contact with the vaginal mucosa next to the cervix. The pH device was maintained in the same position until two stable measurements were completed. The pH measurements were done in duplicate, in the uterus and vagina, and the mean was calculated.

After uterine pH measurements, the perineal region was washed again three times with povidone-iodine scrub, rinsed with clean water and dried with clean paper towels. A Jackson uterine biopsy forceps (60-cm length, 4 mm × 28 mm basket, Jorgensen Laboratories, Inc.) was guarded in a sterile gloved hand and passed through the cervix into the uterus. A uterine biopsy was collected from the base of the uterine horn. The sample was removed from the instrument, with a sterile needle (NIPRO medical corporation) and preserved in RNAlater (Thermo Fisher Scientific) at 4ºC overnight and then at -80ºC until RNA isolation (Herrera et al., 2018). Mares received dinoprost tromethamine (5 mg, IM; Lutalyse; Pfizer, New York, NY) to help with uterine clearance after intrauterine procedures.

Endometrial samples were processed for RNA extraction using a RNeasy Mini Kit (Qiagen, Gaithersburg, MD) following the manufacturer's recommendations. After extraction, RNA concentration and quality were analyzed using a Nanodrop 2000 spectrophotometer Thermo Fisher Scientific) and a Bioanalyzer® (Agilent, Santa Clara, CA). Samples had a 260/280 ratio > 2.0, 28S:18S rRNA ratios >1.8 and RNA integrity number (RIN) was 9.78 ± 0.06 (mean ± SEM).

3.3.1. mRNA library preparation and Next Generation Sequencing

Total endometrial RNA was assessed from a subset of mares (n= 6 mares/group) by RNA sequencing at the University of Louisville Center for Genetics and Molecular Medicine. A TruSeq Stranded mRNA library prep kit (Illumina, San Diego, CA) was used to prepare libraries for mRNA sequencing. A Poly A purification and RNA fragmentation were performed on total RNA. Superscript II was used to generate cDNA and then the RNA template was removed. Second strand synthesis was performed with incorporation of dUTP to ensure stranded libraries, and double-stranded cDNA was purified with AMPure XP beads. The 3' ends were adenylated, indexing adapters were ligated onto the ends, and libraries once again purified with AMPure beads. Fifteen cycles of PCR were used to enrich DNA fragments, followed by two AMPure bead clean-up steps. Libraries were loaded onto an Agilent DNA 1000 chip and validated on an Agilent 2100 Bioanalyzer (Agilent). Quantitation was performed with the Illumina Library Quantification Kit, ABI Prism qPCR Mix from Kapa Biosystems. Three dilutions were tested in triplicate. Libraries were diluted to 10nM, pooled, further diluted and denatured to single strand and run on a NextSeq 500 v2 (Illumina) 300cycles High Output kit in a 2x150 base pairs with paired-end reads.

The Fastq files were evaluated for read quality using FastQC (0.11.4) (Andrews, 2010a). Subsequently, TrimGalore (0.4.1) (Krueger, 2012) was used for adapter and read quality (Phred score threshold of 30) trimming. We mapped our reads to the equine genome EquCab 3.0 using the software STAR (2.5.3.a) (Dobin et al., 2013) then used the equine reference annotation from NCBI with Cufflinks (2.2.1) (Trapnell et al., 2012), using fragments per kilobase per million (FPKM) to determine the expression level of

genes. Lastly, we used Cuffdiff (2.2.1) (Trapnell et al., 2012) to calculate differentially expressed genes (DEG) between samples from the control and urea groups. Significance level was set at FDR-adjusted p-value of the test statistic < 0.1 using a Benjamini-Hochberg correction.

3.3.2. Functional genomics

The Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.8) (https://david.ncifcrf.gov/home.jsp) was used to annotate DEG in relation to biological process and molecular function (Huang da et al., 2009). A functional classification was performed based on the Official Gene Symbol of *Equus caballus* genes in DAVID. The R package GOplot (http://wencke.github.io/) was used to illustrate the results. DAVID uses information about gene functions based on public genomic resources through gene-set enrichment, helping to elucidate characteristics and biological relevance of the DEG.

A core analysis of the DEG was conducted using Ingenuity Pathway Analysis (IPA, QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). Diseases and biological functions analyses were done to show networks of biological interest. Additionally, an upstream regulator analysis and molecule type were conducted. This software uses networks based on cause and effect relationships that have been previously published (Kramer et al., 2014) and served to give additional information regarding the mechanisms of action of the DEG.

Protein-protein interactions related to the DEG between the urea-treated and control animals were analyzed through a correlation prediction of expression level using the

STRING database (http://www.string-db.org/, version 10.5) to characterize the interactions between the proteins coded by the DEG (Szklarczyk et al., 2017) as we expected that the proteins coded by the DEG would have a high interaction.

The FANTOM5 (http://fantom.gsc.riken.jp) curated database consists of 2,558 unique ligand-receptor interactions (Kawaji, Kasukawa, Forrest, Carninci, & Hayashizaki, 2017). A ligand-receptor interaction analysis was done by identifying ligands that were DEG between the urea and control groups, which will help characterize the connected signaling network in the endometrium of mares that was altered by urea supplementation.

3.3.3. Quantitative Real-Time PCR

Expression levels of seven DEG were evaluated with quantitative real-time PCR (RT-qPCR) using the same RNA samples that were analyzed by RNA Sequencing (n=6 mares per group). Genes were chosen based on known functional importance to the research question and based on DEG identified in a previous study in our lab (Chapter 2). The extracted RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit and random hexamers (Invitrogen, Carlsbad, CA). The cDNA was kept frozen at -20°C until quantitative real-time PCR (RT-qPCR) was performed. Primers for the selected transcripts were designed using the Primer-BLAST (National Center for Biotechnology Information, NCBI) function (Table 3.1). The RT-qPCR was conducted using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, Foster City, CA) with the program: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and 55–95°C for dissociation cycling conditions. Each reaction was performed in duplicate.

Efficiency of RT-qPCR was determined using LinRegPCR (version 2012.0) to ensure that it was between 1.8 and 2.2 (Ruijter et al., 2009). Mean threshold cycle (CT) was used to show changes in the mRNA expression and then normalized for the housekeeping genes (Livak & Schmittgen, 2001) Beta-2-Microglobulin (*B2M*) and Eukaryotic Translation Elongation Factor 1 Alpha 1 (*EEF1A1*) to calculate delta CT values (ΔCT). The two housekeeping genes were chosen with GeNORM as the most stably expressed genes in the endometrial samples (De Spiegelaere et al., 2015).

Table 3.1 Forward (F) and reverse (R) primers used for Quantitative Real-Time PCR analysis.

Gene symbol	Forward primer sequence	Reverse primer sequence	Acession number	Product size
B2M	GTGTTCCGAAGG TTCAGGTT	ATTTCAATCTCAG GCGGATG	NM_001082502.3	103
EEF1A1	CAACATCGTCGT CATTGGGC	CAGCAGCCTCCTT CTCGAAT	NM_001081781.1	119
INHBA	GAGGATGACAT CGGCAGGAG	CGACAGGTCACTG CCTTCTT	NM_001081909.1	135
LAMC2	CTGGAGAGCGC TGTGATAGG	GGTACAGCCCTGA GGGTTTC	NM_001081768.1	71
MUC6	TGCCGTACAAG ACTCGCAAT	TGTACACCTGGAA CACAGGC	XM_014729569.1	117
PIGR	GTTTTGGCAGCA GCATCCAG	ACTCCTTGCGAGG GATGTTT	XM_014739411.2	134
SERPINA14	CTGACAGATGC AAAGAGCAGC	AAAAGTCCGCAGA GGGTGAG	XM_014735597.1	131
SIK1	GACTTCCAACGG GCACCTAA	CAGCAACAGGTTC TCGGTCT	XM_023630253.1	127
SPINK7	TTTTCCCTCGTG TTGGCTGA	AGGGCACAACAAC CTTCTCC	XM_003362870.2	88

Primers were generated using the National Center for Biotechnology Information (NCBI) primer-BLAST tool. Key: beta-2-Microglobulin (B2M), eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A1), Inhibin Subunit Beta A (INHBA), laminin subunit gamma 2 (LAMC2), Mucin 6 (MUC6), polymeric immunoglobulin receptor (PIGR), serine peptidase inhibitor clade A (alpha-1 antiproteinase, antitrypsin) member 14 (SERPINA14), serum/glucocorticoid regulated kinase 1 (SGK1), serine peptidase inhibitor, Kazal type 7 (SPINK7).

3.3.4. Statistical analyses

BUN, uterine and vaginal pH were tested for normality with a Shapiro-Wilk test. The data were normally distributed. The BUN was tested using the Fit Least Squares model with fixed factors as day, treatment and interaction between day and treatment, and mare as a random effect. The Tukey post-hoc test was used for pairwise comparisons. Uterine and vaginal pH were tested using a one-tailed paired t-test. A Pearson's correlation coefficient was used to determine the correlation between the -ΔCT (negative delta CT) from RT-qPCR results and the FPKM from RNA sequencing results.

A probability of $P \leq 0.05$ indicated significant difference, and a probability of $P > 0.05$ to ≤ 0.10 indicated a trend toward significance. Data are reported as mean ± SEM. All statistical analyses were carried out using JMP Pro 14 (SAS Institute Inc., Cary, NC).

3.4. Results

3.4.1. Blood urea nitrogen

The effect of group was not significant ($P = 0.57$); there was a trend for the effect of day ($P = 0.10$), and the interaction between group and day was significant ($P < 0.0001$). On the first day of treatment (day of ovulation, D0), the two groups did not show a statistical difference in BUN ($P > 0.05$). There was a significant increase in BUN on D1 through D7. On the last day of treatment, D7, the mean BUN was of 12.75 ± 0.99 and 28.57 ± 2.25 mg/dL in the control and urea-fed mares, respectively ($P < 0.05$) (Figure 3.1).

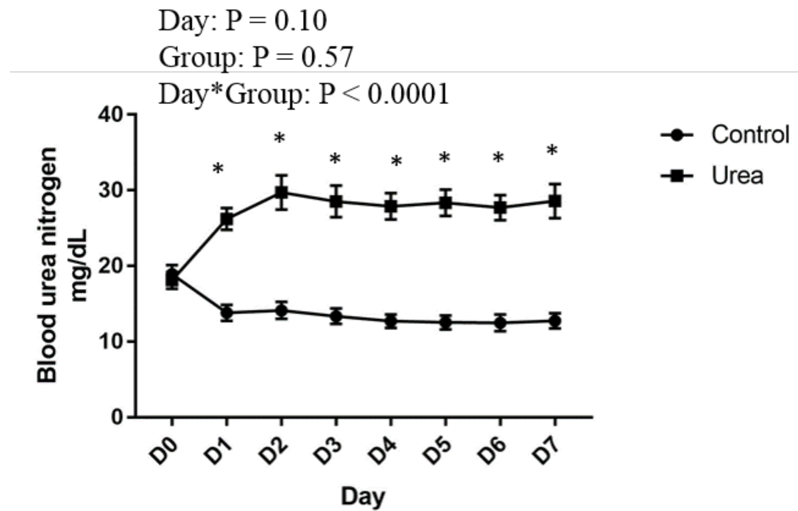


Figure 3.1. Blood urea nitrogen (mg/dL) analyzed in diestrus mares receiving an oral control or urea diet over 7 days. Results are shown as mean ± SEM, D= day, * = P < 0.05.

3.4.2. Uterine and vaginal pH

There was no significant difference in uterine pH in the treated and control groups (P = 0.69). Uterine pH was 6.86 ± 0.03 and 6.89 ± 0.09 pH in the control and treated group, respectively (Figure 3.2). Similarly, there was no significant difference in vaginal pH in the treated and control groups (P = 0.43) with a 7.12 ± 0.07 and 7.12 ± 0.05 pH in the control and treated group, respectively (Figure 3.2).

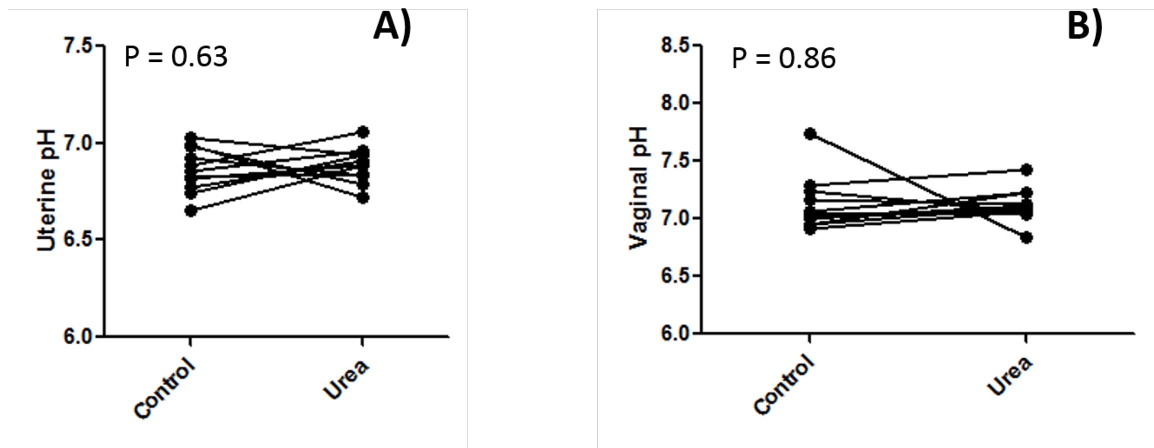


Figure 3.2. A) Uterine and B) vaginal pH analyzed in diestrus mares receiving an oral control or urea diet over 7 days. Results are shown as mean (n=11 mares/group), lines connect the values for each mare after respective treatment.

3.4.3. RNA sequencing

The RNA sequencing analysis performed on 12 endometrial samples (n=6 mares/group) resulted in 22,659 genes analyzed. The average of input reads was 6,152,866, the average input read length was 150, and 5,199,472.5 (84.45%) uniquely mapped reads were obtained for the samples sequenced (Table 3.2).

Table 3.2 Summary of RNA sequencing data for 12 endometrial samples.

Sample	Group	Number of input reads	Average input read length	Uniquely mapped read number	Uniquely mapped reads %
1	Control	5,757,633	150	4,855,414	84.33
2	Urea	7,826,472	150	6,633,286	84.75
3	Control	6,341,747	150	5,351,409	84.38
4	Urea	6,740,392	150	5,711,795	84.74
5	Control	5,388,562	150	4,567,111	84.76
6	Urea	6,892,700	150	5,860,698	85.03
7	Control	7,272,455	150	6,166,986	84.8
8	Urea	6,260,867	150	5,353,307	85.5
9	Control	4,750,575	150	3,935,409	82.84
10	Urea	5,770,651	150	4,852,787	84.09
11	Control	5,584,354	150	4,666,585	83.57
12	Urea	5,247,984	150	4,438,883	84.58

3.4.4. Differentially expressed genes (DEG)

A total of 60 genes were differentially expressed between the urea and control groups (FDR<0.1). A total of 25 genes were upregulated and 35 genes were downregulated in the urea group in comparison to the control group (Table 3.3). The gene *LOC100066131* was uncharacterized in the NCBI database for *Equus caballus* and had its nucleotide sequence (FASTA format) identified in the NCBI database (http://www.ncbi.nlm.nih.gov/), then the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST) (Camacho et al., 2009) was used to identify its ortholog in the *Equus przewalskii* as proline rich 4 (lacrimal) (*PRR4*).

Table 3.3. List of differentially expressed genes between the control and urea-fed mares.

Gene name	Gene symbol	Chromosome location	Log2(Fold Change)	FDR
ADAM metallopeptidase with thrombospondin type 1 motif 6	ADAMTS6	Chr 21:8724863-9083230	-1.181	0.065
apolipoprotein L domain containing 1	APOLD1	Chr 6:41698890-41704045	1.398	0.015
Rho guanine nucleotide exchange factor 19	ARHGEF19	Chr 2:37069152-37087346	-1.801	0.015
ADP ribosylation factor like GTPase 4C	ARL4C	Chr 6:21037228-21041190	-1.295	0.015
activating transcription factor 3	ATF3	Chr 5:23305133-23358402	1.956	0.015
BTG anti-proliferation factor 2	BTG2	Chr 5:73328-75820	1.239	0.040
divergent protein kinase domain 2A	DIPK2A	Chr 16:79271667-79305319	-1.445	0.095
chromosome 1 C14orf28 homolog	C1H14orf28	Chr 1:182248886-182258585	0.996	0.015
C1q and TNF related 6	C1QTNF6	Chr 28:35552205-35559679	-1.821	0.015
claudin 10	CLDN10	Chr 17:65282708-65303020	-1.618	0.015
collagen type XXI alpha 1 chain	COL21A1	Chr 20:54291646-54465931	-1.887	0.015
cysteine and serine rich nuclear protein 1	CSRNP1	Chr 16:47804552-47816648	1.141	0.090
connective tissue growth factor	CTGF	Chr 10:79323385-79435012	0.969	0.015
catenin alpha 3	CTNNA3	Chr 1:55166715-56671260	-1.940	0.028
cysteine rich angiogenic inducer 61	CYR61	Chr 5:75205288-75208196	1.297	0.015
dishevelled binding antagonist of beta catenin 2	DACT2	Chr 31:2139337-2150951	-1.409	0.060
iodothyronine deiodinase 2	DIO2	Chr 24:25068789-25083162	-1.539	0.065
dihydropyrimidinase like 5	DPYSL5	Chr 15:70266697-70358386	0.756	0.090
early growth response 3	EGR3	Chr 2:51783523-51788806	1.428	0.015
fragile histidine triad	FHIT	Chr 16:29154068-30541103	-2.330	0.015
FXYD domain containing ion transport regulator 4	FXYD4	Chr 1:71353201-71357540	1.125	0.015
glutaredoxin and cysteine rich domain containing 2	GRXCR2	Chr 14:31114370-31131400	1.103	0.015
H19, imprinted maternally expressed transcript	H19	Chr 12:34334438-34336980	-2.445	0.015
insulin like growth factor binding protein 3	IGFBP3	Chr 4:16218772-16226910	-1.537	0.015
inhibin subunit beta A	INHBA	Chr 4:12793421-12811345	1.038	0.065
kinesin family member 5C	KIF5C	Chr 18:31398786-31545685	-1.641	0.065
laminin subunit gamma 2	LAMC2	Chr 5:17461883-17513382	-2.305	0.015
olfactory receptor 51G2-like	LOC100053865	Chr 7:75162813-75163755	0.879	0.028

Table 3.4 (continued). List of differentially expressed genes between the control and urea-fed mares.

myelin and lymphocyte protein	MAL	Chr 15:13416153-13439781	-1.974	0.074
proline rich 4 (lacrimal)	LOC100066131 (PRR4)	Chr 6:40554454-40598891	-1.520	0.015
gasdermin-C	GSDMC	Chr 9:73048799-73107940	-2.600	0.015
lecithin retinol acyltransferase	LRAT	Chr 2:79363321-79371844	1.046	0.028
lactotransferrin	LTF	Chr 16:41879029-41907637	-1.852	0.015
monocyte to macrophage differentiation associated	MMD	Chr 11:30310267-30336756	-1.617	0.015
mucin 6, oligomeric mucus/gel-forming	MUC6	Chr 12:35968104-35990415	-4.972	0.015
matrix remodeling associated 5	MXRA5	Chr X:1511212-1541902	-2.441	0.015
neutral cholesterol ester hydrolase 1	NCEH1	Chr 19:15089707-15155042	-1.996	0.015
nephroblastoma overexpressed	NOV	Chr 9:64549295-64570362	-1.626	0.015
nuclear receptor subfamily 4 group A member 1	NR4A1	Chr 6:70094775-70111308	1.561	0.015
nuclear receptor subfamily 4 group A member 2	NR4A2	Chr 18:37574464-37592463	1.528	0.015
peptidyl arginine deiminase 2	PADI2	Chr 2:36681246-36722159	0.935	0.015
pappalysin 1	PAPPA	Chr 25:21035848-21279423	0.905	0.015
procollagen C-endopeptidase enhancer 2	PCOLCE2	Chr 16:78287175-78353319	-1.486	0.095
phosphodiesterase 11A	PDE11A	Chr 18:55973758-56365248	-1.108	0.060
polymeric immunoglobulin receptor	PIGR	Chr 5:3143483-3161575	-2.349	0.015
phospholipase A1 member A	PLA1A	Chr 19:41761268-41786327	-1.125	0.060
pleckstrin and Sec7 domain containing 3	PSD3	Chr 27:1222175-1862961	-1.059	0.028
retinol dehydrogenase 10	RDH10	Chr 9:13539334-13565466	-1.098	0.040
RP1 like 1	RP1L1	Chr 2:59021135-59144653	-1.270	0.060
serine peptidase inhibitor clade A (alpha-1 antiproteinase, antitrypsin) member 14	SERPINA14	Chr 24:37509403-37519502	1.238	0.065
secreted frizzled related protein 1	SFRP1	Chr 27:4059691-4105413	-1.434	0.015
serum/glucocorticoid regulated kinase 1	SGK1	Chr 10:81297093-81405022	1.025	0.015
salt inducible kinase 1	SIK1	Chr 26:40014586-40026987	2.161	0.015
serine peptidase inhibitor, Kazal type 7 (putative)	SPINK7	Chr 14:28699405-28702860	1.698	0.015
transcriptional and immune response regulator	TCIM	Chr 27:5024674-5025975	0.757	0.083
thrombospondin 1	THBS1	Chr 1:150310064-150326658	1.402	0.015
transmembrane protein 86A	TMEM86A	Chr 7:89063186-89070145	0.889	0.015

Table 3.5 (continued). List of differentially expressed genes between the control and urea-fed mares.

two pore channel 3	TPC3	Chr 15:14142046-14199717	-1.508	0.052
tubulin tyrosine ligase like 6	TTLL6	Chr 11:24838293-24886582	-1.512	0.015
UDP glycosyltransferase 8	UGT8	Chr 2:112387729-112467843	-1.332	0.095

3.4.5. Functional genomics analyses

Gene ontology analysis using DAVID indicated overrepresentation of the biological processes of cell adhesion, negative regulation of cell death, skeletal muscle cell differentiation, and cellular response to corticotropin-releasing hormone stimulus were overrepresented after treatment. The overrepresented molecular functions included heparin and integrin binding. The cellular components included extracellular space, proteinaceous extracellular matrix, and intracellular membrane-bounded organelle (Figure 3.3). The IPA diseases and biological functions analysis with the DEG showed categories of biological interest, such as necrosis, lipid concentration, and invasion of cells ($P < 0.05$, Figure 3.4).

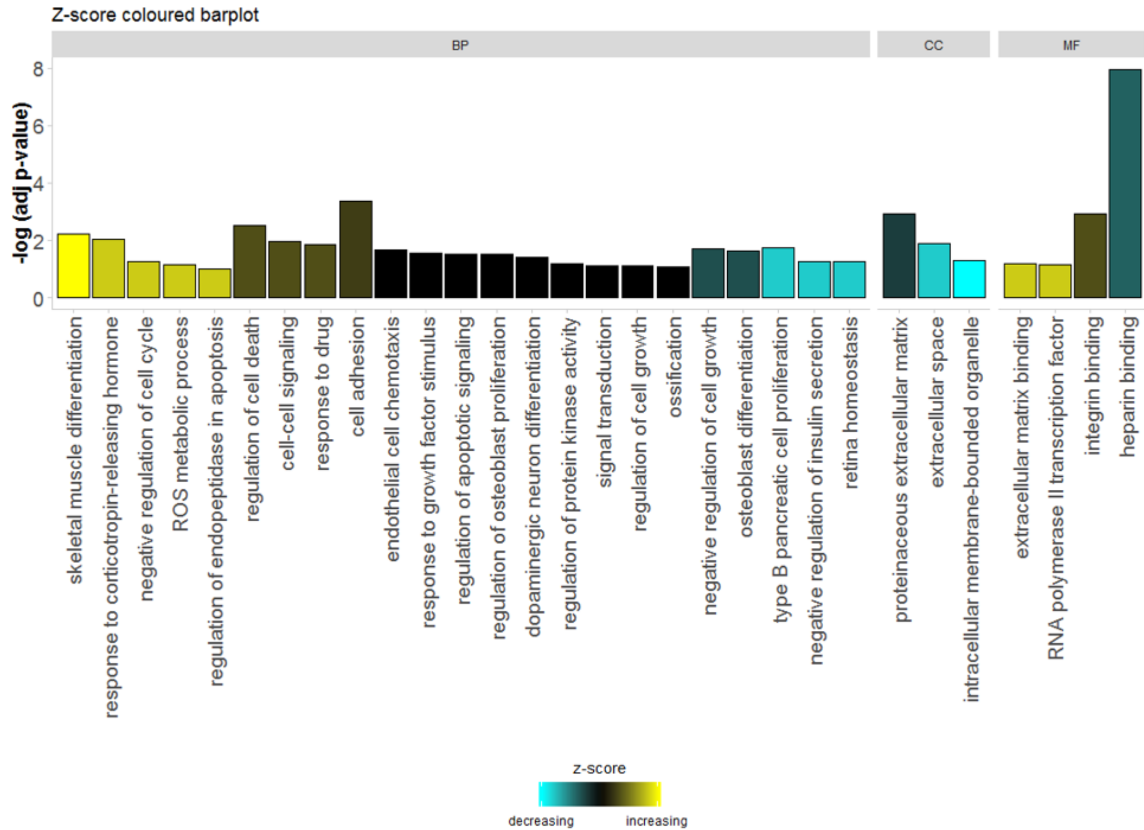


Figure 3.3. Gene ontology of differentially expressed genes analyzed by DAVID and the GOplot package. The y-axis represents the –log of the adjusted p-value and the x-axis represents the gene ontology results indicating the z-score in the color of the bars, blue indicates an increase and yellow indicates a decrease. BP= biological process, CC= cellular component, MF= molecular function.

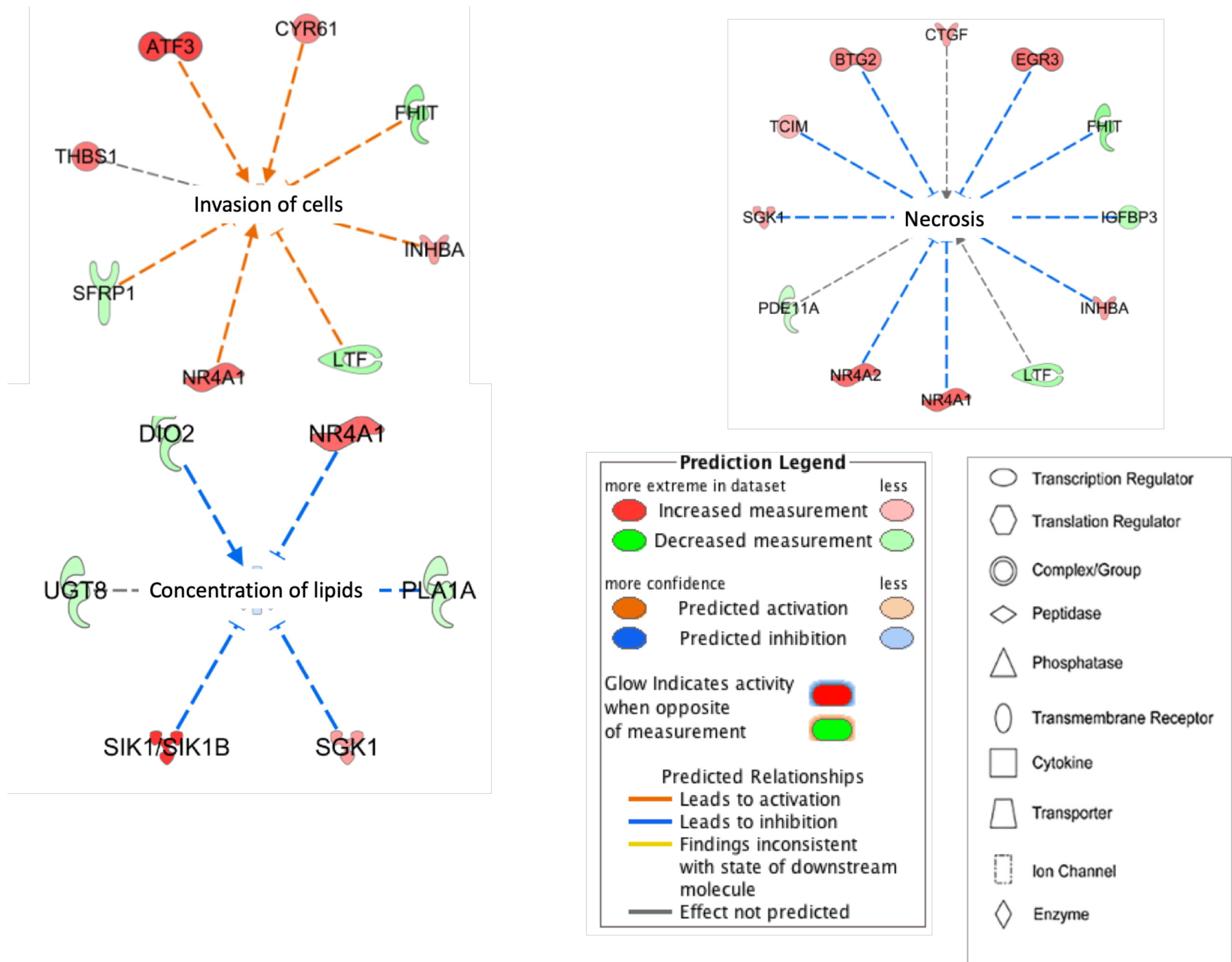


Figure 3.4. Ingenuity Pathway Analysis (IPA) of diseases and biological functions of the differentially expressed genes displayed as nodes (genes) and edges (biological relationship between nodes). The color intensity of each node represents fold change expression, red (upregulated) and green (downregulated). The edges connecting the genes to the respective functions represent the predicted relationships, blue representing inhibition and grey effect not predicted based on the IPA activation z-scores, combination of directional information encoded by the gene expression with information curated from the literature.

3.4.6. Upstream regulators

The IPA upstream regulator analysis indicated a total of 459 genes as upstream regulators. From these, retinol dehydrogenase 10 (*RDH10*), connective tissue growth

factor (*CTGF*), inhibin subunit beta A (*INHBA*), cysteine rich angiogenic inducer 61 (*CYR61*) and thrombospondin 1 (*THBS1*) were differentially expressed in our dataset. Their respective target molecules in our dataset are shown in Table 3.4.

Table 3.6 Upstream regulators from the IPA analysis when comparing the urea and control groups.

Upstream Regulator	Expr Log Ratio	Molecule Type	p-value of overlap	Target molecules in dataset
RDH10	-1.098	Enzyme	0.016	SFRP1
CTGF	0.969	Growth Factor	0.015	CTGF,NOV
INHBA	1.038	Growth Factor	0.009	CTGF,INHBA,NR4A2
CYR61	1.297	Other	0.000	CYR61,NR4A1,SFRP1
THBS1	1.402	Other	0.003	CTGF,THBS1

3.4.7. Protein-protein interactions

The protein-protein interaction (PPI) analysis showed a significant PPI enrichment score ($P < 1.0e\text{-}^{16}$) with a total of 53 nodes (genes) and 34 edges (interactions). The average node degree; how many interactions a protein has on average in the network; was 1.28 and the average local clustering coefficient; how connected the nodes in the network are; was of 0.33. The resulting network is shown in Figure 3.5.

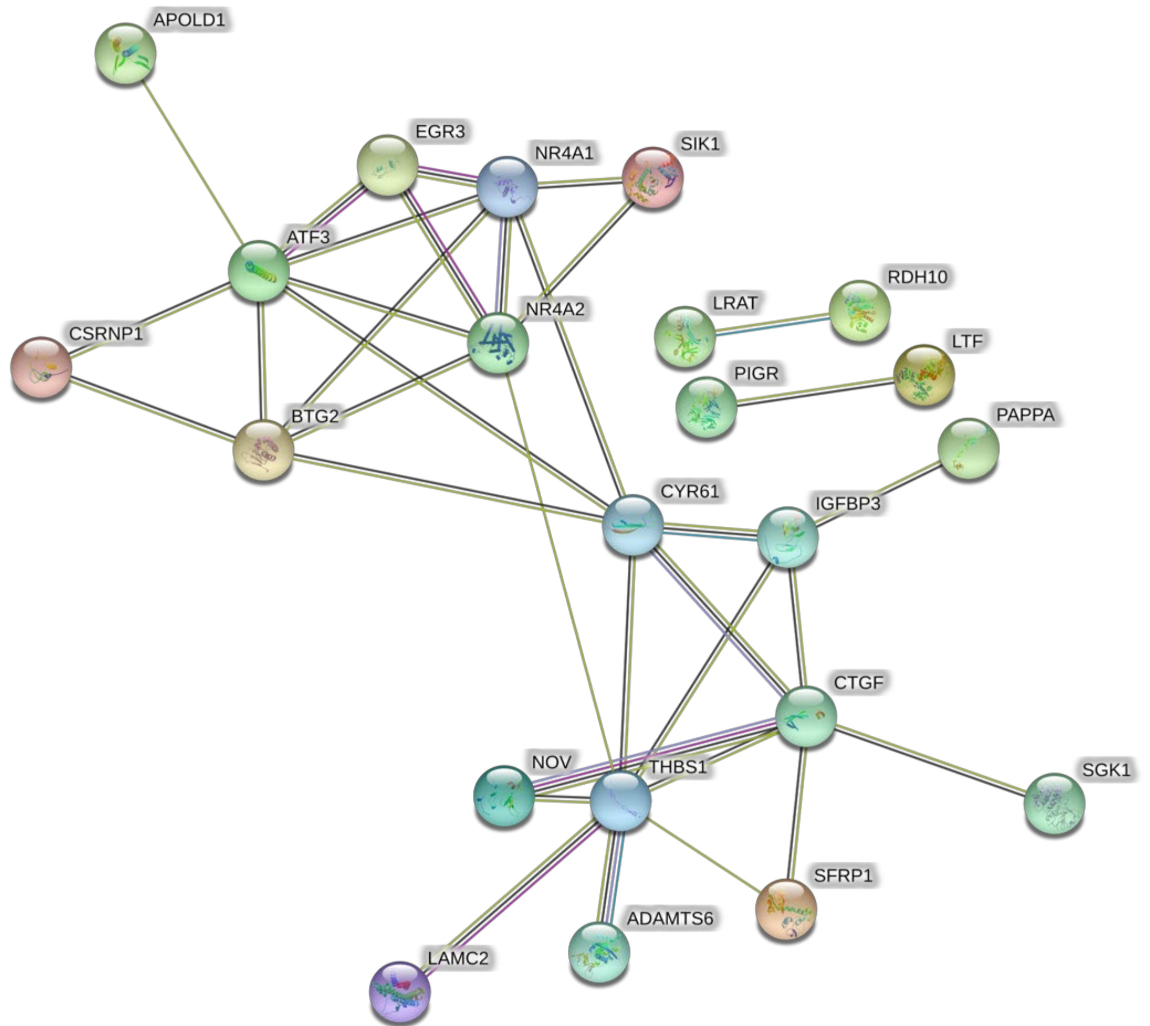


Figure 3.5. Protein-protein interaction analysis with the STRING algorithm showing nodes (proteins) and edges (protein-protein association) of proteins in the differentially expressed genes. The functional interaction network represents how the proteins coded by the differentially expressed genes between the urea and control groups are related. Thicker edges indicate stronger protein-protein interactions, each node represents a protein produced by a single, protein-coding gene locus, node colors represent proteins that are first interactors, nodes that have shapes inside indicate that the protein has a known or predicted 3D structure.

3.4.8. Ligand-receptor interactions

The expression of ligands was decreased in the urea group, such as laminin subunit gamma 2 (*LAMC2*), lactotransferrin (*LFT*), nephroblastoma overexpressed (*NOV*), secreted frizzled related protein 1 (*SFRP1*). Additionally, the following ligands had an

increased expression in the urea group: connective tissue growth factor (*CTGF*), inhibin subunit beta A (*INHBA*), cysteine rich angiogenic inducer 61 (*CYR61*) and thrombospondin 1 (*THBS1*) (Table 3.5).

Table 3.7 List of ligand-receptor pairs in which ligands were differentially expressed between the urea and control groups.

Downregulated ligands	
Ligands	Receptors
LAMC2	CD151, COL17A1, ITGA2, ITGA3, ITGA6, ITGB1, ITGB4
LTF	GP9, LRP1, LRP11, TFRC
NOV	NOTCH1, PLXNA1
SFRP1	FZD2, FZD6
Upregulated ligands	
Ligands	Receptors
CTGF	ITGA5, ITGAM, ITGB2, LRP1, LRP6, NTRK1, ERBB4
INHBA	ACVR1, ACVR1B, ACVR2A, ACVR2B, BAMBI, ENG, TGFBR3
CYR61	CAV1, ITGA5, ITGAM, ITGAV, ITGB2, ITGB3, ITGB5
THBS1	CD36, CD47, ITGA2B, ITGA3, ITGA4, ITGA6, ITGB1, ITGB3, LRP1, LRP5, SCARB1, SDC1, SDC4, TNFRSF11B

3.4.9. Quantitative Real-Time PCR

Analysis of the correlation between genes with RT-qPCR (-ΔCT) and the RNA sequencing results (FPKM) showed significant correlation between the two methods (Table 3.6).

Table 3.6 Pearson's correlation of RNA Sequencing (FPKM) and Quantitative Real-Time PCR (-ΔCT).

Gene	Correlation	P-value
PIGR	0.92	0.000
SIK1	0.92	0.000
MUC6	0.91	0.000
INHBA	0.89	0.000
LAMC2	0.87	0.001
SPINK7	0.87	0.001
SERPINA14	0.77	0.009

3.5. Discussion

This is the first study to show that an oral supplementation with urea changes the endometrial transcriptome of mares during diestrus. The results revealed alterations of the expression of genes associated with necrosis, invasion of cells and concentration of lipids. The DEG genes identified after urea treatment in this dataset serve to formulate possible molecular effects of this metabolite in the endometrium of animals that might be related to fertility.

The oral supplementation of urea developed for this study was an effective model to increase BUN in mares, as BUN increased at the first day after the start of the urea-treatment and remained high. The higher BUN concentration in the urea-treated mares did not result in changes in the uterine pH. This outcome is different from mares that received urea intravenously over 6 hours, which resulted in a decrease in uterine pH (Chapter 2). In the current study, the oral urea treatment was fed during a longer period when compared to the previous study. Possibly, with this chronic model of urea-treatment, there was time for the intrauterine environment to regain its homeostasis, hence returning to a physiological uterine pH.

Genes that were identified as differentially expressed after the urea treatment have been previously reported to be important for pregnancy. For example, lactotransferrin (*LTF*), also known as lactoferrin, has been shown to modulate the inflammatory response when infused after mares were bred (Fedorka et al., 2018). Furthermore, there was mRNA expression of *LTF* in uterine epithelial cells in mice during early pregnancy suggesting that *LTF* might have a role in the preimplantation uterus (McMaster, Teng, Dey, & Andrews, 1992). In our dataset, the urea-treatment decreased the expression of *LTF*

causing a disruption in the uterine environment. The potassium ion channel FXYD domain containing ion transport regulator 4 (*FXYD4*) was downregulated in the endometrium of pregnant mares (Gebhardt et al., 2012). In contrast, *FXYD4* was upregulated in our data. It has been suggested that Claudin 10 (*CLDN10*) prepares the endometrium of cows for embryo implantation by regulating the endometrial structure, and was upregulated in early pregnant heifers (S. Bauersachs et al., 2006). Our current treatment caused a decrease in *CLDN10* in the endometrium of mares. Additionally, the uterine epithelium is an important source of *CTGF*, which regulates physiological uterine functions during early pregnancy in pigs (Moussad, Rageh, Wilson, Geisert, & Brigstock, 2002) and cows (Forde & Lonergan, 2012). Also, the mRNA expression of *CTGF* was lower during early pregnancy in mares (C. Klein et al., 2010); and the IPA analysis identified *CTGF* as an upstream regulator in our dataset. Overall, the change in the expression of genes involved with pregnancy establishment might indicate that urea treatment alters the ability of the equine endometrium to maintain pregnancies, but this hypothesis requires further investigation.

Ligand-receptor interactions mediate cell-cell communication, thus the expression of the ligands and receptors is an important aspect for the physiological functioning of a tissue (Zhou, Taramelli, Pedrini, Knijnenburg, & Huang, 2017). The DEG that were identified as ligands in the interaction analysis; CTGF, *CYR61*, *NOV*, *INHBA* and *THBS1*; were also seen identified as ligands in the endometrium and embryo of cows, signaling that the interaction between these ligands and receptors is possibly related to maternal recognition of pregnancy (Mamo, Mehta, Forde, McGettigan, & Lonergan, 2012). Furthermore, *CTGF*, related to cell adhesion (D. Ball, Rachfal, Kemper, & Brigstock, 2003); *NOV*, an angiogenic regulator (Lin et al., 2003); *CYR61*, an angiogenic protein

related to cell adhesion, migration and proliferation (Mo et al., 2002) are all members of the CCN family of secreted matricellular proteins related to reorganization and transformation of tissues during reproductive events through interaction with their ligands (Mo et al., 2002; Winterhager & Gellhaus, 2014). Additionally, diabetic *CTGF* heterozygous mice had a lower BUN when compared to diabetic wild-type mice (James, Le, Doherty, Kim, & Maeda, 2013), although the reasons for this difference in BUN are unclear, it shows that a relationship between diminished expression of *CTGF* and BUN. Although the expression of these ligands in the endometrium does not necessarily guarantee that they will secrete proteins to compose the uterine histotroph (Mamo et al., 2012), proteomics evaluation of the uterine luminal fluid of mares (M. Hayes et al., 2012) and cows (Muñoz et al., 2017) showed the presence of *CTGF*, *PIGR* and serine peptidase inhibitor clade A member 14 (*SERPINA14*), for example, in uterine fluid during early pregnancy.

Urea caused oxidative stress to murine renal medullary collecting duct cells in culture (Z. Zhang, Yang, & Cohen, 1999); as necrosis is associated with oxidative stress, it is not surprising that necrosis is identified in the IPA diseases and biological functions analysis. Necrosis involves intracellular events such as production of reactive oxygen species, swelling of mitochondria, disruption of calcium ion homeostasis, and plasma membrane rupture (Golstein & Kroemer, 2007). Several DEG related to necrosis had an increased expression, such as *INHBA*, nuclear receptor subfamily 4 group A member 1 and 2 (*NR4A1* and *2*), serum and glucocorticoid-regulated kinase 1 (*SGK1*), transcriptional and immune response regulator (*TCIM*), BTG anti-proliferation factor 2 (*BTG2*), and early growth response 3 (*EGR3*). *SGK1*, for instance, is of interest as it is an early response gene

that is induced in response to cellular stressors. *SGK1* is an important cell survival signal as it attenuates necrotic cell death induced by calcium ions (Brickley et al., 2013). The members of the *NR4A* nuclear receptor family, *NR4A1* (also known as *Nur77*) and *NR4A2* (also known as *Nurr1*), upregulated in our dataset, act as necrosis promoters. *NR4A1* has been reported as upregulated in other abnormal endometrial states, such as in thin endometrium from women, which is associated with implantation failure (Maekawa et al., 2017). *NR4A2* is necessary for necrosis after its translocation from the nucleus to the cytoplasm *in vitro* (Watanabe, Sekine, Naguro, Sekine, & Ichijo, 2015). A transcription regulator, *EGR-1*, was shown to be upregulated in murine renal medullary cells in culture after urea treatment (Z. Zhang et al., 1999), similarly we saw that a transcription regulator of the EGR family, *EGR-3*, was upregulated in the endometrium of mares after oral urea supplementation. Additionally, *BTG2* had an increased expression in cardiomyoblasts after oxidative damage, resulting in necrosis (Choi, Park, Kim, & Lim, 2013). Overall, the urea-treatment altered the expression of genes related to necrosis, which might serve as evidence of the oxidative stress caused by urea in the endometrium.

Invasion of epithelial cells by macromolecules is a process that occurs in normal and pathological conditions through the basement membrane, an extracellular-matrix membrane that separates tissue compartments (Caceres et al., 2018). Urea-supplementation changed the expression of genes related to invasion of cells; *SFRP1*, *LTF*, *FHIT* (decreased expression) and *NR4A1*, *INHBA*, *CYR61*, *ATF3* (increased expression). Supporting the current finding that an increase of *ATF3* in the endometrium of mares would allow for a more pronounced invasion of epithelial cells by macromolecules, the overexpression of *ATF3* in colon cancer and esophageal epithelial cells *in vitro* and *in vivo*

showed an increase in cell migration and invasive ability (Wu, Wei, Sun, Yuan, & Jiao, 2014; Xie et al., 2014). Similarly, lower expression of secreted frizzled related protein 1 (*SFRP1*) were associated with cell proliferation, migration, and invasion of human immortalized nasopharyngeal epithelial cell lines *in vitro* (Ren et al., 2015). Although not evaluated in this study, the changes in these genes related to invasion of cells might be due to a disruption in the basement membrane of the endometrium.

Urea addition in cultures of mouse kidney tissue caused an increase in osmolality, with a decrease of the synthesis of fatty acids from glucose via acetylCoA (Bojesen, 1980). The oral urea treatment altered the expression of certain genes related to concentration of lipids: increased the expression of *NR4A1*, *SGK1*, and salt inducible kinase 1 (*SIK1*) while it decreased the expression of iodothyronine deiodinase 2 (*DIO2*), phospholipase A1 member A (*PLA1A*), and UDP glycosyltransferase 8 (*UGT8*). An overexpression of *SIK1* was accompanied by a reduction of lipogenic gene expression; furthermore, in hepatic knockdown of *SIK1* there was an alteration in lipogenic genes transcription, thus disrupting lipid homeostasis. Overall, *SIK1* regulates endogenous fatty acid synthetic gene expression (Yoon, Seo, Lee, Kim, & Koo, 2009). The expression of *DIO2* in adipose tissue was inversely related to markers of fatty acid oxidation and synthesis, which could be an attempt, even if inadequate, from the adipocyte to minimize lipid accumulation (Bradley et al., 2018). The gene *PLA1A* is responsible for producing free fatty acids (Sonoda et al., 2002), additionally it was a DEG in the endometrium of cattle fed with a n-3 polyunsaturated fatty acid supplement, showing that it is related to lipid metabolism (Waters, Coyne, Kenny, MacHugh, & Morris, 2012).

Moreover, there were nine DEG in common between the current study characterized by a chronic oral urea supplementation and our previous study characterized by an acute intravenous urea infusion (Chapter 2). The genes *IGFBP3*, kinesin family member 5C (*KIF5C*), *LAMC2*, proline rich 4 (lacrimal) (*PRR4*), *PIGR*, and tubulin tyrosine ligase like 6 (*TTLL6*) were decreased, whilst serine peptidase inhibitor Kazal type 7 (*SPINK7*) was increased in both datasets. The genes *SERPINA14* and *SGK1* had an inverse change in mRNA expression, increasing in the oral urea study and decreasing in the intravenous urea experiment. With the diseases and biological functions analysis (IPA), we observed that treatment with urea through both routes of administration elevated BUN of mares, leading to an alteration in endometrial transcriptome with genes related to abnormal growth in endometrium and migration of cells. We believe that the difference in the list of DEG seen in the two studies is mainly due to the acute versus chronic exposure to urea and increased BUN. Possibly, the acute increase in BUN obtained with the first experimental design, had a more pronounced change in the endometrial transcriptome, resulting in a larger number of DEG, because the mares did not have time to adjust to such changes and regain homeostasis. As these studies did not test fertility in mares, subsequent studies need to be done to determine if an acute or chronic increase in BUN is more detrimental to pregnancy rate and early pregnancy loss in mares.

In conclusion, most of the DEG from this dataset are associated with necrosis, cell movement, tissue remodeling and lipid concentration. These results are a starting point to elucidate novel mechanisms through which a high systemic BUN, after oral urea supplementation, might alter the endometrial transcriptome in mares. Additional studies

need to be done to evaluate how the changes in endometrial transcriptome seen in the present study will influence physiological and reproductive function in mares.

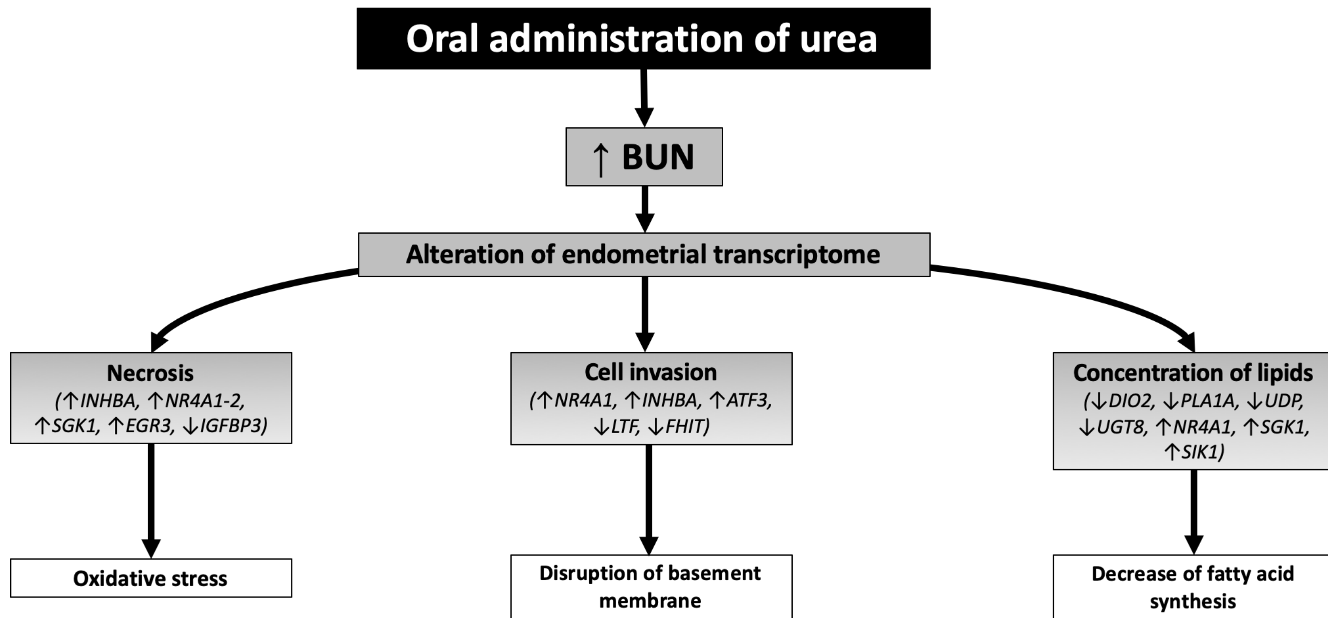


Figure 3.6. Representation of proposed mechanism of action of oral supplementation of urea on the endometrium of mares.

CHAPTER 4. Effect of oral urea administration on the transcriptome of the equine embryo

4.1. Abstract

Maternal diet has an important effect on the development of embryos. High blood urea nitrogen (BUN) in cows and ewes has been reported to have negative impacts on embryo development; however, no studies on this relationship have been published in mares. Therefore, this study evaluated the effects of a high BUN on blastocoele fluid urea concentrations and analyzed the transcriptome of day-14 equine embryos based upon RNA sequencing. When a 25 ± 3 mm follicle was detected, mares were randomly allocated to a urea (n=9) or control treatment (n=10). The urea treatment consisted of an oral supplementation of urea (0.4 g/kg of body weight), mixed with sweet feed and molasses. The control treatment was sweet feed and molasses alone. Blood samples were collected every other day for BUN analysis, one hour after feeding. Mares were artificially inseminated in the presence of a 35-mm follicle and ovulation was detected (D0). Ultrasonographic exams for pregnancy detection started at D11, and embryo collection was done at D14 (n=5 urea-treated embryos; n=7 control embryos). Blastocoele fluid was collected and stored separately for urea nitrogen concentration analysis. RNA was extracted from embryos for RNA sequencing. Cuffdiff(2.2.1) was used to calculate differentially expressed genes (DEG) between urea and control groups. There was an increase in BUN in the urea treatment group. DEG genes involved in neurological development, cell proliferation, vascular remodeling, and adhesion were identified in embryos from urea-treated mares. In summary, oral urea treatment in mares caused transcriptomic changes on D14 equine embryos that might have deleterious effects to their development.
KEYWORDS: High protein diet, blood urea nitrogen, horse, embryo development, blastocoele fluid urea nitrogen.

4.2. Introduction

Maternal diet has been shown to influence embryonic development, pregnancy outcomes, and change gene expression in bovine and human embryos (Chmurzynska, 2010; Fahey et al., 2001; D. S. Hammon et al., 2005; Penagaricano et al., 2013). A balance between a suitable uterine environment and a properly programmed embryo is paramount to a successful pregnancy (Khatib & Gross, 2019). More specifically, high protein diets or urea-treatment, a metabolite of protein metabolism, have been used to increase BUN and evaluate effects on reproductive function (W. R. Butler et al., 1996; C. Elrod et al., 1993; Ferguson et al., 1993). Hence, studies show a positive correlation between systemic BUN and urea nitrogen concentrations in follicular fluid and uterine fluid in cows and ewes (C. Elrod et al., 1993; Fahey et al., 2001; D. S. Hammon et al., 2005; Ellen R Jordan et al., 1983; T. McEvoy et al., 1997). Embryos collected from cows with high BUN (≥19 mg/dL) resulted in lower pregnancy rates when compared to embryos collected from donor cows with moderate BUN (<19 mg/dL) (Rhoads, Rhoads et al. 2006). Similarly, when ewes received urea orally, resulting in increased BUN, fewer and less well-developed embryos were collected four days after ovulation. Additionally, embryos cultured *in vitro* from ewes with high BUN had lower embryonic cell proliferation and survival rates (McEvoy, Robinson et al. 1997), and there was a lower rate of blastocyst hatching *in vitro* collected from cows fed diets resulting in high BUN (Ferreira, Gomez et al. 2011). Overall, these results indicate detrimental effects of a high BUN on the follicular fluid and uterine environments that would lead to a dysregulation in oocyte and embryo development and consequently lower pregnancy rates in cows and ewes. However, the effects of a high BUN

on equine embryos are unknown and studies need to be done in order to fill the gaps in knowledge regarding these possible effects.

Previously, we have shown that there was an increase in BUN and a decrease in uterine pH as well as significant changes to the endometrial transcriptome after urea treatment in mares (Chapter 2 and 3). We revealed the effects of a high BUN on the endometrium of mares; however, the effects of high urea nitrogen concentrations on equine embryos remain unknown.

Therefore, we hypothesize that oral ingestion of urea will elevate BUN and change gene expression of embryos. Our objectives were to determine the effects of a high BUN on embryonic growth rate, blastocoele fluid urea concentrations, and perform RNA sequencing on day-14 equine embryos collected from urea-fed mares and control mares.

4.3. Material and methods

All animal procedures were completed in accordance with the Institutional Animal Care and Use Committee of the University of Kentucky (Protocol #2011-0876). Clinically healthy mares of different breeds, ranging from 5 to 15 years of age were used in this study. All mares underwent a reproductive examination and transrectal ultrasonography for reproductive tract evaluation.

When a 25 ± 3 mm follicle was detected, mares were randomly allocated to a urea (n=9) or control treatment (n=10). The treatment group was fed 0.4 g of feed-grade urea (Hallway Feeds, Lexington, KY) per kg of body weight, mixed with 2.4 kg of sweet feed (Poize 10% crude protein, Hallway Feeds), molasses and mixed grass hay (8.4% crude protein). The control group received identical sweet feed, molasses and hay. More

specifically, the oral treatment supplemented the mares with grain (90% dry matter) and hay (89% dry matter). Considering a daily 2% dry matter total feed intake of body weight for a 500 kg mare, each animal received 2.4 kg of grain and 8.8 kg of hay. The amount of nitrogen supplied by the grain was of 34.56 g and by hay was of 107 g (considering a 16% percentage of nitrogen in the crude protein). The feed grade urea supplied a total of 84 g of nitrogen (considering a 42% of nitrogen). Therefore, in the urea-treatment the mares received a total of 225.56 g of nitrogen and in the control treatment received a total of 141.56 g of nitrogen. Daily meals were divided in two equal amounts given in the morning and afternoon using individual feeding pens. Mares had *ad libitum* access to water.

When a 35-mm follicle and pronounced uterine edema were determined by transrectal ultrasonography (ExaGo ultrasound; ECM Co., Angouleme, France), the mares were artificially inseminated with 500-million progressively motile sperm pooled together from two fertile stallions. Human chorionic gonadotropin (hCG) 2,500 IU (Chorulon; Intervet, Millsboro, DE) was administered intravenously at the time of insemination to induce ovulation. Daily ultrasound examinations were performed until ovulation detection (Day 0). The mares continued to receive their respective treatment until D14, over a total of 18.63 ± 0.23 (mean ± SEM) days.

4.3.1. Embryo collection and analyses

Ultrasonographic exams for pregnancy detection started at D11. Non-pregnant mares were evaluated daily for embryo detection until D14. Embryos were evaluated daily by measuring the vesicle height and width from an ultrasonic image at its maximum size

obtaining the average diameter until D14. Embryo area as an ellipse was calculated with the following formula:

$$[(\text{Embryo width} / 2) * (\text{Embryo height} / 2)] * 3.1416$$

Mares were restrained in palpation stocks, and their tails were wrapped and tied. Feces were removed from the rectum manually, and the perineal region was washed three times with povidone-iodine scrub, rinsed with clean water and dried with clean paper towels. Conceptus recovery was performed on day 14 by transcervical uterine lavage with Hartman's solution and a sterile endotracheal tube of 24-mm diameter (Jorgensen Laboratories, Loveland, CO). Mares were sedated with xylazine hydrochloride (0.1-0.2 mg/kg of body weight, IV; AnaSed; Lloyd, Shenandoah, IA). The cervix was dilated manually in order to fit the endotracheal tube until it reached the uterine body. Fluid collected in a sterile palpation sleeve was infused until it filled the uterus to recover the embryo.

The embryo was rinsed with Hartman's solution three times. The capsule was ruptured using a sterile needle (NIPRO medical corporation, Miami, FL), and the blastocoele fluid was retrieved with a syringe and needle (NIPRO medical corporation). Fluid was frozen at -20ºC for later analyses. After the blastocoele fluid was removed, embryos were preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA) at 4ºC overnight and then kept at -80ºC until RNA isolation.

4.3.2. Blood collection

Blood samples were collected every other day, one hour after the mares finished eating in the afternoon, with vacutainer tubes with sodium heparin (BD Vacutainer, Franklin Lakes, NJ). Blood samples were promptly centrifuged at 1500 x *g* for 10 minutes at 4ºC, and plasma was stored at -20ºC.

4.3.3. Urea nitrogen analyses

Concentrations of urea nitrogen in the plasma and blastocoele fluid were measured with a spectrophotometric assay following an adapted protocol previously described (Mok et al., 2018). All reagents were purchased from Sigma-Aldrich. The standard curve ranged from 5.6 mg/dL to 56.0 mg/dL. The researcher diluted urea (8M after constitution with 16 mL high purity water) to 5.6 mg/dL and 56.0 mg/dL to be used as low and high controls. The reaction consisted of analyzing urea by enzymatic hydrolysis to ammonia at room temperature. The reaction (in duplicate) was done in microcentrifuge tubes (2 mL) with 10 μL of each plasma sample, and 125 μL urease buffer was added with incubation of the samples for 20 min. The urease enzyme hydrolyzes urea to produce carbon dioxide and ammonia (CH_4N_2O + Urease buffer $\rightarrow$ CO_2 + $2NH_3$). Then, 250 μL of phenol nitroprusside solution, 250 μL of alkaline hypochlorite solution (0.2%), and 1000 μL of distilled water were added (NH_3 + phenol nitroprusside + alkaline hypochlorite + H_2O $\rightarrow$ Indophenol blue) (Tabacco & Meiattini, 1985). After a 25-min incubation, a 200-μL aliquot was transferred to a 96-well plate and absorbance (570 nm) was determined with an Epoch microplate spectrophotometer (Biotek, San Francisco, CA) at 570 nm. The intra- and

interassay coefficients of variation for BUN concentrations were 0.5% and 9.8%, respectively. The lower limit of detection of the assay was 0.11 mg/dL.

4.3.4. Progesterone analysis

Progesterone concentrations were determined for D4, D10, and D14 (D0 = day of ovulation) in duplicates with an Immulite 2000 Analyzer (Siemens Healthcare Diagnostic Products, Ltd., Malvern, PA) using the Progesterone Test L2KPW2, a solid phase competitive chemiluminescent enzyme immunoassay. Low and high progesterone adjustors were used, with concentrations of 1.54 and 5.48 ng/mL, respectively. The lower limit of detection of the assay is 0.1 ng/mL. Intra- and inter-assay coefficients of variation (% CVs) were 7% and 3.78%, respectively.

4.3.5. Blastocoele fluid pH and osmolality

The blastocoele fluid pH was measured using an adapted epoxy pH probe (model number 911600, Thermo Fisher Scientific, Waltham, MA) attached to a portable pH meter (Accumet AP115, Thermo Fisher Scientific). Immediately before pH measurements, the probe was calibrated with calibration solutions at pH 4, 7 and 10 (Thermo Fisher Scientific). The pH readings were done in duplicate, and an average was calculated. The blastocoele fluid osmolality was determined by freezing point osmometry (Model 5004: Precision System Inc., Natick, MA) as recommended by the manufacturer. Calibration of the osmometer was done with osmometry standards 100 and 500 mOsm (Precision System Inc.).

4.3.6. RNA extraction

Total cellular RNA was extracted from embryos using TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer's recommendations. After extraction, RNA concentration and quality were analyzed using a NanoDrop DP-1000 spectrophotometer (Agilent Technologies, Palo Alto, CA) and a Bioanalyzer® (Agilent, Santa Clara, CA). All samples had a 260/280 ratio > 2.0, a 28S:18S rRNA ratios >1.8, and RNA integrity number (RIN) > 8 (8.95 ± 0.4, mean ± SEM). A total of 1 µg of RNA was treated with DNase I (Ambion Inc., Austin, TX) for 30 minutes at 37ºC to remove genomic DNA according to manufacturer's instructions. The extracted RNA was kept at -20ºC until further analyses.

4.3.7. RNA sequencing

The total extracted RNA from embryos, as described above, was used for RNA sequencing. Samples from five embryos from the urea group and seven embryos from the control group were used. Total RNA sample quality was evaluated with agarose gel electrophoresis to test the RNA degradation and potential contamination before library construction. The mRNA was enriched using oligo(dT) beads. The mRNA was fragmented randomly in fragmentation buffer, followed by first strand cDNA synthesis using random hexamers and reverse transcriptase. After first-strand synthesis, a custom second-strand synthesis buffer (Illumina, San Diego, CA) was added with dNTPs, RNase H and *Escherichia coli* polymerase I to generate the second strand by nick-translation. Double-stranded cDNA was purified using AMPure XP beads (Beckman Coulter, Beverly, CA). In order to select cDNA fragments of 150 base pairs in length, the library fragments were

purified with AMPure XP system (Beckman Coulter). The final library was obtained by PCR amplification and purification of PCR products by AMPure XP beads (Beckman Coulter, Beverly, USA). The library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies), and then diluted to 1 ng/µl before checking insert size on an Agilent 2100 and quantifying to greater accuracy by quantitative PCR, to ensure the library quality. Sequencing was done with a NovaSeq 6000 instrument (Illumina) in 2x150 base pairs with paired-end reads. A total of 592 million reads were produced. Raw image data file from the high-throughput sequencing was transformed to Raw Reads by CASAVA (1.8 Illumina) base recognition.

4.3.8. RNA Sequencing data analysis

The Fastq files were evaluated for read quality using FastQC 0.11.4 (Andrews, 2010a). Subsequently, Trim Galore 0.4.1 (Krueger 2012) was used for adapter and read quality trimming (Phred score threshold of 30). Reads were mapped to the *Equus caballus* reference genome (EquCab 3.0) using the software STAR 2.5.3a (Dobin et al., 2013), then annotated with the equine reference annotation from NCBI using Cufflinks 2.2.1 (Trapnell et al., 2012). Fragments per kilobase per million (FPKM) were used to determine the expression level of genes. Lastly, we used Cuffdiff 2.2.1 (Trapnell et al., 2012) to calculate differentially expressed genes (DEG) between samples from the control and urea groups. Significance level was set at FDR-adjusted p-value of the test statistic < 0.1 using a Benjamini-Hochberg correction.

4.3.9. Functional annotation and pathway analysis

PANTHER (version 13.1, http://www.pantherdb.org/) annotated DEG in relation to biological process, molecular function, cellular component, and pathways (Huang da et al., 2009). As PANTHER describes the functions of DEG based on public genomic resources through gene-set enrichment, it provided a better understand and described the transcriptomic changes on embryos after urea supplementation.

4.3.10. Quantitative Real-Time PCR

Expression levels of a subset of DEG determined by RNA sequencing between the control and urea groups were confirmed with RT-qPCR. The extracted RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit and random hexamers (Thermo Fisher Scientific). The cDNA was kept frozen at -20°C until quantitative real-time PCR (RT-qPCR) was done. Primers for the selected transcripts were designed using the Primer-BLAST (National Center for Biotechnology Information, NCBI) function (Table 4.1). The RT-qPCR was done using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) with the program: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and 55–95 °C for dissociation cycling conditions. Each reaction was done in duplicate.

Table 4.1 Forward and reverse primers used for quantitative Real-Time PCR analysis.

Gene symbol	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Accession number	Product size
GAPDH	AGAAGGAGAAAGGCCCTCAG	GGAAACTGTGGAGGTCAGGA	NM_001163856	87
APLNR	GATCCGAGAGAAGCCTGGTG	GAAGGTGCCCTCACACTACC	XM_005598133.3	84
GSTA1	CCATTCGCAACTACATCGCC	TTTCATCACGTGGGGTCATGG	NM_001284532.1	141
LOC102149479	CGGCCTTCTCTGTTTCAGACT	TGGTGCCGTTCCTGGTGATA	XM_005599320.3	127
NFASC	TTCAGAACGAGCTGTCCCAG	CCCTTCGCCTCACACTCAAT	XM_023640364.1	103
PCSK1	TGCTGGATGGCATTGTGACT	AAGCCTTCTGGGCTAATCGG	XM_001504608.5	144
PSCA	GGCGTGTAAGATCCCAGGAG	GCTAAGCCAGTGGGCCTTTA	XM_001505016.4	126

Primers were generated using the National Center for Biotechnology Information (NCBI) primer-BLAST tool. Key: GAPDH: glyceraldehyde 3-phosphate dehydrogenase, APLNR: apelin receptor, GSTA1: glutathione S-transferase alpha 1, LOC102149479: sperm-associated acrosin inhibitor, NFASC: neurofascin, PCSK1: proprotein convertase, PSCA: prostate stem cell antigen.

The RT-qPCR efficiency was determined using LinRegPCR (version 2012.0) to ensure that it was between 1.8 and 2.2 (Ruijter et al., 2009). Mean threshold cycles (CT) were used to show changes in the mRNA expression and then normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (C. Klein, Rutllant, & Troedsson, 2011) to calculate delta CT values (ΔCT) (Livak & Schmittgen, 2001).

4.3.11. Statistical analyses

Data were tested for normality with a Shapiro-Wilk test. The BUN concentration, progesterone concentration, embryo area as an ellipse, and blastocoele fluid osmolality were not normally distributed and a normal quantile transformation was done. The blastocoele fluid urea concentration and pH data were normally distributed. A Standard Least Squares with fixed factor as treatment and day and random effect as horse was

performed for: BUN, progesterone, and embryo area as an ellipse. A Standard Least Squares with fixed factor as treatment and random effect as horse was performed for: blastocoele fluid osmolality, blastocoele fluid urea concentration and pH. A Student's t test was used for pairwise comparison. Significance of difference between embryo recovery in the control and urea groups was assessed applying the Chi-Square test.

A Pearson's correlation was done to determine the relationship between BUN and blastocoele fluid urea nitrogen concentrations and for BUN and progesterone.

Data are reported as median (range) when not normally distributed and mean ± SEM when normally distributed. Significance was set at $P < 0.05$ and trend at $0.1 \geq P > 0.05$. JMP Pro (version 14; SAS Institute, Cary, NC, USA) was used for all statistical tests.

4.4. Results

4.4.1. Blood urea nitrogen concentrations

There was an effect of treatment day ($P = 0.013$) and group ($P < 0.001$) for BUN (Figure 4.1). Immediately before treatment (FEEDD0), the BUN of the control and urea groups were 13.35 (9.73-17.28) and 12.56 (10.11-17.77) mg/dL, respectively. At the day of ovulation (OVD0) the BUN of the control group was 12.27 (8.52-16.06) compared to the BUN of the urea group of 19.80 (13.94-24.02) mg/dL ($P < 0.05$). At 14 days after ovulation (OVD14) the BUN of the urea group was also higher than that of the control group, 24.56 (15.47-28.48) and 12.31 (9.10-16.80) mg/dL, respectively ($P < 0.05$).

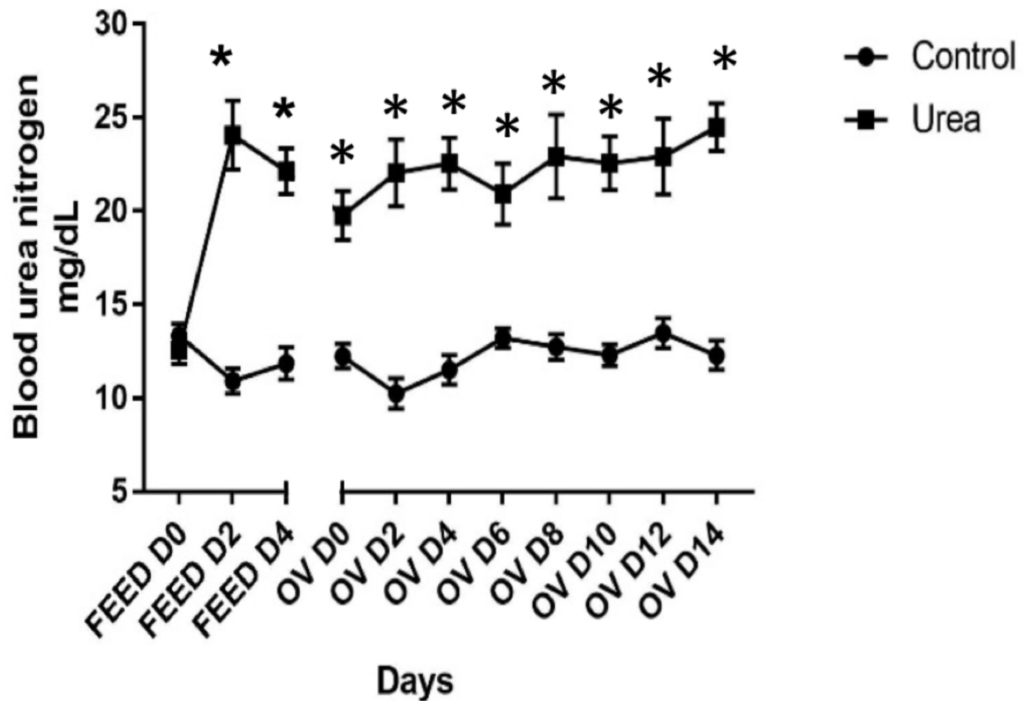


Figure 4.1. Blood urea nitrogen (mg/dL) analyzed in mares receiving a control or urea diet over a total of 18.63 ± 0.23 (mean ± SEM) days. Results are shown as mean and SEM, * $P < 0.05$.

4.4.2. Progesterone concentrations

A total of five mares (56%) from the urea and six mares (60%) from the control group produced an embryo, with one of the control mares having a twin pregnancy. There was no effect of day ($P = 0.41$) or interaction between group*day ($P = 0.56$), but there was a group effect ($P = 0.03$), with lower progesterone concentrations in the urea-treated mares (Figure 4.2). Although the progesterone values from the mare with twin embryos (at D4, D10 and D14) were not identified as outliers (values 3 times the interquantile range past the lower and upper quantiles), if we exclude this animal from the statistical analyses, as mares with twin pregnancies have a higher progesterone concentration, there were no differences in the progesterone concentrations between the groups (Day: $P = 0.47$; Group:

P = 0.47; Group*Day: P = 0.63). The Pearson's correlation between progesterone and BUN of all the mares were not significant (D4: R = -0.20, P = 0.57; D10: R = 0.43, P = 0.21; D14: R = 0.08, P = 0.82).

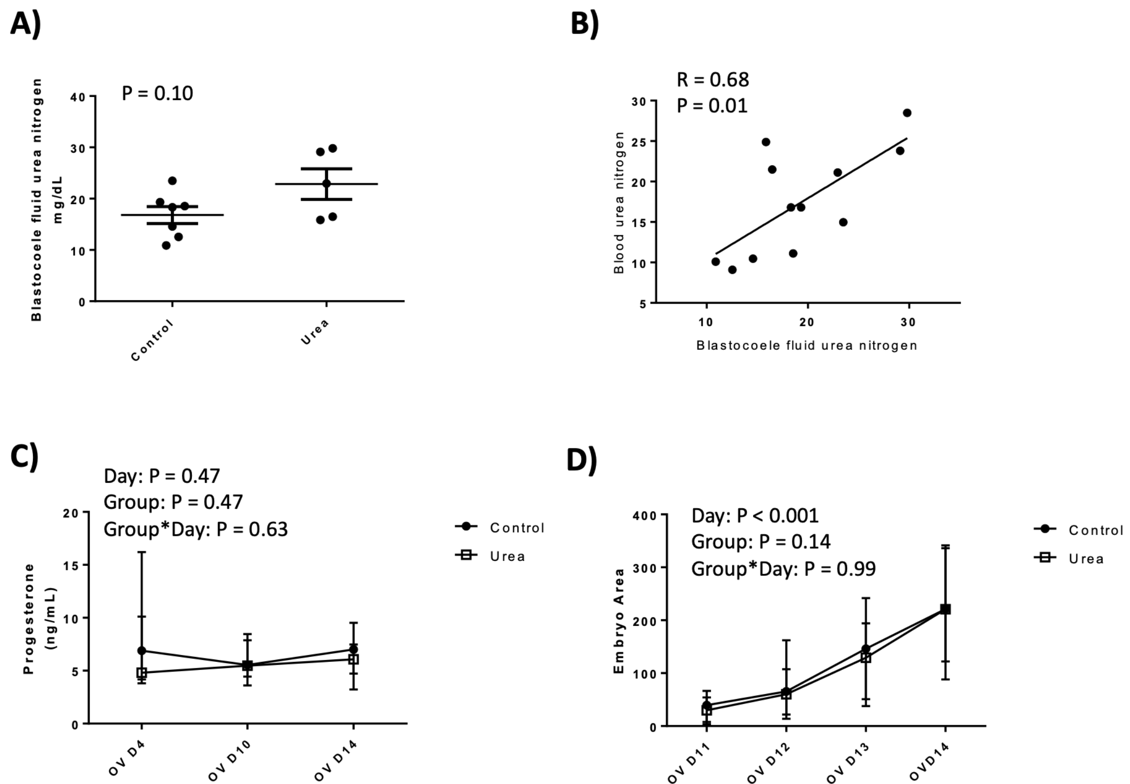


Figure 4.2. A) Blastocoele fluid urea nitrogen concentrations analyzed in mares receiving a control or urea diet. Results are shown as mean and SEM. B) Relationship between plasma BUN and blastocoele fluid urea nitrogen concentration in control and urea mares after oral urea treatment. Results are shown as mean. C) Progesterone concentrations of mares with recovered embryos receiving a control or urea diet, with the exception of the control mare that had twin embryos. Results are shown as median and range. D) Embryo area calculated as an ellipse from Day 11-14. Results are shown as median and range.

4.4.3. Embryo parameters

There was no difference between the recovery or not of an embryo in the control and urea treated groups (P = 0.71). There was an effect of day (P < 0.001) but no effect of group (P = 0.14) or group by day interaction (P = 0.99) for the embryo area as an ellipse calculated from D11-D14 (Figure 4.2). There was no difference between the urea and

control group for: osmolality (P = 0.73) with 132 mOsm (117-171) and 139 mOsm (118-196), for control and urea, respectively or pH (P = 0.56) with 8.46 pH (7.82-8.81) and 8.54 pH (7.89-8.88), for control and urea, respectively of the blastocoele fluid on D14.

Blastocoele fluid urea nitrogen concentration showed a tendency to be higher in embryos from the urea group when compared to the control group, 22.84 ± 2.97 and 16.81 ± 1.65 mg/dL, respectively (P = 0.10). The Pearson's correlation analysis showed a strong positive relationship between plasma BUN and blastocoele fluid urea nitrogen concentration across all samples (R = 0.68, P = 0.01) (Figure 4.2).

4.4.4. RNA sequencing

Characteristics of the RNA sequencing analysis of the data are summarized in Table 4.2. The average of input reads was 52,169,145; the input read length for paired end reads was 150, and 87% of uniquely mapped reads were obtained for the samples sequenced.

Table 4.2 Summary of RNA sequencing data for 12 embryo samples.

Sample	Group	Number of input reads	Uniquely mapped read number	Uniquely mapped reads
1	Urea	55,301,214	48,678,110	88.02%
2	Urea	58,619,132	50,633,657	86.38%
3	Urea	48,519,308	41,477,499	85.49%
4	Urea	51,559,296	44,983,757	87.25%
5	Urea	52,999,962	46,225,781	87.22%
6	Control	49,692,326	44,014,661	88.57%
7	Control	51,049,824	44,759,739	87.68%
8	Control	51,136,796	44,991,192	87.98%
9	Control	46,088,700	40,275,043	87.39%
10	Control	56,581,538	48,557,995	85.82%
11	Control	55,379,410	47,879,277	86.46%
12	Control	49,102,238	41,994,083	85.52%

4.4.5. Differentially expressed genes

A total of fourteen genes were differentially expressed in the embryos from the urea and control groups. Ten genes were upregulated and four genes were downregulated in the urea group in comparison to the control group. The genes that were uncharacterized for *Equus caballus* in the NCBI database had their nucleotide sequence (FASTA format) identified in the NCBI database (http://www.ncbi.nlm.nih.gov/), then Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST) (Camacho, Coulouris et al. 2009) identified their orthologs in other species (*Bos taurus*, *Equus przewalskii*) (Table 4.3).

Table 4.3. List of differentially expressed genes of embryos between the urea and control-treated mares.

Gene name	Gene symbol	Orthologous gene (Species)	Accession number	Log2 (Fold Change)	P-value	FDR-adjusted p-value
proline rich 35	PRR35		XM_023616733.1	1.028	0.000	0.048
glutathione S-transferase alpha 1	GSTA1		XM_001503029.4	1.108	0.000	0.048
keratin 4	KRT4		NM_001346204.2	1.185	0.000	0.048
JPX transcript	JPX	*Bos taurus*	XR_002805540.1	1.873	0.000	0.048
prostate stem cell antigen	PSCA		XM_001505016.4	2.045	0.000	0.048
sperm-associated acrosin inhibitor	LOC102149479		XM_005599320.3	2.056	0.000	0.048
neurofascin	NFASC		XM_023640364.1	-1.505	0.000	0.048
carbohydrate sulfotransferase 1	CHST1		XM_023653932.1	-1.477	0.000	0.048
serpin family G member 1	SERPING1		XM_001498338.6	-0.750	0.000	0.048
G protein-coupled receptor 155	GPR155		XM_001917170.4	0.619	0.000	0.048
fibrinogen gamma chain	FGG		XM_001914798.5	0.820	0.000	0.048

Table 4.3 (continued). List of differentially expressed genes of embryos between the urea and control-treated mares.

G elongation factor, mitochondrial 1	GFM1	*Equus przewalskii*	XR_002805905.1	0.824	0.000	0.048
proprotein convertase subtilisin/kexin type 1	PCSK1		XM_001504608.5	2.281	0.000	0.083
apelin receptor	APLNR		XM_005598133.3	-1.048	0.000	0.083

4.4.6. Functional analyses

To better understand the effects of a high urea concentration on the embryonic transcriptome a GO analysis with PANTHER was done. The GO terms significantly enriched in the molecular functions by DEG in the ontology were binding, catalytic activity, molecular function regulator, and molecular transducer activity. In the biological processes, the categories that were enriched were biological adhesion and regulation, cellular and metabolic process, and multicellular organismal process. Enriched pathways were related to Alzheimer disease, blood coagulation, endothelin signaling pathway, and plasminogen activating cascade (Figure 4.3).

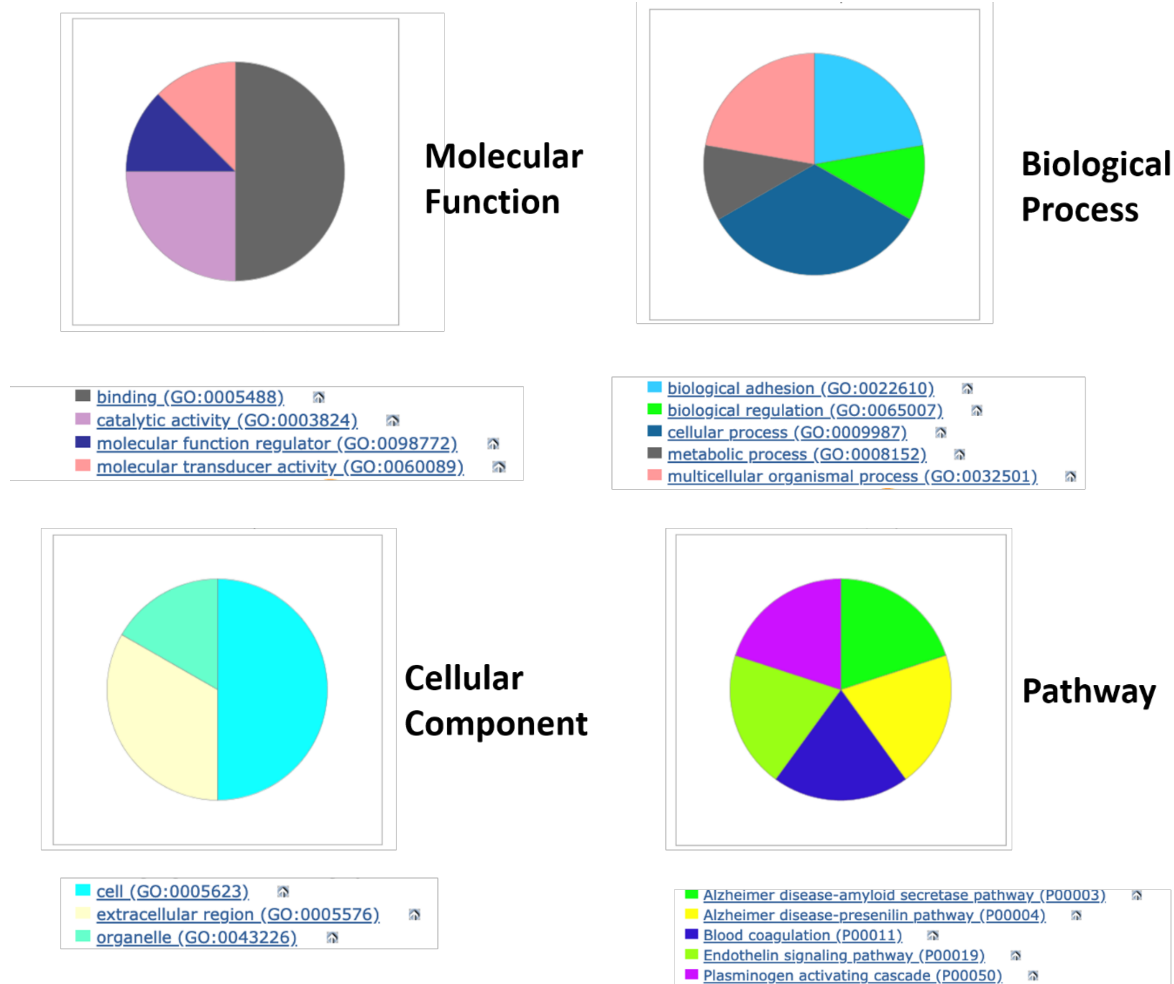


Figure 4.3. Functional annotation analysis of the differentially expressed genes using PANTHER (version 13.1) statistical overrepresentation test. Molecular function, biological process, cellular component, and pathway shown in pie charts identified by the GO terms in different colors.

4.4.7. Quantitative Real-Time PCR

Analysis of the correlation between genes with RT-qPCR (-ΔCT) and the RNA sequencing results (FPKM) showed that three genes had a significant correlation and two had a trend between the two methods (Table 4.4).

Table 4.4 Pearson's correlation of RNA Sequencing (FPKM) and Quantitative Real-Time PCR (-ΔCT) to confirm RNA Sequencing results.

Gene	Correlation (R)	P-value
APLNR	0.560	0.058
GSTA1	0.613	0.034
LOC102149479	0.780	0.003
NFASC	0.350	0.264
PCSK1	0.547	0.066
PSCA	0.490	0.106

4.5. Discussion

Effects of high BUN on D14 equine embryos were identified for the first time. Embryos from urea-treated mares tended to have higher blastocoele fluid urea nitrogen concentration. Genes and pathways related to nervous system development, cell proliferation, endothelial remodeling, detoxification, and adhesion from the treated embryos were changed. The gene ontology analysis of the DEG revealed that blood coagulation and endothelin signaling pathways were enriched. Thus, suggesting for the first time that high concentrations of urea in maternal circulation will have an impact on early equine embryos (D14). Overall, there was a similar pattern of regulation with a significant correlation between –ΔCT and FPKM, showing concordance between RNA Sequence and qPCR results.

Urea is a small molecule that can diffuse through circulation into the maternal reproductive tract; thus elevated BUN in cows and ewes resulted in an increase in intrauterine urea concentration (Fahey et al., 2001; Ellen R Jordan et al., 1983; T. McEvoy et al., 1997). Although the intrauterine urea concentration was not measured in this study, based on the previously mentioned study in ruminants, we can hypothesize that the urea in circulation diffused to the uterus and then to the blastocoele fluid of the early embryo,

leading to a higher blastocoele fluid urea concentration in embryos recovered from urea-treated mares.

The reported relationship between progesterone values and BUN in cows differs between studies. In some investigations, cows with a high BUN had higher progesterone concentrations (Jordan & Swanson, 1979), while other reports showed no difference in progesterone concentrations in relation to BUN (Rhoads et al., 2004). Although the relationship between progesterone and BUN has not been reported in mares, in the current study, an increase in BUN did not result in significant changes in serum progesterone values. What was seen in the present study was a higher progesterone concentration in the mare with a twin pregnancy, as was expected (Urwin & Allen, 1983). Additionally, there was no correlation between BUN and progesterone. Our results on relationship of BUN and progesterone values, indicate that in mares, an increase in BUN was not associated with an increase in peripheral progesterone.

In the current study, urea-treatment altered expression of genes related to neurological and brain development on D14 embryos. For example, the G protein-coupled receptor 155 (*GPR155*) has an important role in the brain, possibly by acting in the limbic system (Nishimura et al., 2007). Also, neurofascin (*NFASC*) was observed in the embryonic mouse hindbrain, being responsible for clustering voltage-gated sodium channels to form the node of Ranvier, an axonal domain in myelinated nerves that allow for rapid nerve conduction (A. Zhang et al., 2015). On the other hand, the oral urea treatment altered the expression of serpin family G member 1 (*SERPING1*). The *SERPING1* binds and inhibits the three activation arms of the complement system: the classical pathway, the lectin pathway, and the alternative pathway (Gorelik, Sapir,

Woodruff, & Reiner, 2017). Additionally, *SERPING1* is important for the proliferation of neuronal stem cells in mice during cerebral cortex development (Gorelik et al., 2017), the current urea-treatment resulted in a downregulation of this gene related to neurological embryo development which might lead to developmental impairment of the D14 embryos. The change in expression of these genes, involved in neurological processes, after urea-treatment on D14 embryos, will likely result in an abnormal development of the nervous system, which starts at day 20 in equine embryos (Franciolli et al., 2011).

As the early embryo is in a stage of intense and precise cell division and proliferation, changes to genes related to cell division can have serious consequences. For instance, prostate stem cell antigen (*PSCA*) is used as a molecular marker for abnormal cell proliferation and differentiation in cancers (Raff, Gray, & Kast, 2009). *PSCA* was upregulated in embryos from urea-treated mares, indicating an alteration in the normal cell division process that happens in D14 embryos.

The apelin receptor gene (*APLNR*) is a G-protein-coupled receptor highly expressed in vasculature. *APLNR* modulates the polarization and vascular remodeling of endothelial cells in zebrafish and humans during development (Kwon et al., 2016). More specifically, *APLNR* was required for proper cardiac development in zebrafish (Deshwar, Chng, Ho, Reversade, & Scott, 2016). Thus, the identification of *APLNR* as a downregulated gene in the urea-treated embryos, might have negative effects in the cardiac development, which is physiologically observed in equine embryos at 16 days (Franciolli et al., 2011).

Three polypeptide chains (Aα/Bβ/Gγ) form fibrinogen, which is the precursor of fibrin, essential for the hemostatic process (Kant et al., 1985). Thus, the identification of

fibrinogen gamma chain (*FGG*) as an upregulated DEG in our dataset, as well as the enrichment of the blood coagulation and plasminogen activating cascade pathways, indicate that the oral urea treatment resulted in a disruption in the coagulation properties of the early equine embryo. Additionally, the embryo derived *FGG* is involved in the adhesion of the conceptus to the endometrium in mares (C. Klein, 2016; C. Klein et al., 2010); the change in the expression of *FGG* associated with the high BUN might indicate that it will interfere with normal fixation of the urea-treated embryos to the endometrium.

Glutathione S-transferase A1 (*GSTA1*) was upregulated in our dataset, and it encodes enzymes responsible for adding glutathione to products of oxidative stress, thus detoxifying these compounds and protecting cells from reactive oxygen species and the products of peroxidation (J. D. Hayes & McLellan, 1999; Raza, 2011). In preimplantation development of in vitro-matured/in vitro-fertilized bovine embryos, the intracytoplasmic concentration of glutathione increased during development, showing that it plays an important intracellular role at specific stages for bovine embryos (Lim, Liou, & Hansel, 1996). Additionally, in mice glutathione increased the antioxidant ability of embryos (Nasr-Esfahani, Aitken, & Johnson, 1990). The upregulation of *GSTA1* in the embryos from urea-treated mares might be due to the increase in urea concentrations in the blastocoele fluid, which would lead to oxidative stress; therefore, the antioxidant effects of glutathione could be an important attempt of the embryo to compensate for changes caused by the urea treatment.

Our results have characterized the effects of systemic high BUN on equine embryos at D14. A trend for higher urea nitrogen concentrations in the blastocoele fluid of embryos from mares with a high BUN was seen, along with transcriptomic changes

related to neurological development, cell proliferation, vascular remodeling, embryo adhesion, and detoxification in the D14 embryos. These findings will improve knowledge regarding the effects of high BUN in equine embryonic development. However, whether similar changes to embryos will occur and result in lower pregnancy rates when mares receive a high protein diet needs to be investigated.

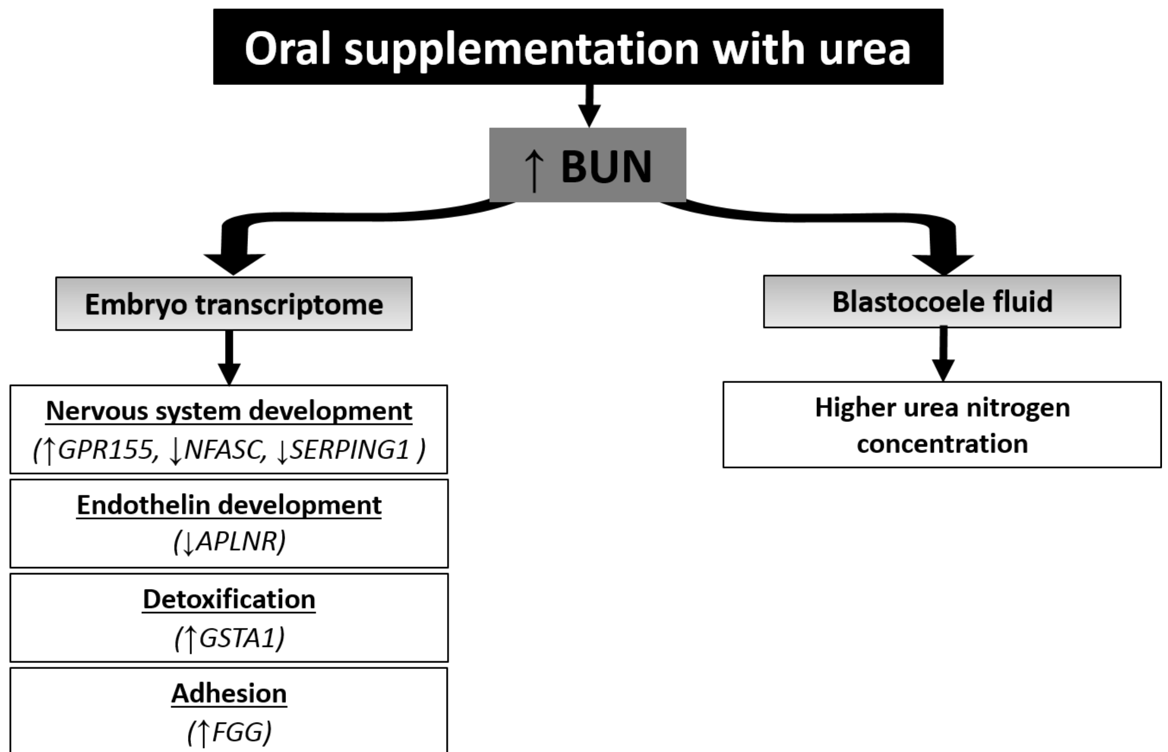


Figure 4.4. Schematic overview of the main findings on equine embryonic transcriptome after oral urea treatment in mares. BUN- blood urea nitrogen.

CHAPTER 5. Relationship of urea nitrogen concentrations on follicular fluid and embryos from mares

5.1. Abstract

High blood urea nitrogen values (BUN) have been related to adverse effects in embryos from cows and ewes; however, this phenomenon has never been reported in mares. The current study aimed to investigate the relationship between peripheral BUN and oocyte environment (follicular fluid) during follicle growth and to study the impact of BUN on pregnancy outcome of equine embryos. In experiment one, we collected follicular fluid and blood samples from mares with growing follicles (15-20 mm) and pre-ovulatory follicles (35 mm) to investigate the relationship between BUN and follicular fluid urea nitrogen (FUN). In a second experiment, blood urea nitrogen concentrations of embryo donor mares were evaluated to determine the relationship between BUN and pregnancy rates after embryos were transferred to recipient mares. In experiment one, there was a strong positive correlation between BUN and FUN ($R=0.83$; $P<0.0001$), with a higher BUN from mares with growing compared to preovulatory follicles ($P=0.004$) and higher FUN in growing compared to preovulatory follicles ($P=0.031$). In experiment two, there was an effect of BUN ($P=0.02$) and of age ($P=0.01$) for pregnancy outcome. Additionally, there was a high area under the curve (0.74) calculated with a receiver operating characteristic analysis. Therefore, these experiments showed that BUN had an effect on the follicular fluid environment and on pregnancy rates of embryos collected from mares with high BUN.

KEYWORDS: High protein diet, horses, pregnancy rates, embryonic development.

5.2. Introduction

Maternal diet may have an important effect on embryo development and pregnancy establishment. For example, a high protein diet is associated with high blood urea nitrogen (BUN) concentrations in cows and ewes that has detrimental effects to early oocytes, embryos, and pregnancy rates (Butler, 2000b; C. Elrod et al., 1993; Ferguson et al., 1993; T. McEvoy et al., 1997). A high urea concentration during *in vitro* oocyte maturation resulted in more bovine degenerated ova and a decreased number of oocytes that developed into blastocysts after *in vitro* fertilization (D. Hammon, Wang, & Holyoak, 2000; Ocon & Hansen, 2003). Additionally, high BUN *in vivo* in cows and ewes resulted in a reduction of oocyte cleavage and blastocyst development and an overall 20% reduction in pregnancy rates at 40-50 days after breeding (W. R. Butler et al., 1996; D. Hammon et al., 2000; Sinclair, Kuran, Gebbie, Webb, & McEvoy, 2000).

The effects of a high BUN on embryos is not fully understood, but as urea is a small molecule that can move between cellular compartments through diffusion (Jozwik et al., 2006), a high BUN is believed to elevate tissue concentrations of urea nitrogen in the reproductive tract (Collins et al., 1997; D. S. Hammon et al., 2005; Jozwik et al., 2006; Nandi et al., 2007). Follicular fluid urea nitrogen (FUN) concentrations are correlated with BUN in women (Jozwik et al., 2006), cows (D. S. Hammon et al., 2005), ewes (Nandi et al., 2007), and mares (Collins et al., 1997). Follicular fluid is composed of secretions from theca and granulosa cells, and from plasma transudate; including electrolytes, proteins, hormones, and water (Rodgers & Irving-Rodgers, 2010). This composition is an important factor, as it is in direct contact with the oocyte-cumulus complex before ovulation (Jozwik

et al., 2006; Nandi et al., 2007). Follicular fluid composition changes as follicles grow (Leroy et al., 2004), possibly due to an increase in follicular vascularity (Acosta et al., 2005; Gastal et al., 2007), a dilution caused by an increase in follicular fluid volume (Nandi et al., 2007), and an increase in the blood-follicle barrier permeability (Bagavandoss et al., 1983).

Moreover, an increase in BUN caused a decrease in uterine pH and an increase in urea nitrogen concentrations in the uterus of cows (C. Elrod et al., 1993; D. S. Hammon et al., 2005). Additionally, a high BUN caused changes in the endometrial transcriptome of mares (Chapter 2 and 3). These changes in the intrauterine environment could also affect early embryos when they enter the uterus, as they are dependent upon the uterine histotroph for nutrition (C Klein & Troedsson, 2011). Although high urea nitrogen concentrations will cause changes in the follicular fluid, in the uterine environment, and on endometrial transcriptome, no studies have investigated the effects of elevated BUN on developing oocytes and embryos in mares.

Our hypothesis was that there would be a positive linear relationship between the BUN and FUN and that high BUN concentrations in embryo donor mares would be associated with a decreased pregnancy rate after embryo transfer to recipient mares. The objectives of this study were to: 1) evaluate the relationship between BUN and follicular fluid urea nitrogen, 2) investigate the follicular fluid urea nitrogen concentrations in growing and dominant follicles, and 3) evaluate the relationship between BUN in embryo donor mares at the day of ovulation and subsequent embryo survival after transfer to recipient mares.

5.3. Material and methods

5.3.1. Experiment 1: Relationship between plasma and follicular fluid urea nitrogen concentrations

The current experiment aimed to study the relationship between concentrations of urea nitrogen in peripheral blood and in follicular fluid. All animal procedures in this experiment were performed in compliance with the animal use protocol approved by the Institutional Animal Care and Use Committee at the University of Kentucky (#2010-067). Clinically healthy mares of mixed breeds, with mean age of 15.08 ± 1.55 years were used in this study. All mares underwent a reproductive evaluation and transrectal ultrasonography for reproductive tract evaluation (vulva, cervix, uterus and ovaries) before the experiment. Researchers only used mares with no detectable abnormalities of the reproductive system. Mares were kept at the University of Kentucky's Farm on grass pasture with grain supplementation and access to water and trace mineralized salt *ad libitum*.

Experimental procedures were previously reported (Claes et al., 2016). In brief, animals were examined by transrectal ultrasonography (Sonoscape S8, Universal Medical Systems Inc., Bedford Hills, New York). Beginning at ovulation, two dimensions (width and height) of the six largest follicles were recorded on an ovarian map every other day to track follicular waves. When follicular growth was identified, ultrasonographic examinations were performed daily until ovaries were collected for subsequent follicle isolation.

A blood sample was collected in heparinized tubes (BD Vacutainer, Franklin Lakes, NJ) by venipuncture at the day of tissue collection and kept on ice until centrifugation at 1200x *g* for 10 min and storage at -20°C until analysis for BUN.

Mares were euthanized either before follicle deviation (n = 16 mares) when follicular growth was identified and the largest growing follicle reached a diameter of 15–20mm or during estrus (n = 10 mares) when endometrial edema was present and the dominant follicle reached a diameter of 35-mm. Ovaries were removed after euthanasia and kept on ice. Follicular fluid was aspirated without blood contamination, snap frozen, and stored at -80°C until further analysis (Claes et al., 2016).

5.3.1.1. Urea nitrogen concentrations

Urea nitrogen concentrations in plasma (BUN) and follicular fluid (FUN) were measured with a colorimetric spectrophotometric assay following an adapted protocol described previously (Mok et al., 2018). The standard curve ranged from 5.6 mg/dL to 56.01 mg/dL. All reagents for this assay were purchased from Sigma-Aldrich (St. Louis, MO). Urea was diluted (8M after reconstitution with 16 mL high purity water) to 5.6 mg/dL and 56.01 mg/dL to be used as low and high controls. The reaction consisted of analyzing urea by enzymatic hydrolysis to ammonia at room temperature. The reaction (in duplicate) was done in microcentrifuge tubes (2 mL) with 10 µL of each plasma sample, and 125 µL urease buffer was added with incubation of the samples for 20 minutes. The urease enzyme hydrolyzes urea to produce carbon dioxide and ammonia (CH_4N_2O + Urease buffer → $CO_2 + 2NH_3$). Then, 250 µL of phenol nitroprusside solution, 250 µL of alkaline hypochlorite solution (0.2%), and 1000 µL of distilled water were added (NH_3 +

phenol nitroprusside + alkaline hypochlorite + H_2O → Indophenol blue) (Tabacco & Meiattini, 1985). After a 25-min incubation, a 200-µL aliquot was transferred to a 96-well plate and absorbance (570 nm) was determined with an Epoch microplate spectrophotometer (Biotek, San Francisco, CA). The intra- and interassay coefficients of variation for BUN concentrations were 0.5% and 9.8%, respectively. The lower limit of detection of the assay was 0.11 mg/dL.

5.3.2. Experiment 2: Pregnancy rates from embryos collected from donor mares with an elevated BUN

The main aim of this experiment was to evaluate the relationship of BUN in embryo donor mares at the day of embryo recovery (D7 or D8) with subsequent embryo survival after transfer to synchronized recipient mares. Clinically healthy mares (median age: 14 years; age range: 3-25 years) from an embryo collection breeding program were used in this study. All mares were visually examined, palpated and transrectal ultrasonography was done for reproductive tract evaluation before the experiment. Donor mares (n=170 mares) were kept at different private farms, on grass pasture with grain supplementation, and access to water, and trace mineralized salt *ad libitum*. A total of 20 donor mares were lactating at the time of sample collection. Mares were presented to an equine hospital for embryo collection. Recipient mares were all kept at the equine hospital (with the exception of three animals kept at private farms) and received grass pasture with grain supplementation and access to water and trace mineralized salt *ad libitum.*

Embryo donor mares were examined by transrectal ultrasonography (Sonosite M-Turbo; SonoSite, Inc., Bothell, WA) and when a preovulatory follicle and uterine edema

were observed, mares were artificially inseminated. Ultrasound examinations were performed until ovulation detection (D0). Embryo recovery was performed by nonsurgical uterine lavage at D7 or D8, as previously described (Jacob et al., 2012). Blood samples were collected from donor mares immediately before embryo recovery, and blood samples were centrifuged at 1200 x *g* for 10 min and stored at -20°C until analysis for BUN

Recipient mares (n=88 mares) had their estrous cycles monitored by transrectal ultrasonography to synchronize ovulation with donor mares, such that recipient mares ovulated between one day before and two days after the donor mare. Mares were selected as recipients according to their uterine echodensity and uterine and cervical tone at the day of embryo transfer (Jacob et al., 2012). Embryos were transferred using a nonsurgical embryo transfer technique. Ultrasonographic exams on recipient mares for pregnancy checks were done at D14, and pregnancy status was recorded.

5.3.2.1. Statistical analyses

The BUN and FUN were tested for normality with a Shapiro-Wilk test and were normally distributed. Standard Least Squares with FUN and BUN for follicle type and mare as random effects were done. The relationship between BUN and FUN values was examined with a Pearson's correlation analysis.

The BUN values for donor mares were also tested for normality with a Shapiro-Wilk test and were normally distributed. A nominal logistic fit for pregnancy outcome was used considering the effects of BUN, age, lactational status and the interaction between lactation and age. A receiver operating characteristic (ROC) analysis was done to evaluate the accuracy of the BUN values in predicting the pregnancy outcome through the area

under the curve (AUC). Additionally, a Standard Least Squares model of the donor mares BUN was done, with age, lactational status, pregnancy outcome and interaction between lactation and age and between pregnancy and age as effects. A reduced model with lactation and pregnancy outcome was used, as there were no effects for age or the interactions.

Data was reported as mean ± SEM. Significance was set at $P < 0.05$ and trend at $0.1 > P > 0.05$. JMP Pro (version 14; SAS Institute, Cary, NC, USA) was used for all statistical tests.

5.4. Results

5.4.1. Experiment 1: Relationship between plasma and follicular fluid urea nitrogen concentrations

BUN concentrations from mares with growing follicles were higher when compared to mares with preovulatory follicles (17.42 ± 0.60 and 14.35 ± 0.78 mg/dL, $P = 0.0045$). Similarly, FUN concentrations from growing follicles were higher compared to FUN concentrations in preovulatory follicles (17.36 ± 0.54 and 15.13 ± 0.88 mg/dL, $P = 0.031$). BUN was significantly and positively correlated with FUN ($R = 0.83$; $P < 0.0001$) (Figure 5.1).

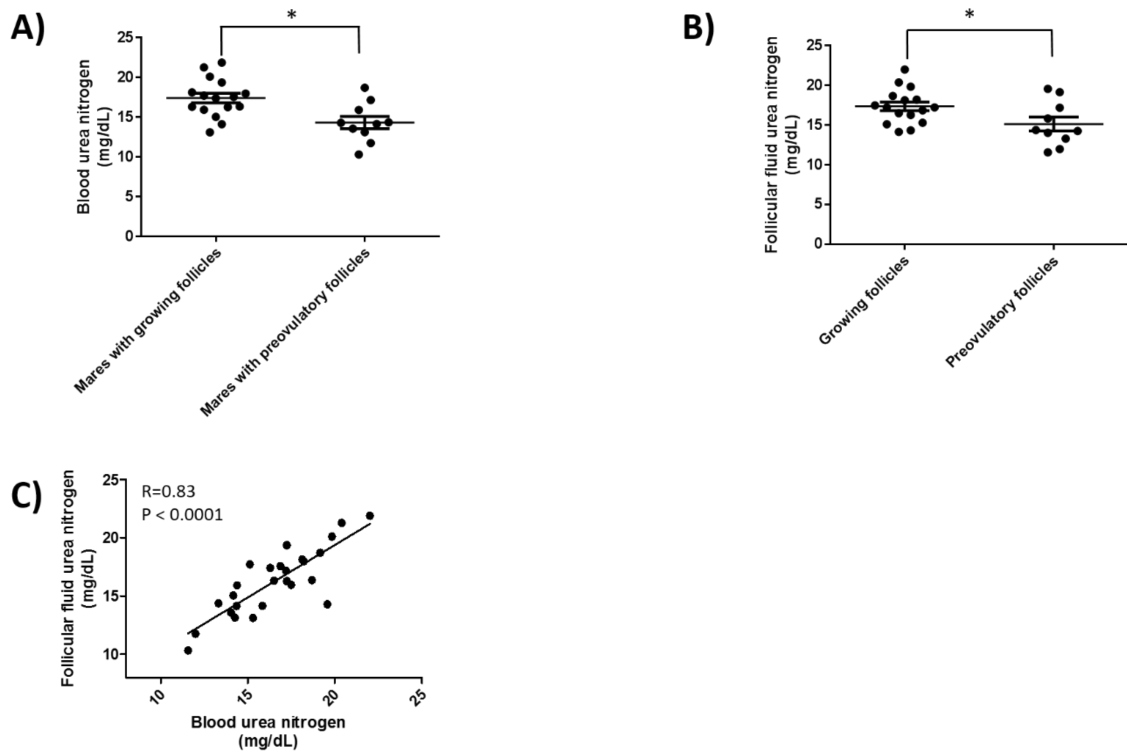


Figure 5.1. A) Blood urea nitrogen concentrations (mg/dL) from mares with growing follicles (15–20 mm) or pre-ovulatory follicles (35 mm). B) Follicular fluid urea nitrogen concentrations (mg/dL) in mares with growing follicles (15–20 mm) or pre-ovulatory follicles (35 mm). C) Correlation between the follicular fluid and plasma urea nitrogen concentrations (mg/dL) from mares. Middle horizontal lines represent mean and error bars represent standard error of the mean. * P < 0.05.

5.4.2. Experiment 2: Pregnancy rates from embryos collected from donor mares with an elevated BUN

There was a 52% embryo recovery rate, with 88 embryos recovered from donor mares and subsequently transferred to synchronized recipient mares. There were 71 (81%) recipient mares that became pregnant after embryo transfer, compared to 17 (19%) recipient mares that were not pregnant at D14.

With the nominal logistic fit for pregnancy outcome there was a significant effect of BUN (P= 0.02), age (P = 0.01) and interaction between lactation and age (P = 0.009).

The effect of lactation was not significant (P = 0.50). The ROC analysis showed an AUC of 0.74.

Eight donor mares had double ovulations and had two embryos recovered. Each embryo was transferred to a different recipient mare. Twin embryos from six donor mares had the same pregnancy outcome, embryo transfer to two of the donor mares resulted in one pregnant recipient and one non-pregnant recipient. Therefore, we designed the Standard Least Squares model of donor mares BUN using each embryo as an experimental unit. There was a trend for lactation (P = 0.05) and a significant effect of pregnancy outcome (0.03). Donor mares that were lactating had a higher BUN mean concentration than those that were not lactating, 19.71 ± 0.64 mg/dL and 17.53 ± 0.31 mg/dL, respectively (Figure 5.2). Also, the mean BUN from donor mares that had embryos which resulted in positive pregnancies in the recipient mares was lower when compared to those that had embryos that did not result in pregnancies at D14, 17.16 ± 0.43 mg/dL and 19.46 ± 0.83 mg/dL (Figure 5.2).

Figure 5.2. A) Blood urea nitrogen concentrations (mg/dL) from embryo donor mares that were not lactating and from mares that were lactating. B) Blood urea nitrogen concentrations (mg/dL) from embryo donor mares that underwent embryo flushing related to pregnancy outcome in recipient mares (non pregnant or pregnant). Horizontal lines represent mean, whiskers represent minimum and maximum values * $P < 0.05$.

5.5. Discussion

There was a strong positive relationship between urea nitrogen concentrations in follicular fluid and plasma from cyclic mares. Moreover, it showed that FUN had a higher concentration in growing follicles when compared to preovulatory follicles. In experiment two, embryos recovered from donor mares with higher BUN resulted in lower pregnancy rates after transfer to recipient mares. Together, the current findings might suggest an influence of elevated systemic urea nitrogen concentrations on follicular fluid and pregnancy outcome in mares.

The higher concentrations of BUN when mares had a growing follicle compared to mares with a pre-ovulatory follicle might be related to changes in BUN during the estrous cycle. Both ovarian steroids, progesterone and estradiol, fluctuate during the estrous cycle thus affecting liver metabolism (Kuhl, 1990; Kushwaha, Guntupalli, Jackson, & McGill, 1996; O'Donohue & Williams, 1997). As the liver also metabolizes protein and urea (Reitnour, 1978; Reitnour et al., 1970), the changing concentrations of ovarian steroids during the estrous cycle might affect BUN concentrations. In ewes, the BUN values were higher during diestrus when compared to estrus, 28.5 ± 0.76 mg/dL and 22.0 ± 0.97 mg/dL, respectively (Singh & Dutt, 1974). There was a similar result in the current study, with higher BUN when there were follicles with 15-20-mm diameter when compared to 35-mm diameter follicles in mares. In female water buffaloes, there was no difference between BUN during the estrous cycle (Baki Acar et al., 2013), indicating that

there might be differences in BUN during the estrous cycle between species that need further investigation.

Values of FUN were also higher in growing follicles than in the pre-ovulatory follicles from the mares observed. In agreement to our study, there was a tendency for the FUN to decrease as follicles from mares grew (Collins et al., 1997). Studies with cows and ewes also presented a decrease in FUN as the follicles grew (Leroy et al., 2004; Nandi et al., 2007). However, there was no difference in FUN during the estrous cycle in buffaloes (Baki Acar et al., 2013; Tabatabaei & Mamoei, 2011). This decrease in FUN as follicles grow in most species, might be due to the negative effects of higher concentrations of urea in follicular fluid to the development of oocytes (D. Hammon et al., 2000; Ocon & Hansen, 2003; Sinclair et al., 2000), so that as follicles approach ovulation there would be a decrease in FUN. One of the mechanisms responsible for this decrease in urea nitrogen concentrations can be the increase in vascularity as follicle grows (Acosta et al., 2005; Gastal et al., 2007) by increasing the elimination of urea through the follicle wall to the exterior environment. Other ways that the FUN can change, might be through a dilution of the follicular fluid as it increases in volume during follicular growth and/or a higher permeability of the blood-follicle barrier in larger follicles (Bagavandoss et al., 1983; Nandi et al., 2007).

Not surprisingly, there was a strong positive correlation between the FUN and BUN, as has been shown in mares (Collins et al., 1997), women (Jozwik et al., 2006), cows (D. S. Hammon et al., 2005; Leroy et al., 2004), ewes (Nandi et al., 2007) and female buffaloes (Baki Acar et al., 2013). Because urea is a small molecule, it diffuses across

tissues (Jozwik et al., 2006), resulting in this high correlation between the urea concentrations in follicular fluid and systemic circulation.

Our study found a difference in the mean age of donor mares with embryos that resulted in pregnancy. This is similar to what has been previously shown, with older mares having embryos with a lower morphological quality that resulted in lower pregnancy rates, when compared to younger mares (Carnevale, Griffin, & Ginther, 1993). Also, lactating mares had a higher BUN concentration, possible because recommendations of crude protein for mares during lactation are higher when compared to non-lactating mares (NRC, 2007). The early exposure of the oocyte and embryo to higher urea concentrations did have deleterious effects on embryonic development, with lower pregnancy rates observed with embryos collected from donors with high BUN. Similar deleterious effects of high urea concentrations on embryo development have been reported in ewes, such as a reduction in mean number of cells per embryo recovered at day 4 from animals with elevated BUN (Fahey et al., 2001; T. McEvoy et al., 1997). Cows with high BUN also had a lower percentage of recovered embryos with good morphological quality (Dawuda et al., 2002). An interesting finding in this experiment was the high area under the curve value (0.74), indicating that the increase of donor mares BUN is related to the decrease of pregnancy rates in recipient mares. Overall, these findings indicate that BUN from donor mares at the day of embryo collection was associated with subsequent embryo survival with a lower pregnancy rate in recipient animals at D14.

In conclusion, these experiments show for the first time that there is a negative effect of high BUN on early embryos from mares. Higher systemic urea nitrogen concentrations and follicular fluid urea concentrations are positively related, and embryos

collected from donor mares with a higher systemic urea nitrogen concentration resulted in a lower pregnancy rate in recipient mares.

CHAPTER 6. General conclusions

The studies presented in this dissertation evaluated the effects and relationship of high blood urea nitrogen (BUN) concentrations on follicular fluid, uterine environment, endometrium, and embryos in mares. We used a clinical approach and molecular techniques to identify how elevated BUN can affect reproductive functions in mares. More specifically, transcriptomic analyses identified genes of interest in the endometrium and embryos from mares with elevated BUN concentrations compared to controls. The observed disruption in the mRNA expression of these genes, might help elucidate mechanisms through which urea changes reproductive functions in mares. Additionally, clinical studies showed alterations in follicular fluid urea concentrations during follicular growth, identifying higher concentrations in smaller follicles and a strong relationship between follicular fluid urea concentration and BUN in mares. Finally, BUN was higher in donor mares whose embryos failed to establish pregnancies subsequent to embryo transfer to recipient mares.

We developed two experimental methods to serve as a high protein diet model. The first was an intravenous acute urea-treatment over 6 hours, and the other was an oral chronic urea-treatment over several days. As these methodologies resulted in a significant increase in BUN, they appear to be effective methodologies to study the effects of elevated BUN on reproductive functions in mares.

It is important to remember that using urea-treatments as model for a high protein diet does have limitations. As urea is only one of the metabolites of protein digestion, these treatments do not completely identify the effects that a high ingestion of protein might have on the reproductive tract function and reproductive functions in mares. However,

urea-treatment has the advantage of elevating BUN while providing the same energy content, which can be difficult to achieve with diets that contain different concentrations of dietary crude protein. Another limitation of this dissertation is that other conditions can be responsible for elevation of systemic BUN values other than a high protein diet or urea-treatment. For instance, renal disease results in an elevation of BUN due to an impaired capacity of the kidney to eliminate urea from circulation through urine. Also, when animals are dehydrated an elevation of BUN can occur, as there will be a reduction of fluid volume to eliminate urea and other waste products. Additionally, when animals are in a negative energy state and start losing weight going into a catabolic state, with skeletal muscle breakdown, there is an elevation of BUN. Therefore, the current findings are the first to elucidate the effects of an elevation of BUN on reproductive function of mares; however, additional studies with larger number of mares done in a field study setting would be beneficial.

Although both urea-treatments elevated BUN, results were considerably different. The acute increase of BUN resulted in a larger number of differentially expressed genes from the endometrium between the urea and control groups when compared to the chronic model. Furthermore, only mares treated with an intravenous administration of urea had a detectable decrease in uterine pH. However, there were a group of genes differentially expressed in both studies, mainly related to abnormal cell growth and cell migration. We believe that the more chronic BUN increase allowed the endometrium to compensate and reestablish a more normal physiological environment.

More specifically, when mares had an acute BUN increase, changes involved alterations in genes related to pH of cells. After this urea-treatment, the expression of

solute carriers, sodium channels, ion channels, and enzymes were changed leading to modifications of the normal uterine environment. There was also a change in the expression of genes involved in fatty acid synthesis, which are important in the composition of cell membranes and are precursors of steroid and eicosanoid synthesis. Although not tested in this study, such changes will likely result in a less suitable environment for pregnancy development; thus, resulting in lower pregnancy rates and/or early embryonic losses in broodmares that have sudden diet changes resulting in an elevated BUN.

Furthermore, the oral urea-treatment resulted in changes to genes related to necrosis, due to the oxidative stress properties of urea, and genes that disrupt the basement membrane of cells, allowing the transport of macromolecules between cellular components. Additionally, there were changes in genes involved with fatty acids synthesis, possibly indicating a different concentration of fatty acids in the endometrium of the treated mares. This chronic treatment would be more similar to animals receiving a high protein diet over a longer period of time, which probably would allow the endometrium of the mare to regain homeostasis.

Moreover, high BUN also resulted in transcriptomic changes in day-14 embryos. The developing embryos had variations in a gene related to adhesion of the embryo to the endometrium, therefore having a significant importance at this early stage of development. There were also alterations in genes associated with cell division, angiogenesis, and cardiac development. We hypothesize that these changes in embryonic transcriptome, due to high BUN, would result in developmental complications leading to early pregnancy loss.

As previously mentioned, our first studies consisted of molecular changes in response to experimental increases in BUN. Hence, we designed the following studies to elucidate clinical aspects of BUN on reproductive functions. We identified a strong relationship between follicular fluid urea nitrogen and BUN in mares. In addition, follicular fluid urea nitrogen was lower in preovulatory follicles than in growing follicles. In another study, embryos collected from donor mares with higher BUN resulted in lower pregnancy rates in recipient mares. We believe that there are detrimental effects to embryo viability when the reproductive tract of mares is exposed to higher urea nitrogen concentrations. These clinical data suggest that different diets and management protocols that mares receive routinely result in a wide range of BUN values, affecting the preovulatory follicular environment as well as the uterine environment as evidenced in the studies presented here; thus, these effects might influence oocytes and developing embryos.

These are the first studies that reported effects of elevated BUN on the reproductive tract in mares. These elevated BUN concentrations caused changes in the mRNA expression in the endometrium and embryos from mares. Additionally, BUN was higher in donor mares whose embryos failed to establish pregnancies subsequent to embryo transfer to recipient mares. Overall, these results show that the effects of a urea-treatment, which will result in high BUN, might have adverse consequences to pregnancy rates in mares.

APPENDIX 1.

Chapter 2- List of the differentially expressed genes in the endometrium of mares from the urea compared to the control group in a crossover design.

Gene name	Gene symbol	Orthologous gene (Species)	Log2(fold_change)	P-value	Adjusted p-value
arylacetamide deacetylase	AADAC		-2.476	0.000	0.010
abhydrolase domain containing 15	ABHD15		-0.966	0.001	0.056
abhydrolase domain containing 17C	ABHD17C		-1.424	0.000	0.031
actin binding LIM protein family member 3	ABLIM3		-1.995	0.000	0.031
acyl-CoA dehydrogenase short/branched chain	ACADSB		0.978	0.001	0.064
acyl-CoA synthetase long chain family member 4	ACSL4		-1.102	0.000	0.018
actin, gamma 2, smooth muscle, enteric	ACTG2		3.964	0.000	0.010
ADAMTS like 2	ADAMTSL2		-2.262	0.001	0.100
alpha-fetoprotein	AFP		INFINITE	0.001	0.067
1-acylglycerol-3-phosphate O-acyltransferase 5	AGPAT5		-0.873	0.001	0.086
aldo-keto reductase family 1 member C23	AKR1C23		INFINITE	0.000	0.010
aldolase, fructose-bisphosphate B	ALDOB		-4.479	0.000	0.010
angiopoietin like 4	ANGPTL4		-1.510	0.000	0.036
aquaporin 5	AQP5		1.075	0.000	0.018
ADP ribosylation factor guanine nucleotide exchange factor 1	ARFGEF1		-0.758	0.001	0.089
Rho GTPase activating protein 5	ARHGAP5		-0.837	0.001	0.067
Cdc42 guanine nucleotide exchange factor 9	ARHGEF9		-0.963	0.001	0.073
AT-rich interaction domain 5B	ARID5B		-1.062	0.001	0.070
antioxidant 1 copper chaperone	ATOX1		0.872	0.001	0.094
ATPase 13A3	ATP13A3		-0.923	0.000	0.018
ATPase H+ transporting V0 subunit a4	ATP6V0A4		-2.171	0.000	0.018
BMP and activin membrane bound inhibitor	BAMBI		-1.972	0.000	0.010
BRCA1 associated RING domain 1	BARD1		-1.103	0.001	0.100

B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	BDP1		-0.775	0.001	0.086
baculoviral IAP repeat containing 3	BIRC3		-0.959	0.001	0.056
BRCA1 interacting protein C-terminal helicase 1	BRIP1		-2.145	0.000	0.010
complement C3-like	C3		-1.274	0.000	0.049
complement component 4 binding protein alpha	C4BPA		-1.833	0.000	0.041
carbonic anhydrase 2	CA2		-2.977	0.000	0.010
Cdk5 and Abl enzyme substrate 1	CABLES1		-2.137	0.000	0.010
calcium/calmodulin dependent protein kinase II beta	CAMK2B	*Homo sapiens*	-2.487	0.000	0.010
caveolin 2	CAV2		-1.125	0.000	0.018
cadherin 16	CDH16		-1.610	0.001	0.060
cilia and flagella associated protein 74	CFAP74		-1.041	0.000	0.052
C-type lectin domain containing 11A	CLEC11A		1.067	0.001	0.067
C-type lectin domain containing 20A	CLEC20A		-3.145	0.000	0.010
cytokine dependent hematopoietic cell linker	CLNK		-4.270	0.000	0.010
CKLF like MARVEL transmembrane domain containing 4	CMTM4		-1.000	0.001	0.094
contactin 1	CNTN1		-2.026	0.000	0.024
cordon-bleu WH2 repeat protein	COBL		-1.157	0.000	0.052
cytoplasmic polyadenylation element binding protein 4	CPEB4		-1.056	0.000	0.010
calcium release activated channel regulator 2B	CRACR2B		0.927	0.001	0.067
CREB3 regulatory factor	CREBRF		-0.814	0.001	0.087
cysteine rich transmembrane BMP regulator 1	CRIM1		-1.143	0.000	0.024
colony stimulating factor 3 receptor	CSF3R		-1.768	0.001	0.098
cytochrome b-245 alpha chain	CYBA		0.892	0.001	0.087
doublecortin domain containing 2	DCDC2		-1.078	0.000	0.010
integrator complex subunit 6-like	DDX26B		-0.921	0.001	0.056

DENN domain containing 4A	DENND4A	-2.420	0.000	0.036
dickkopf WNT signaling pathway inhibitor 1	DKK1	-0.941	0.001	0.087
dihydropyrimidine dehydrogenase	DPYD	-0.975	0.001	0.060
dual specificity phosphatase 9	DUSP9	-3.352	0.000	0.010
EF-hand domain containing 2	EFHC2	-2.092	0.000	0.018
epidermal growth factor	EGF	-3.834	0.000	0.010
ETS homologous factor	EHF	-1.688	0.000	0.010
elongation factor for RNA polymerase II 2	ELL2	-1.000	0.000	0.044
endonuclease domain containing 1	ENDOD1	-1.969	0.000	0.010
ectonucleotide pyrophosphatase/phosphodiesterase 1	ENPP1	-0.913	0.000	0.031
ectonucleotide pyrophosphatase/phosphodiesterase 4	ENPP4	-0.848	0.000	0.052
EPM2A interacting protein 1	EPM2AIP1	-1.276	0.000	0.010
endoplasmic reticulum to nucleus signaling 1	ERN1	-1.157	0.000	0.018
ERBB receptor feedback inhibitor 1	ERRFI1	-0.871	0.000	0.041
ETS variant 1	ETV1	-2.041	0.000	0.044
ETS variant 4	ETV4	-5.332	0.000	0.010
ETS variant 5	ETV5	-1.441	0.000	0.010
fatty acid desaturase 1	FADS1	-1.602	0.000	0.036
fatty acid desaturase 2	FADS2	-0.998	0.001	0.056
family with sequence similarity 81 member A	FAM81A	-2.252	0.000	0.010
FCH domain only 2	FCHO2	-0.877	0.000	0.044
free fatty acid receptor 4	FFAR4	-3.122	0.000	0.041
focadhesin	FOCAD	1.035	0.001	0.087
growth arrest and DNA damage inducible gamma	GADD45G	0.827	0.001	0.073
polypeptide N-acetylgalactosaminyltransferase 15	GALNT15	-2.339	0.000	0.010

polypeptide N-acetylgalactosaminyltransferase 4	GALNT4		-1.353	0.001	0.084
GLIS family zinc finger 3	GLIS3		-1.070	0.001	0.060
geminin coiled-coil domain containing	GMNC		-1.401	0.000	0.018
granulysin	GNLY		-1.136	0.000	0.031
G protein-coupled receptor 152	GPR152		-1.554	0.000	0.010
G protein-coupled receptor 176	GPR176		-2.491	0.000	0.010
homeodomain interacting protein kinase 3	HIPK3		-0.913	0.001	0.084
heparan sulfate-glucosamine 3-sulfotransferase 1	HS3ST1		-1.317	0.000	0.044
heparan sulfate 3-O-sulfotransferase-4	HS3ST4	*Homo sapiens*	-0.952	0.000	0.052
iduronate 2-sulfatase	IDS		-0.816	0.001	0.086
insulin like growth factor binding protein 3	IGFBP3		-1.206	0.000	0.010
insulin receptor	INSR		-1.154	0.000	0.010
insulin receptor substrate 2	IRS2		-1.253	0.000	0.010
integrin subunit beta 8	ITGB8		-2.041	0.000	0.010
inositol 1,4,5-trisphosphate receptor type 1	ITPR1		-0.974	0.000	0.044
joining chain of multimeric IgA and IgM	JCHAIN		-0.945	0.001	0.084
KN motif and ankyrin repeat domains 4	KANK4		-1.838	0.001	0.070
potassium voltage-gated channel subfamily A member 3	KCNA3		-1.854	0.001	0.080
potassium voltage-gated channel subfamily C member 4	KCNC4		-2.445	0.000	0.010
KIAA1217	KIAA1217		-0.956	0.001	0.094
kinesin family member 12	KIF12		-2.167	0.000	0.010
kinesin family member 5C	KIF5C		-1.011	0.000	0.036
keratin 4	KRT4		1.564	0.000	0.010
keratin 78	KRT78		1.066	0.000	0.010
laminin subunit beta 3	LAMB3		-1.178	0.000	0.036
laminin subunit gamma 2	LAMC2		-1.496	0.000	0.024

lipocalin 2	LCN2		-2.985	0.000	0.049
LIF, interleukin 6 family cytokine	LIF		-4.828	0.000	0.010
MHC class I antigen pseudogene	LOC100054536		0.964	0.000	0.024
xanthine dehydrogenase/oxidase	LOC100054688		-0.980	0.000	0.041
proline rich 4 (lacrimal)- PRR4	LOC100066131	*Equus przewalskii*	-1.684	0.000	0.010
gasdermin-C	LOC100068406		-2.597	0.000	0.010
homeobox protein MSX-3-like	LOC100146619		0.973	0.000	0.041
metallothionein-1A-like	LOC100630794		0.914	0.001	0.073
T-lymphocyte surface antigen Ly-9	LOC102147390		-0.850	0.000	0.041
short/branched chain specific acyl-CoA dehydrogenase, mitochondrial pseudogene	LOC102149005		0.998	0.001	0.067
leucine rich alpha-2-glycoprotein 1	LRG1		-3.278	0.000	0.010
leucine rich repeat containing 26	LRRC26		1.467	0.000	0.010
mannosidase alpha class 1C member 1	MAN1C1		-1.631	0.000	0.010
mannosidase alpha class 2A member 1	MAN2A1		-1.206	0.000	0.024
mitogen-activated protein kinase kinase kinase 5	MAP3K5		-1.087	0.001	0.070
mediator complex subunit 13 like	MED13L		-0.998	0.000	0.018
MET proto-oncogene, receptor tyrosine kinase	MET		-1.074	0.000	0.010
methyltransferase like 17	METTL17		0.807	0.001	0.070
membrane metalloendopeptidase like 1	MMEL1		1.111	0.001	0.100
N-acetyltransferase 8B	NAT8B		-2.576	0.001	0.056
N-myc downstream regulated 1	NDRG1		-1.082	0.001	0.073
nuclear receptor subfamily 1 group D member 2	NR1D2		-1.048	0.000	0.010
oxidation resistance 1	OXR1		-0.856	0.001	0.064
pantothenate kinase 3	PANK3		-1.333	0.000	0.010
papilin, proteoglycan like sulfated glycoprotein	PAPLN		-1.107	0.000	0.010

progestin and adipoQ receptor family member 5	PAQR5		-3.706	0.000	0.010
pyruvate dehydrogenase kinase 4	PDK4		-1.401	0.000	0.010
PDZ and LIM domain 3	PDLIM3		-2.099	0.000	0.010
paternally expressed 10	PEG10		-1.028	0.001	0.077
progastricsin	PGC	*Canis lupus dingo*	0.942	0.000	0.049
polyhomeotic homolog 3	PHC3		-0.829	0.001	0.089
pleckstrin homology like domain family B member 2	PHLDB2		-2.082	0.000	0.010
polymeric immunoglobulin receptor	PIGR		-1.328	0.000	0.010
phosphatidylinositol transfer protein cytoplasmic 1	PITPNC1		-1.725	0.000	0.044
phospholipase C beta 1	PLCB1		-1.283	0.000	0.010
phosphatidylinositol specific phospholipase C X domain containing 3	PLCXD3		-1.396	0.001	0.086
PPARG coactivator 1 alpha	PPARGC1A		-2.211	0.000	0.010
protein phosphatase 4 regulatory subunit 4	PPP4R4		-2.053	0.000	0.010
phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 2	PREX2		-1.656	0.000	0.010
prolactin receptor	PRLR		-0.995	0.001	0.080
prostaglandin reductase 1	PTGR1		-1.176	0.000	0.024
RAP1 GTPase activating protein 2	RAP1GAP2		-1.474	0.000	0.024
RAS and EF-hand domain containing	RASEF		-1.939	0.000	0.010
RAS guanyl releasing protein 1	RASGRP1		-1.877	0.000	0.010
retinol binding protein 1	RBP1		1.113	0.000	0.031
ring finger protein 208	RNF208		0.848	0.001	0.092
RAR related orphan receptor A	RORA	*Homo sapiens*	-1.585	0.000	0.010
RP11-401A10	RP11-401A10	*Homo sapiens*	-1.212	0.001	0.100
relaxin family peptide receptor 1	RXFP1		-1.518	0.000	0.010
scinderin	SCIN		-1.674	0.000	0.010
sodium channel epithelial 1 beta subunit	SCNN1B		-1.543	0.000	0.010

sodium channel epithelial 1 gamma subunit	SCNN1G		-1.738	0.000	0.010
short chain dehydrogenase/reductase family 16C member 5	SDR16C5		-1.462	0.001	0.089
short chain dehydrogenase/reductase family 42E, member 1	SDR42E1		-1.054	0.000	0.044
semaphorin 4G	SEMA4G		-1.286	0.001	0.080
serine peptidase inhibitor clade A (alpha-1 antiproteinase, antitrypsin) member 14	SERPINA14		-2.851	0.000	0.010
serpin family B member 5	SERPINB5		-2.064	0.000	0.036
serpin family I member 1	SERPINI1		-4.225	0.000	0.010
serum/glucocorticoid regulated kinase 1	SGK1		-0.939	0.000	0.052
serum/glucocorticoid regulated kinase family member 3	SGK3	*Homo sapiens*	-7.348	0.000	0.010
solute carrier family 25 member 36	SLC25A36		-0.862	0.001	0.067
solute carrier family 37 member 1	SLC37A1		-1.079	0.000	0.018
solute carrier family 45 member 3	SLC45A3		-2.007	0.000	0.010
solute carrier family 52 member 3	SLC52A3		-3.983	0.000	0.018
solute carrier family 6 member 20	SLC6A20		-4.538	0.000	0.010
SLIT and NTRK like family member 4	SLITRK4		-3.646	0.000	0.010
antileukoproteinase	SLPI		-2.955	0.001	0.092
Sp4 transcription factor	SP4		-1.394	0.000	0.018
sphingosine kinase 1	SPHK1		1.107	0.000	0.018
alpha-1-antiproteinase 2-like	Spi2-1		-4.304	0.000	0.010
serine peptidase inhibitor, Kazal type 7 (putative)	SPINK7		2.035	0.000	0.010
SPARC (osteonectin), cwcv and kazal like domains proteoglycan 2	SPOCK2		-0.990	0.001	0.094
spondin 2	SPON2		0.882	0.001	0.089
signal peptide peptidase like 2A	SPPL2A		-1.115	0.000	0.010
sprouty RTK signaling antagonist 2	SPRY2		-1.776	0.000	0.010

sperm specific antigen 2	SSFA2		-0.910	0.001	0.087
ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 1	ST6GALNAC1		-1.499	0.001	0.086
STEAP family member 1	STEAP1		-1.573	0.000	0.024
sulfotransferase 1C4	SULT1C4		0.949	0.001	0.087
synaptotagmin like 5	SYTL5	*Equus asinus*	-1.649	0.000	0.052
transcription factor EB	TFEB		0.894	0.001	0.056
thyroid hormone responsive	THRSP		1.018	0.001	0.077
transducin like enhancer of split 6	TLE6		1.309	0.000	0.049
transmembrane protein 154	TMEM154		-1.045	0.000	0.044
transmembrane serine protease 2	TMPRSS2		-3.022	0.000	0.010
transmembrane serine protease 4	TMPRSS4		-3.654	0.000	0.010
transient receptor potential cation channel subfamily M member 5	TRPM5		1.846	0.000	0.044
tetraspanin 7	TSPAN7		-1.317	0.000	0.010
tubulin tyrosine ligase like 6	TTLL6		-1.879	0.000	0.010
ubiquitin specific peptidase 53	USP53		-0.984	0.000	0.052
villin like	VILL		-1.049	0.001	0.089
V-set and transmembrane domain containing 5	VSTM5		-1.264	0.000	0.018
XK related 5	XKR5		-1.819	0.000	0.010
XK-related protein 5-like	XKR5-like	*Equus przewalskii*	-6.294	0.000	0.010

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Publications

Journal Articles

Boakari, Y. L.; Alonso, M. A.; Riccio, A. V.; & Fernandes, C. B. Are mule pregnancies really longer than equine pregnancies? Comparison between mule and equine pregnancies. Reproduction in Domestic Animals, 2019, Epub ahead of print.

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Book chapter

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