THE EFFECTS OF INJECTABLE TRACE MINERAL SUPPLEMENTS IN DONOR COWS

AT THE INITIATION OF A SUPEROVULATION PROTOCOL ON EMBRYO OUTCOMES

AND PREGNANCY RATES IN RECIPIENT FEMALES

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ABSTRACT

Concentrations of trace minerals within the body are known to impact reproductive processes. Thus, the current study analyzed the effects of using an injectable trace mineral supplement containing selenium, zinc, copper, and manganese during a superovulation protocol on embryo outcomes in donor beef cows and further effects on pregnancy rate in recipient females. We hypothesized that an injectable trace mineral (TM) supplement provided to cows fed to meet known nutrient requirements would increase TM status and influence superovulation, embryo characteristics, and enhance pregnancy rates. Our findings indicate that the injectable TM increased concentration of Se within the liver. However, superovulatory response, embryo production, quality grade, and developmental stage were not influenced by TM status. In addition, embryo treatment did not influence pregnancy rate, gestation length, or calf body weight.

DEDICATION

In memory of Rodney E. Schmidt, for his unconditional help and support throughout my work at

CGREC with a smile on his face. For his friendship and caring, keep looking after us from above

bud.

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LIST OF ABBREVIATIONS

AI ....Artificial insemination

BCS ....Body condition score

BW ....Body weight

BUN ....Blood urea nitrogen

Ca ....Calcium

CGREC ....Central grasslands research and extension center

CIDR ....Controlled internal drug release

CL ....Corpus luteum

°C ....Celsius

cm ....Centimeter

cm^3 ....Cubic centimeter

Co ....Cobalt

CON ....Control

Cu ....Copper

d ....Days

DM ....Dry matter

DNA ....Deoxyribonucleic acid

DPP ....Days post-partum

ET ....Embryo transfer

Fe ....Iron

FTAI ....Fixed-time artificial insemination

g ....Grams

GH ....Growth hormone

GnRH ....Gonadotrophin-releasing hormone

GPX..Glutathione peroxidase

h...Hour

LH ....Luteinizing hormone

I ....Iodine

ICP-MS ....Inductively coupled plasma mass spectrometry

IGF ....Insulin-like growth factor

ITM ....Injectable trace mineral

Kg...Kilogram

mg ....Milligram

mL...Milliliter

Mn..Manganese

Mo..Molybdenum

Na...Sodium

NASEM...National academies of sciences, engineering, and medicine

ng..Nanogram

NEFA ....Non-esterified fatty acid

NRC ....National research council

P ....Phosphorus

Pb ....Lead

PGF$_{2\alpha}$ ....Prostaglandin

ppm ....Parts per million

P4 ....Progesterone

RDP...Rumen degradable protein

RNA ....Ribonucleic acid

S ....Sulfur

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Cow-calf operations within the United States are primarily based on pasture systems, normally in areas not needed or suited for crop production. Thus, they rely on weather conditions (average rainfall and temperature) for beef production (USDA, 2017). The beef industry expects dams to produce a calf per year (USDA, 2017). In pastoral livestock systems, farming is managed to avoid or keep under control fluctuations on the system such as commodities prices and seasonal weather patterns. Approaches such as nutritional and forage management are used to achieve the production goal of a calf per year (Romera et al., 2014).

According to National Cattlemen's Beef Association in 2016, the calf crop in the United States was 35 million head. In addition, from 2016 to 2017 the number of replacement heifers increased 1.3 %. An efficient livestock production allowed the United Sates to be ranked as the biggest beef producer in the world, being responsible for producing 25.2 billion pounds of commercial carcass weight (CattleFax, 2016). Not surprisingly, in 2015 the Agriculture sector (crops and livestock) was responsible for a 5.5 % share to U.S. gross domestic product (USDA, 2017).

Mineral supplementation is an important tool used to foster greater livestock productivity as most of the cow herd grazes pastures or rangelands with very little, if any, grain supplementation. Mineral deficiency in grasses varies from different geographic regions and different stages of plant growth. In a survey reported by NAHMS (1997) in 23 U.S. states, 77.0 % of the forage samples were deficient or marginally deficient in Zn, 66.7 % in Cu, and 69.5% in Se. Minerals can be supplemented in several different ways from mineral blocks to water-soluble products, allowing ranchers several options to provide mineral supplementation.

Adequate nutrition is required throughout reproductive processes; influencing processes ranging from gamete production all the way to fertility of offspring (Robinson et al., 2006). Proper nutritional management, combined with reproductive techniques such as fixed-time AI, embryo transfer, and in vitro fertilization can lead to greater reproductive rates and subsequent genetic improvements (Robinson et al., 2006).

Although it is common in the U.S. for herds to have *ad libitum* (free-choice) access to minerals, the amount that will be absorbed is still unknown due to dietary antagonist and formation of insoluble complexes (Spears, 1996). In addition, a major issue to overcome when supplying free choice minerals is variation in consumption (McDowell, 1985; Tait et al., 1992; Greene, 2000). Mineral deficiency is correlated to reproductive impairments (Rabiee et al., 2010), decreased performance (Spears and Kegley, 2002), and lower immunologic response (Shankar and Prasad, 1998).

An option to avoid poor absorption within the gastrointestinal tract or poor bioavailabity of bound minerals is giving injectable trace mineral supplements (ITM). Past research showed that concentration levels of Se and Cu in the liver were increased for at least 15 d and plasma Zn and Mn were increased for several h after ITM administration (Pogge et al., 2012).

The literature review on the following pages will discuss mineral requirements in beef cattle, impacts of trace minerals on female reproduction, impacts on follicular growth and superovulation protocols, effects on embryonic and fetal development, and adequate nutrition and fetal programming.

1.2. Factors affecting requirements and supplementation methods

According to McDowell (1996), within macrominerals and trace minerals, grazing ruminants are more prone to be deficient in Ca, P, Na, Co, Cu, I, Se, and Zn. Many variables will

influence mineral status under grazing conditions, such as: plant species, water and soil ingestion, forage stage of maturity, and soil characteristics. Older, more acid, and sandy soil formations are more likely to be deficient in trace minerals compared to younger and alkaline geological formation (McDowell, 1992). In addition, concentrations of minerals like Cu, Co, Fe, Se, Zn, and Mo normally decrease in the leaves as plants matures, mostly due to nutrient translocation to the roots and natural dilution process (Reid and Horvath, 1980).

Mineral requirements will vary according to level and stage of production (NASEM, 2016), but commonly Ca and P are the main nutrients driving supplementation strategies. Calcium levels in forages are generally adequate particularly in legume grasses, contrary to P that is the mineral mostly likely to be deficient in grazing ruminants (McDowell, 1996). A balance between these nutrients is required to avoid impaired growth, skeletal malformations, and reduced reproduction efficiency. The optimal ratio (Ca:P) vary from 1.5:1 to 2:1, even though animal performance is normally becomes negatively impacted when the Ca:P ratio exceeds 6:1 (Ward et al., 2005).

Calcium and P importance is due to its high amount within the body composition, mainly in bones where 99% of Ca and 80 to 85% of P are located. Besides, P plays a role from cell wall composition (phospholipids), genetic material (DNA and RNA) to energy metabolism. Because of it broad participation in body processes when supplemented, P is the mineral that provides the greatest economic return (McDowell, 1996). Even though minerals are required in various processes, under grazing conditions, supplementation is justified economically viable when energy and protein in the diet are meeting their requirements (McDowell, 1996).

Minerals can be supplemented through direct and indirect methods; indirect methods would be utilizing improved pasture, soils fertilizers, and altering soil pH. Ranchers can provide direct mineral supplementation through mineral blocks, water-soluble mixes, drenches, oral boluses,

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free-choice supplementation, and injections (Underwood, 1981; McDowell et al., 1992). Specific mineral supplementation programs will vary according to individual production scenarios.

Among all direct methods of supplementation, the most widely used is a free-choice mineral (Greene, 2000), mainly because it is not as laborious and does not require handling animals through a chute as other supplementation practices articulated below. Even though, minerals that are given orally are subjected to negative interactions in the gastrointestinal tract, meaning that the amount that will be absorbed is still unknown due to dietary antagonist and formation of insoluble complexes (Spears, 1996). In addition, free choice supplementation allows consumption variation, which can range from 60 to 330 g per head/d (Tait et al., 1992). Similarly, Coppock et al. (1972) showed a daily consumption variation ranging from 0 to 1 kg of dicalcium phosphate per d by lactating dairy cows. Thus, mineral content in free choice products can be pointless if the supplement is not consumed in the recommended dosage (Greene, 2000). Therefore, regulating consumption turns into a key point. Consumption regulation can be achieved applying intake limiting compounds such as salt, calcium chloride, fats and oils, and phosphoric acid; in combination to physical limiter factors as blocks, tubs, liquids, and different feeders (Kunkle et al., 2000).

Popular beliefs among producers normally confuse pica (depraved appetite), which is the action of ingesting objects that are not commonly present in the diet, with the belief that cattle are able to know which mineral is lacking in the diet (McDowell, 1996). Thus, it is common among producers to erroneously correlate bone consumption with phosphorus deficiency (McDowell, 1996). However, research has shown that most mammals do not have nutritional wisdom, selecting diets according to palatability over nutritional content (Arnold, 1964). Therefore, there is a high need of a close monitoring regarding consumption, as it is common to have animals that will

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underconsume or overconsume the mineral supplement. Based on consumption, producers will be able to adapt the mineral source to supply the requirements of the herd (Greene, 2000).

An alternative for free choice supplements is drenching or oral dosage, the advantage is that each animal is receiving a controlled amount of supplement. While administering drenches or oral doses by producers is a laborious duty it can be conducted at the same time as vaccination or deworming protocols (McDowell, 1996). Oral drenching is known to be efficient when minerals can be stored for long periods in body reserves (e.g. Cu in the liver and Se in the muscle) rather than being readily metabolized (e.g. Co salts; McDowell, 1996).

Injections have the same advantage of drenches; allowing for accurate control of the dosage each animal will receive. In addition, intramuscular injections avoid any antagonist's effect that may occur in the gastrointestinal tract (Suttle, 1986) and fluctuations in the consumption that normally occurs when using free-choice supplements and result in each animal receiving the target amount (Arthington and Swenson, 2004). Researchers utilizing injectable sources of trace minerals (Cu, Se, Zn, and Mn) have shown increased liver concentration of Se and Cu for at least 15 d and increased plasma concentration of Zn and Mn for several h (Pogge et al., 2012).

Oral boluses have been an efficient method to provide the required Cu, Co, Se, and I (McDowell, 1996; Greene, 2000). However, as macrominerals are required in greater amounts, oral boluses can be not applicable because of the size of the boluses required to deliver adequate amounts to meet animal requirements (Greene, 2000). All methods cited above, except free choice supplementation, require handling animals and trained personnel to administer the supplements; nevertheless, they allow precise control of supplements administered to each animal (McDowell, 1996; Greene, 2000; Olson, 2007).

1.3. Trace minerals (TM)

Trace minerals are those that are essential to bodily processes but required in small amounts (lower than 100 mg/kg diet) by several species, including: iron (Fe), zinc (Zn), iodine (I), selenium (Se), manganese (Mn), chromium (Cr), copper (Cu), molybdenum (Mo), fluorine (F), boron (B), cobalt (Co), silicon (Si), aluminum (Al), arsenic (Ar), tin (Sn), lithium (Li) ,and nickel (Ni; Mahan and Escott-Stump, 2005). Among all TM, Cr, Co, Cu, I, Fe, Mn, Mo, Ni, Se, and, Zn are known to play an important role in beef cattle production (NASEM, 2016).

With advances in genetics and cross breeding with continental breeds, trace mineral deficiencies started to be more prominent, mostly leading to reproductive and growth impairments (Corah, 1996). Trace minerals are components of hormones of the endocrine system, enzyme cofactors, and metalloenzymes (NASEM, 2016). Deficiencies in TM can cause decreased growth performance (Spears and Kegley, 2002), impaired enzyme activity (Boyne and Arthur, 1981; Ward and Spears, 1997), effects on embryo development (Hostetler et al., 2003), and decreased immune response (Suttle et al., 1989).

Dairy cows supplemented with trace minerals presented increased leukocyte functions (Gyang et al., 1984; Grasso et al., 1990; Hogan et al., 1990). In addition, Zn and Se status were correlated to neutrophil function and superoxide production in postpartum dairy cows (Meglia et al., 2001; Cebra et al., 2003). In dairy cows, TM supplementation was correlated to increased udder health, leading to a decreased somatic cell count and a decreased incidence of clinical mastitis (Machado et al., 2013).

Deficiencies can be caused either by the lack of TM in the diet (primary deficiency) or by compromised absorption, distribution or retention (secondary deficiency; Olson, 2007). The main cause of secondary deficiencies is an antagonist effect; when one mineral interferes with another.

When beef cattle ingest greater levels of Mo and S, thiomolybdates are formed in the gastrointestinal tract and tissues, this complex binds Cu, making it unavailable to the animal (Suttle, 1991). According to a research from Montana State University, grazing cattle from 9 states in the Midwest region had Cu deficiency, directly affecting performance and immune response, mainly correlated with low Cu intake and formation of thiomolybdate–Cu complexes. (Greene, 2000). In addition, Zn absorption can be compromised by greater levels of dietary Cu (Van Campen, 1969), not allowing Zn to be present in a variety of cellular transduction pathways (Predki and Sarkar, 1992; Pena et al., 1999).

1.3.1. Selenium (Se)

Selenium is a nonmetal element that rarely occurs in its elemental state or as pure ore compounds in the Earth's crust. It is normally found bound with others elements, being characterized as organically bound (e.g. selenomethionine and selenocysteine) or inorganically bound (e.g. sodium selenite or selenate). Selenium and Vitamin E have a close relationship (Wagner, 1988), with Se being responsible for sparing Vitamin E, Se will preserve the integrity of pancreas allowing normal lipid digestion and Vitamin E absorption, and the mineral is required in lower amounts in the presence of the vitamin (Suttle, 2010). Vitamin E will work primarily complementing Se protein duties as the vitamin is a lipid-soluble antioxidant acting in the cell membranes and glutathione peroxidase (GPX) is a water-soluble working primarily as an intracellular antioxidant (Suttle, 2010). In addition, Vitamin E will work as first line of defense against lipid peroxidation, with Se being a second line of defense that destroys peroxides before they can cause cell damage (Suttle, 2010). Ruminants have lower absorption of Se (~34% in sheep) compared to monogastric animals (~ 85% in swine) when Se is administrated orally (Wright, 1966). Lower absorption by ruminants could be due to the formation of insoluble forms in the

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rumen such as elemental selenium or selenides (Cousins and Cairney, 1961; Peterson and Spedding, 1963). In addition, concentration of sulfur is known to impair bioavailabity of Se (Spears, 2003).

Selenium deficiency is correlated to reproductive impairments in the herd and can present as early embryonic loss (Corah and Ives, 1991), retained placentas (Trinder et al., 1969), cystic ovaries (Corah, 1996), weak or silent heat periods (Corah, 1996), and weak newborns calves (Smart et al., 1981). In addition, Se deficiency can cause nutritional muscular dystrophy (white muscle disease), which can lead to degeneration and necrosis in the cardiac and skeletal muscle (Underwood and Suttle, 1999). Moreover, as Se is the component of some metalloenzymes (e.g. glutathione peroxidase and iodothyronine 5'-deiodinase), its deficiency can lead to higher oxidative damage to tissues and increased T4 and decreased T3 concentration in the plasma (Arthur et al, 1988).

Selenium is an important component of proteins as it is constituent of selenocysteine, which is known as the 21st amino acid (Rayman, 2000). Mammals contain around 25 selenoproteins, although a couple still do not have an identified action. Selenoproteins have important enzymatic functions (e.g. glutathione peroxidase, mitochondrial capsule selenoprotein, and selenoprotein P; Rayman, 2000). Their actions are known to be related with proper immune response, antioxidant protection, hormonal production and regulation, and sperm maturation (Rederstorff et al., 2006).

Selenium is required for beef cattle at 0.1 mg/kg dietary DM and in ruminants it will be mostly absorbed by the duodenum with little or no absorption in the rumen or abomasum (NASEM, 2016; Table 1.5). Mineral status can be analyzed either by liver biopsies or blood samples, reference levels are contained in the table below (Table 1.1). In general, Se concentrations

in the liver were greater in the fetus compared to the dam (Gooneratne and Christensen, 1989) and Se concentrations in maternal and fetal tissues are highly correlated (Van Saun et al., 1989). Selenium crosses the placenta barrier in ruminants (Jacobsson and Oksanen, 1966; Koller et al., 1984). Selenium can also be toxic when levels in feedstuff exceed 5 mg/kg dietary DM, presenting symptoms as loss of tail hair, sluffing of hooves, loss of appetite, and death (Pulls, 1988); however, other work with sheep has shown no toxicity symptoms when feeding Se at 15 mg/kg of diet DM from early to late gestation (Neville et al., 2008).

Table 1.1. Classification of selenium status in cattle.

Status	Whole blood (ng/mL)	Liver of adults (µg/g DM)
Deficient	< 200	< 1.25
Adequate	210 to 1,200	1.25 to 2.5
High adequate	> 1,200	> 2.5

Adapted from Kincaid (2000).

1.3.2. Zinc (Zn)

Zinc is considered a transition metal that is normally found bonded with other metals in nature such as Cu and Pb. Like selenium, Zn is an important component of metalloenzymes such as retinene reductase, alcohol dehydrogenase, Cu-Zn superoxide dismutase, carbonic anhydrase carboxypeptidase, RNA polymerase, and alkaline phosphatase (Hambidge et al, 1986).

Zinc deficiency has been related to decreased feed intake, feed efficiency, and growth (Miller and Miller, 1962). In addition, in non-deficient ruminants Zn supplementation has been shown to improve growth rate and reproductive performance (as reviewed by Spears, 1989). Extended gestation periods, dystocia, low birth weight, and weak newborns are also associated with Zn deficiency in mammals (Favier, 1992).

The requirement for dietary Zn is 30 mg of Zn/kg dietary DM to avoid deficiency in beef cattle (NASEM, 2016; Table 1.5). Zinc is mostly absorbed through the abomasum and small

intestine (Miller and Cragle, 1965). Moreover, according to Miller (1975) Zn absorption by cattle is homeostatically controlled based on their requirements, and absorption rates vary from 5 to 40% DI (Hambidge et al., 1986). Liver and plasma samples are commonly used to access Zn status; baseline concentration within the body can vary from toxic to deficient levels (Table 1.2). Plasma concentration of Zn varies significantly by animal age. In newborns, circulating Zn concentrations can be as high as 2.3 µg/mL in plasma whether with 12 weeks of age levels are around 1.2 µg/mL (Kincaid and Hodgson, 1989). Moreover, concentrations over 500 mg/kg in feedstuff (NRC, 1980, 2005) can cause toxicity, leading to decreased weight gain, feed intake, and efficiency (Jenkins and Hidiroglou, 1991).

Zinc can possibly affect gestation in at least two different ways; first through prostaglandin ($PGF_{2\alpha}$) production as Zn metalloenzymes are responsible for controlling the arachidonic acid cascade (Chanmugam et al., 1984; Sakuma et al., 1996, 1999; Wauben et al., 1999). In sows, $PGF_{2\alpha}$ is increased in the uterine lumen during pregnancy (Bazer and Thatcher, 1977) and the lack of $PGF_{2\alpha}$ is correlated to early pregnancy loss in both swine and mice (Kraeling et al., 1985; Lau et al., 1973). Second, Zn can impact pregnancy through its action on insulin-like growth factor (IGF) at the cellular levels (Hostetler et al., 2003). Insulin-like growth factor is likely involved in uterine remodeling during implantation and could influence conceptus growth in general (Hostetler et al., 2003). Studies show that Zn participates in decreasing the binding affinity of IGF to IGF-binding proteins, while concomitantly increasing the affinity of IGF to IGF receptors on the cell membrane (Sackett and McCusker, 1998). Thus, more IGF is moved onto the cell surface allowing growth and differentiation (Hostetler et al., 2003) through second messenger systems.

Table 1.2. Classification of zinc status in cattle.

Status	Liver (µg/g DM)	Plasma (µg/mL)
Deficient	< 40	< 0.4
Adequate	25 to 200	0.8 to 1.4
Toxic	> 1,000	3 to 15

Adapted from Kincaid (2000).

1.3.3. Manganese (Mn)

Manganese is a metal normally found in nature bonded to iron and Mn has an industrial importance as it is mostly used to produce metal alloys, particularly stainless steels. In biology Mn is an important component of metalloenzymes such as arginase, pyruvate carboxylase, and superoxide dismutase (Hurley and Keen, 1987). In addition, Mn acts as an enzyme activator for glycosyltransferase, hydrolase, kinase, transferase, and decarboxylases (NASEM, 2016).

Deficiencies of Mn in cattle have been correlated to skeletal abnormalities and decreased bone strength in young animals (Hurley and Keen, 1987). Moreover, in adult cattle deficiency can lead to abortion, low conception rates, stillbirth, low birth weights (NASEM, 2016), and testicular atrophy in males (Hurley and Doane, 1989). Ruminants absorb around 1% or less of dietary Mn (Hidiroglou, 1979; VAN Bruwaene et al, 1984). Little research has been done in recent years regarding Mn deficiency, mainly because it is not considered a major problem in ruminants.

According to the NASEM (2016), 40 mg/kg diet of Mn is recommended for breeding cattle (Table 1.5), Mn status can be assessed either by analyzing the concentration of Mn in blood or liver biopsy (Table 1.3). However, as Mn stored in the liver will be excreted trough the bile, the amount of this mineral in the liver may not reflect an accurate estimate of Mn intake (Kincaid, 2000). Contrary to the majority of TM, Mn is not mainly accumulated in the liver of the conceptus and levels in the fetus tended to be lower than the dam (Meinel et al., 1979). In addition, Mn is transferred from the dam to the conceptus through the placenta (Gamble et al., 1971; Newland and

Davis, 1961). Most of Mn within the body is transported by α2-macroglobulin, being rapidly spread throughout the tissues (Maynard and Cotzias, 1955). Manganese can be toxic to calves when levels are over 2000 mg Mn/kg dietary DM, causing decreased growth and feed intake (Cunningham et al., 1966). In addition, weight gain and feed efficiency were compromised when newborns were fed milk replacer containing 1000 mg Mn/kg dietary DM compared to 40,200, and 500 mg/kg dietary DM (Jenkins and Hidiroglou, 1991).

Table 1.3. Classification of manganese status in cattle.

Status	Serum (ng/mL)	Liver (µg/g DM)
Deficient	< 5	< 7
Adequate	6 to 70	> 13

Adapted from Kincaid (2000).

1.3.4. Copper (Cu)

Copper is a metal with high thermal and electrical conductivity, and is one of the few that can be found in nature without needing to be extracted from an ore. In biology, Cu is a necessary TM that is component of metalloenzymes such as cytochrome oxidase, lysyl oxidase, superoxide dismutase, ceruloplasmin, and tyrosinase (McDowell, 2003).

According to Underwood and Suttle (1999), Cu deficiency is widespread in regions of the United States and Canada. Deficiencies have been correlated to delayed or depressed estrus, diarrhea, fragile bones, cardiac failure, decreased growth, achromotrichia and anemia (NASEM, 2016). Moreover, cardiac hemorrhages were observed in Cu deficient fetuses and newborns (Tinker and Rucker, 1985). Noteworthy, some cases of Cu deficiency have been related to the antagonist effect that Mo and S present in Cu absorption instead of low Cu intake. In addition, in general Cu is poorly absorbed in ruminants with a functional rumen (NASEM, 2016).

Copper requirements will depend on the amount of Mo, S, and Fe present in the diet as these minerals have an antagonist action on Cu absorption, and therefore requirements can vary

from 4 to 15 mg/kg dietary DM (NASEM, 2016; Table 1.5). Moreover, supplementation needs in grazing cattle are greater than in feedlot cattle due to the amount of available Cu in concentrate diets. Taking this information into account, an average of 10 mg/kg dietary DM Cu is required to avoid deficiencies when levels of ingested S and Mo do not exceed 0.25 % and 2mg/kg dietary DM, respectively (NASEM, 2016). Liver and plasma samples are the most commonly used method to assess Cu status in cattle, see reference table below (Table 1.4). Copper is accumulated in the liver of the conceptus and in sheep liver Cu concentrations increase as gestation progresses (Grace et al., 1986; Langlands et al., 1982), additionally, in cattle and pigs conceptuses tissue Cu concentrations were greater than in the dam (Gooneratne and Christensen, 1989). Copper can also be toxic in cattle when levels exceed 1,250 μg/g DM in the liver and 1.2 μg/mL in the plasma (Puls, 1988). Toxic levels of Cu can cause hemolysis, hemoglobinuria, jaundice, widespread necrosis, and death (NRC, 1980, 2005).

Tong and McArdle (1995) demonstrated trophoblast uptake of Cu using a human term placenta as a model. In addition, when female rats were fed diets containing 1.3 or 11.1 ppm of Cu the number of implantation sites and fetuses were the same on d 11, although on d 13 females receiving low Cu diet presented higher number of necrotic fetuses and reabsorption (Beguin et al., 1985).

Table 1.4. Classification of copper status in cattle.

Status	Plasma (μg/mL)	Liver (μg/g DM)
Deficient	< 0.5	< 33
Adequate	0.7 to 0.9	125 to 600
Toxic	> 1.2	> 1,250

Adapted from Kincaid (2000).

Table 1.5. Requirements of Se, Cu, Zn, and Mn for beef cattle.

Trace mineral	Requirement mg/kg dietary DM
Se	0.1
Cu	10
Zn	30
Mn	40

Adapted from NASEM (2016).

1.4. Nutritional impacts on female reproduction

The United States and Canadian beef industries expect heifers to be able to conceive by 13 to 15 months of age and that these dams produce a calf crop a year until they reach 10 years of age. Some factors such as nutrition, genetics, management, and environment can either foster or compromise this expectation (NASEM, 2016). According to Robinson (1996), undernutrition can decrease ovulation rates, follicular development, embryonic and fetal development, age at puberty, dominant follicle size in heifers, period of anestrous, and uterine blood flow. In summary, compromised nutrition will negatively influence the formation of the fetal gonads and their post-natal development and function (Robinson, 1996).

In beef cattle, body condition score (BCS) at calving has been correlated with the length of postpartum anestrus. In addition, beef cows receiving increased feed intake and nursing a single calf expressed shorter anestrus post-calving and higher BCS at subsequent calving compared to lower BCS cows (Wright et al., 1992). Staples et al. (1998) reported that feeding dietary protected fat resulted in greater AI conception rates or overall pregnancies in dairy cows. In their work (Staples et al., 1998), the inclusion of 2 to 3 % of dietary protected fat on a DM bases during the first 30 d post calving led to increased in reproductive performance in the majority of the studies reported. Interestingly, trials that presented a negative effect of fat supplementation on reproductive performance did show an increase in milk production (Staples et al., 1998). However,

feeding greater amounts protected fats can decrease fiber intake and digestion, furthering increasing the negative energy balance and being prejudicial to fertility (Staples et al., 1998).

Cow plane of nutrition can also influence postpartum anestrous by its effect on the hypothalamic/pituitary/ovarian axis. Concentrations of insulin were correlated to progesterone (P4) production by the CL, which is important in the reestablishment of the ovarian activity (Robinson et al., 2006). Gong et al. (2002) showed an increase in plasma insulin followed by shorter interval from calving to first ovulation when supplementing dairy cows with a diet that would increase propionate production in the rumen (260 g starch/kg DM) versus acetate production (100 g starch/kg DM). Moreover, poor nutrition can produce greater levels of GH and decreased levels of IGF-1 that can lead to decreased estradiol production by the dominant follicle and directly impact the preovulatory LH surge (Kadokawa et al., 2000). In addition, Mann and Blache (2002) reported abnormal postpartum reproductive cycles in dams with decreased concentration of leptin, which is a hormone produced by the adipose tissue that contributes to appetite regulation and energy expenditure. Leptin has been reported to be decreased in animals experiencing undernutrition (Mann and Blache, 2002).

Not only can negative energy balance can be responsible for reproductive failure, the lack of specific minerals (either macro or trace) can directly affect reproduction. Copper imbalance will cause prenatal mortality, mainly via early embryonic loss. Selenium deficiency can be responsible for ovarian cysts, retained placenta, and infertility in ewes. Moreover, selenium supplemented cows presented 22% greater levels of P4 compared to non-supplemented counterparts (Kamada and Hodate, 1998). The explanation behind this observation is still unknown although the main theory is that Se degrades peroxides in the CL supporting it function. This hypothesis is possible as Se is a cofactor in glutathione peroxidase (GSHpx) and phospholipid hydroperoxide glutathione

peroxidase (PH-GSHpx; Kamada and Hodate, 1998). Manganese deficiency has been correlated to abortion, low conception rates, stillbirth, and low birth weights (NASEM, 2016). While Zn imbalance was shown to impair reproductive performance (Masters and Fels, 1980; Piper and Spears, 1982).

Many studies over the past years have investigated the effects of injectable Cu, Mn, Zn, and Se on animal health and reproductive performance. Sales et al. (2011) investigated the effect of injectable TM (Cu, Mn, Zn, and Se) administered 17 d prior to embryo transfer on pregnancy rates of crossbred heifers synchronized for TET. Although there was no difference on number of synchronized heifers and pregnancy-loss rate among treatment and control group, females that received the injectable TM presented 1.58 fold and 1.72 fold higher odds of being pregnant (conception rate) at 23 and 48 d after embryo transfer. In addition, conception rates were 48% versus 36% at 23 d and 43% versus 30% at 48 d after TET, for ITM and CON, respectively (Sales et al., 2011).

Brasche et al. (2014) when administering an injection containing Cu, Mn, Se, and Zn to heifers 30 d prior breeding showed an increase in overall pregnancy rates in beef heifers bred by FTAI followed by clean up bulls when comparing control and treatment group, 83 % and 92 % respectively. However, no difference was observed in AI conception rates, both averaging around 63 %. Contrary, Mundell at al. (2012) observed an increase in pregnancy to FTAI in Angus cross cows that were administered injectable TM 105 d before calving and 30 d before FTAI compared to saline, 60.2 % versus 51.2 %, respectively. However, no difference in overall pregnancy rates were observed, interestingly the improved result was unexpected as females had access to free choice minerals following manufacturer recommendations for at least 1 year before trial started.

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Although overall pregnancy rates (82.7 %) did not differ, beef heifers tended to have increased pregnancy rates to AI when receiving injectable TM 33 d prior to breeding compared to control, 62.1 % and 45.2 %, respectively (Stokes et al., 2017). It is noteworthy that heifers were being offered free choice trace mineral supplement that should have been meeting NRC (2005) recommendations during trial and that heifers had previously received mineral incorporated to TMR. Kirchhoff (2015) also noted an increase in pregnancy rates to FTAI when beef heifers received injectable TM 4 weeks prior to breeding (51.28 % for ITM and 25.58 % for CON, respectively). It is important to note that heifers were receiving TM in the ration according to NRC (2005) requirements.

When TM (Cu, Zn, and Mn) were supplemented through a free choice mineral feeder system to crossbred beef cows, Ahola et al. (2014) did not observe any difference in pregnancy rates to AI between three treatment groups in the first year of a two years study. However, in the second year of the study, a difference in pregnancy rates to AI between organically bound and inorganically bound mineral source to control group were observed, 57 %, 58 %, and 34 %, respectively.

These studies suggest that female reproductive status is influenced by source, amount, and combination of minerals. Studies regarding the effects of injectable TM in reproductive outcomes have been inconsistent across literature. However, it is interesting to see that several studies where females were already receiving TM according to requirements in the diet and injectable TM groups had a significant positive impact in reproductive outcomes. Clearly, further research regarding the impact of injectable TM is still needed.

1.5. Nutritional impacts on follicular growth and superovulation protocols

Although TM deficiencies are known to impair reproductive processes, little is known about their influence on follicular growth and superovulation protocols. Selenium deficiency caused cystic ovaries (Corah, 1996) and weak or silent heat periods. Manganese is linked to abortion and low conception rates (NASEM, 2016), and Cu to delayed or depressed estrous (Underwood and Suttle, 1999). In an effort to evaluate the impacts of trace mineral supplementation on superovulatory response Lamb et al. (2008) compared organically bound and inorganically bound sources of trace minerals to a treatment that received no additional TM supplement in Angus heifers. Interestingly, follicle number, size, and number of ovulated follicles were not different among treatments, with 51 % of control heifers responding to superovulation, compared with 60 % of organically bound and 64% of inorganically bound supplemented heifers, respectively (Lamb et al., 2008). In addition, on first d of data collection the number of CL and follicles in ovaries where ovulation did not occur were the same among groups.

In similar study with dairy cows, Hackbart el al. (2010) supplemented the control group with an inorganically bound source of Zn, Cu, Mn, and Co and the treatment group diet was partially inorganically bound and organically bound sources. Overall, there was no difference among groups regarding first-wave dynamics (i.e. ovulation rate, d to first ovulation, and numbers of ovulation at the first ovulation cycle) and first cycle luteal measures. Whereas Boas et al. (2017) when treating dairy heifers with Multimin®90, 17 d prior to embryo recovery noted a tendency to increase the proportion of stage 4 and 5 embryos. The results may be due to a synchrony in the ovulation and embryo development pattern.

A high plane of nutrition prior to ovulation is beneficial to oocyte quality in spontaneously ovulating animals such as cow and sheep, although this information seems not to be true when

animals are used in superovulation protocols and embryo production (Robinson et al., 2006). High concentration of urea nitrogen in plasma (BUN) and ammonia in follicular fluid due to high amount of rumen degradable protein (RDP) are associated with decreased fertility and reduced *in vitro* production of blastocysts in dairy cattle (Robinson et al., 2006). In contrast, undernutrition has shown to be detrimental to reproductive processes as well, oocytes collected from dairy cows treated *in vitro* with non-esterified fatty acid (NEFA) presented reduced proliferation of granulosa cells, impaired blastocyst production and delayed oocyte maturation (Jorritsma et al., 2004). Moreover, cows with high fat mobilization during early lactation (Snijders et al., 2000) and increased contents of hepatic triacylglycerol (Kruip et al., 2001) also produced lower quality oocytes with poor *in vitro* development. In addition, ovarian function in ruminants has been modulated by various metabolites such as insulin, IGF-1, and leptin (Adamiak et al., 2005).

Researchers were evaluating the effects of nutrition in superovulation protocols in ewes, mainly the effect on oocyte quality and development in late follicular phase (Lozano et al., 2003). To test their hypothesis, two experiments were designed where ewes were either fed an ad libitum diet, control (1.5 x maintenance) or low energy diet (0.5 x maintenance). It was observed in experiment 1 a lower superovulation response and ovulation rate per ewe ovulating in the group that received *ad libitum* diet compared to control and low diets. When looking into experiment 2 ewes that received *ad libitum* diet produced fewer oocytes and with inferior quality compared to ewes receiving low energy diet. Also, a tendency was observed for a higher cleavage rate in the low energy group. In summary, the *ad libitum* diet affected superovulation response and oocyte quality negatively when compared to control and low intake diets (Lozano et al., 2003).

Bridges et al. (2012) compared the effects that body condition score (BCS) of donors and recipient cows had on pregnancy rates, ovulatory response, CL diameter, and progesterone levels.

Recipient Angus cows with a BCS 6 presented greater ovulatory follicle size compared to cows with BCS 4, 14.4 ± .3 mm versus 13.2 ± .3 mm, respectively. In addition, BCS 6 recipients had greater CL size (23.7 ± .5 mm versus 21.4 ± .5 mm) and progesterone levels (3.9 ± 0.1 ng/mL versus 3.4 ± 0.2 ng/mL). However, no difference was observed in pregnancy rates.

Still little is known regarding the impacts of trace minerals in follicular growth and superovulatory response in beef cattle, practices that increase reproductive outcomes and improve results of assisted reproductive technologies such as embryo transfer have the potential to increase the efficiency of the system and being highly adopted by producers.

1.6. Nutritional effects on embryonic, fetal development, and fetal programming

The third of pregnancy cannot be overlooked even though 75% of fetus growth occurs within the last 2 months of gestation in cattle, during early stages important steps takes places such as placental growth and development as well as fetal organogenesis (Funston et al., 2010). These early happenings will dictate further fetus development, limb development start as early as 25 d of pregnancy, followed by most of abdominal organs and brain (Hubbert et al., 1972). In addition, testicles and ovarian development starts at 45 and 50 to 60 d of gestation, respectively (Funston et al., 2010).

In the past years, researchers have been investigating the role that trace minerals have in the conceptus development throughout pregnancy in livestock species and carryover effects in the offspring. Studies start as far as 1937 when Bennetts and Chapman correlated Cu deficiency in grazing ewes to enzootic neonatal ataxia, more currently, studies range from their importance in livestock body processes to how they affect fetal development (Hostetler et al., 2003).

In livestock species, most of early embryonic loss occurs during maternal recognition (Moore, 1985; Wilmut et al., 1986a). This period is specie specific, being around 15-17 d in cattle

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(Betteridge et al., 1980; Northey and French, 1980), 12-13 in sheep (Moor and Rowson, 1966; Rowson and Moor, 1967), 12–13 in pigs (Dhindsa and Dzuik, 1968), and 15-17 in goats (Gnatek et al., 1989). There is little information of the cause of early embryonic loss; however, nutrition likely plays a major role (Ashworth and Antipatis, 2001; McArdle and Ashworth, 1999; Wilmut et al., 1986a).

Adequate amount of trace minerals are needed within the body for effective immune system response (Chandra, 1999; Galyean et al., 1999; Madsen et al., 1991), which implies that maternal recognition might be dependent upon trace mineral status. During early d of pregnancy in some species (i.e. pigs, sheep, mice, and humans) immune cells (i.e. macrophages and T lymphocytes) are recruited to the uterus (Bazer and Johnson, 1989; Croy et al., 1987, 1988; Keys and King, 1990; Lee et al., 1988; Starkey et al., 1988), these cells secrete cytokines, which may be responsible to stimulate growth of the fetal-placental structure (Bazer and Johnson, 1989; Starkey et al., 1988).

When investigating the effects of organically bound and inorganically bound TM supplementation in Angus heifers subjected to a superovulation protocol, Lamb et al. (2008) did not find any difference in the number of recovered embryos among treatments. In addition, the number of degenerate embryos were the same across groups, but the number of unfertilized oocytes (UFO) were higher for inorganically bound (2.3 ± 0.4) and control (1.6 ± 0.4) compared to organically bound (0.4 ± 0.4) treatment.

According to Lee et al. (2001), the addition of Se to embryo media in an *in vitro* experiment improved the development to morula (67.1 % versus 57.5 %) and blastocyst (30.1 % versus 20.5 %) stages. This fact may be due the antioxidant effect that Se present through the action of glutathione peroxidase.

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Hansen et al. (2006) fed two groups of heifers with a low (15.8 mg/kg of dietary DM) and high (50 mg/kg of dietary DM) Mn diet throughout gestation. Calves born from the group receiving low Mn presented lower birth weight, superior brachygnathism, unsteadiness, disproportionate dwarfism, and swollen joints.

Recently, several studies have been focused on the effects of poor nutrition on embryonic, fetal and postnatal development. With some findings, that inappropriate maternal nutrition during pregnancy can have effects throughout the offspring life. Maternal nutrition during gestation can lead to alteration in offspring metabolism and physiology which can affect offspring development, this occurrence is known as fetal programming (Barker, 1997). Impairments in fetal nutrition have been correlated to intestinal and respiratory problems, retarded postnatal growth, higher fat deposition, and reduced meat quality (Funston et al., 2010). Maternal nutrition seems to play a role in gene expression in the conceptus (Holland and Rakyan, 2013), thus elucidating mechanisms that could impact gene expression in utero is a key point for food production (Gicquel et al., 2008). Moreover, early embryonic stages cannot be overlooked, during this initial phase the genome is programmed and these changes can impact performance of conceptus throughout its life (Barker, 1997).

Little is known regarding the impacts of fetal programming and epigenetics alterations in the offspring, however, these topics are leading to several novel studies. Trace minerals are among all nutritional factors that could impact offspring performance.

Marques et al. (2017) supplemented beef cows during the last trimester of gestation either with organically bound or sulfate sources of trace mineral (Cu, Co, Mn, and Zn) or no supplemental TM source. Interestingly, calves from cows that received organically bound supplement had greater weaning weight compared to calves from control cows. In addition, weight differences

were observed until slaughter. Moreover, calves from organically bound supplemented cows presented decreased incidence of bovine respiratory disease compared to control and sulfate sources originated calves.

Further research is still necessary to understand the impacts of maternal nutrition on offspring performance. Knowing key points of offspring development and matching these with adequate nutrient supply throughout developmental periods will potentially improve food production. Especially in locations where cattle face harsh conditions and may lack in specific nutrients.

1.7. Statement of the problem

Thus, an experiment was designed with the objectives of investigating the role that trace minerals plays in superovulation and embryo transfer protocols. We hypothesized that an injectable trace mineral (TM) supplement provided to cows fed to meet known nutrient requirements would increase TM status leading to an improved superovulatory response furthering affecting embryo outcomes (i.e. quality and quantity). In addition, we hypothesize that an increased TM status in early stages of development could have long lasting affects in the embryo furthering affecting pregnancy rates and postnatal performance. To test theses hypothesis we conducted two experiments.

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2. EFFECTS OF ADMINISTERING AN INJECTABLE TRACE MINERAL SUPPLEMENT TO DONOR COWS AT THE INITIATION OF A SUPEROVULATION PROTOCOL ON THE SUPEROVULATORY RESPONSE AND EMBRYO OUTCOMES

2.1. Abstract

The objectives of the study were to determine if the administration of an injectable trace mineral (Multimin®90) at the initiation of a superovulation protocol would influence superovulatory response, number of embryos produced, embryo quality, or embryo developmental stage. Thirty-five multiparous Angus-based beef cows (577.9 ± 9.1 kg and BCS 5.75 ± 0.11) were selected from Central Grassland Research and Extension Center (CGREC) herd near Streeter, ND to serve as embryo donors. Donors were randomly assigned to one of two treatments at initiation of a 16 d superovulation protocol; 1) cows received 90 mg Cu (as Cu disodium EDTA), 60 mg Mn (as Mn disodium EDTA), 30 mg Se (as sodium selenite), and 360 mg Zn (as Zn disodium EDTA) (6 ml Multimin®90 s.q. ; ITM; Multimin USA, Fort Collins, CO); or 2) were untreated (CON). All donors were exposed to a common superovulation protocol, embryos were recovered 7 d after AI, graded for quality and developmental stage, and frozen until transfer. Cows were kept in a dry-lot system at CGREC and offered Purina® Wind and Rain® Storm® 7.5 CP Avalia® 4 Original XPC Altosid® (Land O'Lakes, Inc., Arden Hills, MN) at manufacturer recommendation of 113.4 g/d. Minerals were supplemented with grass hay during the first two flushes and incorporated into a TMR during the last two flush. Liver biopsies were collected from a subset of cows (n = 12) on d 0 and 16 of each superovulation period (i.e. the initiation of the superovulation protocol and the day of embryo recovery). Samples were frozen and shipped to Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University for trace mineral analysis via inductively coupled plasma mass spectrometry (ICP–MS). A crossover design was used, with 121

± 2 d between flushes (total n = 70 for CON and 69 for ITM), with each animal being exposed to the same treatment twice. Donors were blocked by lactation status as flushings 1 and 2 cows were lactating and had suckling calves; whereas, at flushings 3 and 4 calves were already weaned. Data were analyzed by the Mixed Procedure of SAS (SAS Institute Inc., Cary, NC) for treatment, period, and block. Significance levels ($P \leq 0.05$) were separated by LSMEANS statement using the PDIFF option and animals were blocked by lactation status. Mineral analyses were tested for carryover effect between replications. At d of embryo recovery, Se levels were greater ($P < 0.0001$) in ITM cows compared to CON, 3.89 ± 0.30 versus 2.77 ± 0.17 ug/g of tissue DM, respectively. In contrast, zinc levels were greater ($P = 0.019$) in CON compared to ITM cows, 139.59 ± 7.66 versus 132.65 ± 6.07 ug/g of tissue DM, respectively. However, no change was observed in Cu and Mn ($P > 0.17$). Number of viable embryos recovered from all treated cows and responding to superovulation was similar ($P = 0.94$) among ITM and CON cows. Moreover, number of degenerate, unfertilized, and total ova collected ($P \geq 0.67$), and number of embryos in respective developmental stages or quality grades ($P \geq 0.24$) were not affected by treatment. In summary, Multimin®90 increased concentrations of liver Se at the time of embryo collection, but not number, quality, or developmental stage of embryos in this experiment.

2.2. Introduction

In 2016, approximately 489,500 bovine in-vivo derived embryos (uterine flush) were recovered in North America, leading the global ranking of bovine in-vivo produced embryos (Perry, 2017). Embryo transfer was primarily introduced to maximize the number of potential offspring produced by high merit females (Jones and Lamb, 2008). The technique has the potential to lower the cost of genetic trading among countries, boost genetic improvement within herds, and increase marketing with purebred cattle (Spell et al., 2001).

Several factors can influence the outcomes of the techniques, however genetics and nutrition are known to be the main single variables affecting the process. Donors are required to be in a positive nutritional balance to allow a proper response to superovulation protocol (Lamb, 2010). One of the key nutritional aspects is mineral requirements, especially trace minerals. As trace mineral deficiency in beef cattle are known to lead to reproductive impairments (Rabiee et al., 2010), decreased performance (Spears and Kegley, 2002), and lower immunologic response (Shankar and Prasad, 1998).

The source of trace mineral (i.e. organically bound or inorganically bound) is known to influence their absorption and bioavailability (Brown and Zeringue, 1994), however little is known regarding the usage of injectable trace minerals. Injectable sources would be beneficial to overcome negative interaction in the gastrointestinal tract (Pogge et al., 2012), increasing their bioavailability to the dam. In donors, increasing the absorption and retention could potentially affect embryo outcomes. Previously, studies have shown a positive effect of injectable TM in pregnancy rates when using artificial insemination (Mundell et al, 2012) and fixed-time embryo transfer (Sales et al., 2011). Moreover, Boas et al. (2017) showed a tendency to reduce the number of unfertilized oocytes per collection; however, no influence in embryo quality was noted.

Although the amount of research done on the topic has been considerable (Hyttel et al., 1991; Stock et al., 1996), little improvement has been made in the superovulation procedure in the past 20 years (Lamb, 2010), yet 20% of donors are expected to not respond to the superstimulation (Velasquez, 2008). Being one of the drawbacks and limiting steps of the technique, researchers that aim to overcome the high variability in ovarian response to gonadotropin stimulation, improving harvested embryo outcomes and minimize cost are crucial. Thus, we hypothesized that an injectable trace mineral supplement provided to cows fed to meet known nutrient requirements

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would increase TM status leading to an improved superovulatory response furthering affecting embryo outcomes (i.e. quality and quantity). Our objectives are to test the efficacy of an injectable TM in improving TM status within the body and its effects on superovulatory response and further embryo production.

2.3. Materials and methods

All procedures involving animals were approved by the North Dakota State University Animal Care and Use Committee (#A16051).

2.3.1. Animals and treatments

Thirty-five multiparous Angus-based beef cows (578 ± 9 kg and BCS 5.75 ± 0.11) were selected from Central Grassland Research and Extension Center (CGREC) herd near Streeter, ND to serve as embryo donors. Donors were randomly assigned to one of two treatments at initiation of a 16 d superovulation protocol; 1) cows received 90 mg Cu (as Cu disodium EDTA), 60 mg Mn (as Mn disodium EDTA), 30 mg Se (as sodium selenite), and 360 mg Zn (as Zn disodium EDTA) as an injectable TM supplement (6 ml Multimin®90 s.q.; ITM; Multimin USA, Fort Collins, CO); or 2) were untreated (CON). Cows were managed in a crossover design and 121 ± 2 d washout period, meaning that each animal received the same treatment twice (ITM and CON) totalizing 139 flushes. Cows were blocked by lactation status, where cows were lactating and had suckled calves at the time of flush 1 and 2, then calves were weaned and cows were non-lactating at the time of flush 3 and 4.

All females were exposed to a common superovulation protocol (Fig 2.1). On d 0 of superovulation protocol at 1800, all cows received a controlled internal drug release insert (CIDR, Eazi – Breed^{TM} CIDR 1.38 g of progesterone, Zoetis Inc., Kalamazoo, MI). On d 2 at 1800, cows received 3 mL i.m of gonadotropin-releasing hormone (GnRH; 150 µg, Factrel, Zoetis Inc.). All

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injections were given in the neck region following the Beef Quality Assurance guidelines. Starting on d 4 at 1800 until d 8 at 0600, cows received decreasing doses of follicle stimulating hormone (20 mg/mL, Folltropin, Vetoquinol, Fort Worth, TX) intramuscularly every 12 h, doses started at 3 mL and decreased by 0.5 mL every 24 h. Additionally, on d 7 at 1800, cows received 5 mL prostaglandin $F_{2\alpha}$ i.m. (25 mg, Lutalyse, Zoetis Inc.) followed in 12 h by CIDR removal, 5 mL of prostaglandin $F_{2\alpha}$ i.m, and application of estrus detection patch (ESTROTECT[TM], Rockway Inc., Spring Valley, WI). Cows were observed for estrus three times daily (0600, 1200, and 1800) with patch score recorded at each estrus detection event. On d 9 at 1800 cows were AI with two semen straws and administered 2 mL i.m. GnRH (100 µg, Factrel, Zoetis Inc.), followed by a second AI 12 h later, cows were flushed on d 16 at 0600 and body condition score (BCS) was recorded. A single sire was used for all inseminations in all flushes.
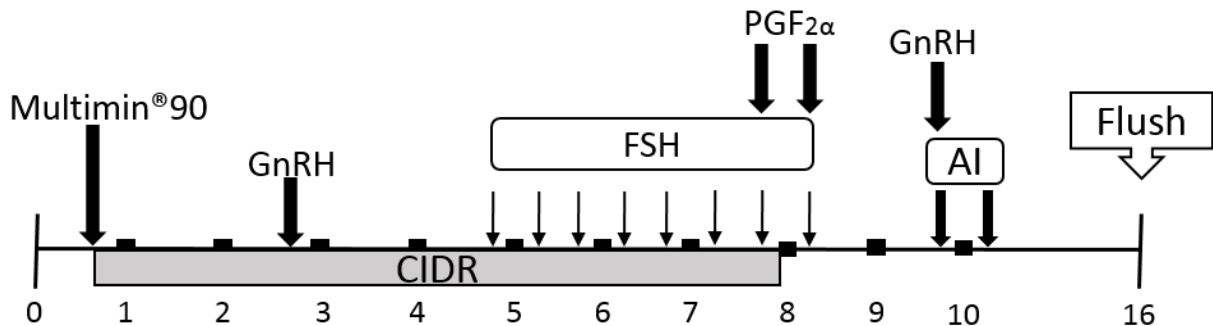
Figure 2.1. Schematic of superovulation protocol for donor cows.
Indicating that on d 0 at 1800 all cows were treated with a CIDR (Eazi – Breed[TM] CIDR; 1.38 g P4; Zoetis Animal Health) insert and ITM cows received 90 mg Cu, 60 mg Mn, 30 mg Se, and 360 mg Zn Multimin®90 (Multimin USA, Fort Collins, CO). On d 2 at 1800, all cows received 150 µg of GnRH (Factrel; gonadorelin hydrochloride; Zoetis Animal Health, Parssipany, NJ). Starting on d 4 at 1800 until d 8 at 0600, cows received decreasing doses of FSH (Folltropin, Vetoquinol, Fort Worth, TX) every 12 h, doses decreased 10 mg every 24 h (from 60 mg to 20 mg). On d 7 at 1800, cows received 25 mg of $PGF_{2\alpha}$ (Lutalyse, dinoprost tromethamine; Zoetis Animal Health) followed 12 h later by CIDR removal and 25 mg of $PGF_{2\alpha}$. On d 9 at 1800, cows were AI and administered 100 µg GnRH. Followed 12 h later by a second AI, cows were flushed on d 16 at 0600. Liver biopsies were collected from a subsample of cows on d 0 and d 16. All donors had their BCS scored on d 16, at CIDR removal females received estrus detection patches (ESTROTECT[TM], Rockway Inc., Spring Valley, WI) that were evaluated at 0600, 1200, and 1800 on d 9.

Cows were kept in a dry-lot system at CGREC and offered Purina® Wind and Rain® Storm® 7.5 CP Avalia® 4 Original XPC Altosid® (Land O'Lakes, Inc., Arden Hills, MN) at manufacturer recommendation of 113.4 g/d. Minerals were supplemented with grass hay during the first two flushes and were incorporated in total mixed ration (TMR) during the last two flushes for a minimum of 60 d before superovulation in each period. For management purpose, cows were sorted into three flushing groups in order to facilitated animal handling and be a feasible number during flushing d. During block 1 cows were offered ad libitum grass hay (Table 2.1) and Purina® Wind and Rain® Storm® 7.5 CP Avalia® 4 Original XPC Altosid® (Land O'Lakes, Inc., Arden Hills, MN) at manufacturer recommendation of 113.4 g/d. The total mixed ration (Table 2.2; Table 2.3) content during block 2 was the same among the three pens and given daily at 0800 totalizing 20 kg/head daily. One donor were excluded from the experiment after the third flush, due to neurological symptoms.

Table 2.1. Nutrient analysis of grass hay[1].

Nutrient	DM, basis %
Ash	10.82
CP	9.52
NDF	62.54
ADF	36.26

[1] *Ad libitum* consumption, containing a mixed variety of cool season grasses and alfalfa.

Table 2.2. TMR[1] composition.

Item	DM, basis %
Silage	40
Chopped Hay	39.16
Ground Corn	14.84
Wet Distillers Grains	6

[1] Fed daily at 20 kg/head daily as fed or 9.73 kg/head daily on a DM basis.

Table 2.3. Nutrient analysis of TMR.

Nutrient	DM, basis %
Ash	9.39
CP	9.38
NDF	54.87
ADF	31.76
P	0.29
Ca	0.28
Trace mineral	mg/kg DM
S	2,114
Fe	368
Mn	61.6
Zn	27.6
Se	< 10
Cu	5
Mo	1.2

2.3.2. Liver biopsies and body condition score

Liver biopsies were collected from a subset of cows (n = 12) on d 0 and 16 of each superovulation period (i.e. the initiation of the superovulation protocol and the d of embryo recovery). An imaginary line was drawn from the tuber coxae (hook) to the olecranon (elbow) and a square area was trimmed exposing the 10th intercostal space between ribs 11th and 12th at this location. The area was then cleaned with 1 % iodine solution (AgriLabs, St. Joseph, MO) and gauze, followed by 70 % isopropyl alcohol (AgriLabs, St. Joseph, MO). An injection of 2% lidocaine hydrochloride (2.5 mL, Vet One®, MWI, Boise, ID) was injected subcutaneously and intramuscularly, followed by a 1cm stab incision using a sterile surgical blade 15 (Butler Schein Animal Health, Dublin, OH). A trochanter needle (14 G x 15 cm Tru-Cut^{TM}, CareFusion, Vernon Hills, IL) was then inserted parallel to the ground, going through the intercostal muscles and diaphragm. Two to three samples were collected and dried with hardened ashless filter papers 541

(Whatman^{TM}, GE Healthcare Life Sciences, North Bend, OH) and then stored in test tubes. Samples were frozen and shipped to Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University for trace mineral analysis via inductively coupled plasma mass spectrometry (ICP–MS). The incision was closed with 1 to 2 surgical staples and covered with an aerosol bandage (alu-spray, Neogen$^®$ Corporation, Lexington, KY). In addition, all animals received 5 ml intravenously (vena jugularis externa) of Flunixin Meglumine (250 mg, Banamine, Merck Animal Health, Whitehouse Station, NJ) to prevent any inflammatory reaction and discomfort.

On the same d, cows had their body condition score recorded. BCS was recorded in a 1 to 9 scale, where 1 means emaciated and 9 obese (Whitman, 1975).

2.3.3. Embryo flushing and sorting

On d 16 embryos were recovered from donor cows via a non-surgical embryo flushing technique. In preparation for flushing, cows received 5 mL of 2 % lidocaine hydrochloride (Vet One$^®$, MWI, Boise, ID) in the epidural space and 1 ml of Acepromazine I.V. (10 mg, Vedco Inc., St. Joseph, MO) as a mild sedative. The vulva and perineal areas where cleaned with paper towels and Isopropyl alcohol 70 % (AgriLabs, St. Joseph, MO) followed by 1 % iodine solution (AgriLabs, St. Joseph, MO). A Foley catheter (Agtech Inc, Manhattan, KS) was passed through the cervix and inflated once correctly placed into the uterine horn. The uterine horn was infused with flushing solution (emP3, Partnar Animal Health, Port Huron, MI). The uterine horn was then massaged and the fluid returned through a collection filter with a 75 micron stainless steel screen to recover the embryos. The flushing procedure was repeated until approximately 1 L of media had been flushed, and then repeated in the other uterine horn. Transrectal ultrasonography of

ovaries was done to check donor response to superovulation protocols, number of CL present in each ovary was recorded.

The embryo filter was taken to the laboratory where embryos were recovered. Embryos were rinsed from the collection filters into petri dishes and placed at a 38°C warm stage. Embryos were located and recovered from petri dishes with the aid of a stereomicroscope (Stemi 305, Carl Zeiss, Jena, Germany) and transferred to a 6-well plate with Vigro^{TM} holding media (Bioniche, Animal Heath USA, Inc., Pullman, WA) for grading. Each embryo was assigned a developmental stage and quality score according to standards set forth by the International Embryo Transfer Society (Savoy, IL). Developmental stages were Morula (4), Compact Morula (5), Blastocyst (6), and Expanded Blastocyst (7). Embryos classified within any of these stages were considered viable in our study and had the potential to be transferred. Quality Grades were Excellent (1), Fair (2), Poor (3), degenerating (DEG), or unfertilized oocyte (UFO). Embryos Graded as 1 had at least 85 % of their cellular material intact, they were symmetrical and spherical with uniform blastomeres in size and color. Embryos Grade 2 had at least 50 % of their cellular material intact, they presented minor irregularities regarding their color and size. Grade 3 embryos had at least 25 % of their cellular material intact with major irregularities in their size and color.

Within 5 h after flushing embryos were transferred to a well with Vigro^{TM} freezing media (Ethylene Glycol with Sucrose; Bioniche, Animal Heath USA, Inc., Pullman, WA). Embryos were loaded in a freezing straw (0.25 cc) and placed in the Cryochamber (Cryologic Pty. Ltd., Victoria, Australia) inside of a Cryobath (Cryologic Pty. Ltd., Victoria, Australia) filled with liquid nitrogen and attached to a freeze control (CL–8000, Cryologic Pty. Ltd., Victoria, Australia). Straws were manually seeded to start ice nucleation and temperature of the straws was reduced at a controlled rate (0.5 °C/min) allowing first stage of dehydration and avoiding cell damage due to ice formation.

Once the freezing cycle was complete (- 32 °C) embryos were plunged into liquid nitrogen and stored until transfer.

2.3.4. Statistical analyses

The experiment was a crossover design. The same animals were used for multiple flushings, with an average washout period of 121 ± 2 d between each flush. Each animal was exposed to the same treatment twice. Data for concentration of mineral in the liver were analyzed using the Mixed Procedure of SAS (SAS Institute Inc., Cary, NC), significance levels ($P \le 0.05$) were separated by LSMEANS statement using the PDIFF option, and cows were blocked by lactation status. Cows were considered the experimental unit for main effects of treatment, period, carryover, and block with respective concentrations on d 0 as covariates for mineral analysis. Mineral differences among flushing period were calculated using a model with treatment and flushing for their main affect and their interaction. Data for embryo analysis were analyzed by the Mixed Procedure of SAS (SAS Institute Inc., Cary, NC), significance levels ($P \le 0.05$) were separated by LSMEANS statement using the PDIFF option, and cows were blocked by lactation status. Data was analyzed for main effects of treatments, period, and block. Embryo outcomes among flushing period were calculated using a model with treatment, flushing, and block. Embryo outcomes according to response to superovulation protocol were calculated using a model with treatment, flushing, block, estrus by 6, estrus by 12, estrus by 18, and estrus observed. The variance–covariance structure were tested for all analysis above and criteria of selection was based on the lowest fit statistics (AIC, AICC, and BIC). The Proc Corr statement of SAS (SAS Institute Inc., Cary, NC) was used to analyze the relationship between number of viable embryos harvested and BCS.

2.4. Results

2.4.1. Baseline mineral status

The mineral baseline status of the donors prior to administering the injectable trace mineral supplement was considered as adequate according to reference ranges set forth by Michigan State University (Table 2.4). The mineral content in the diet during block 2 was according NASEM (2016) requirements based mineral analysis for the diet during block 2 (Table 2.3) and mineral supplementation provided.

Table 2.4. Hepatic mineral concentration prior to administering an injectable trace mineral supplement.

	Treatment	Mean Value	Reference Range[1]
			µg/g tissue DM
Selenium	CON	2.72 ± 0.20	0.60-3.30
	ITM	2.79 ± 0.18	
Copper	CON	193.86 ± 18.84	40.0-650.0
	ITM	187.55 ± 17.15	
Manganese	CON	12.03 ± 0.40	5.50-15.0
	ITM	12.53 ± 0.49	
Zinc	CON	109.31 ± 3.92	90.0-500.0
	ITM	127.99 ± 9.37	

[1]Normal ranges are based on MSU-DCPAH. Levels are determined to be adequate if within listed ranges.

The maintenance requirements of Se, Zn, Mn, and Zn were calculated based on cow intake in the present study (9.73 kg/head daily on a DM basis) and maintenance requirements recommendations set forth by NASEM (2016; Figure 2.2). Intake was estimated based on amount of feed provided and taking in consideration that there was no waste left in the feed bunks, intake variation between cows was not considered.
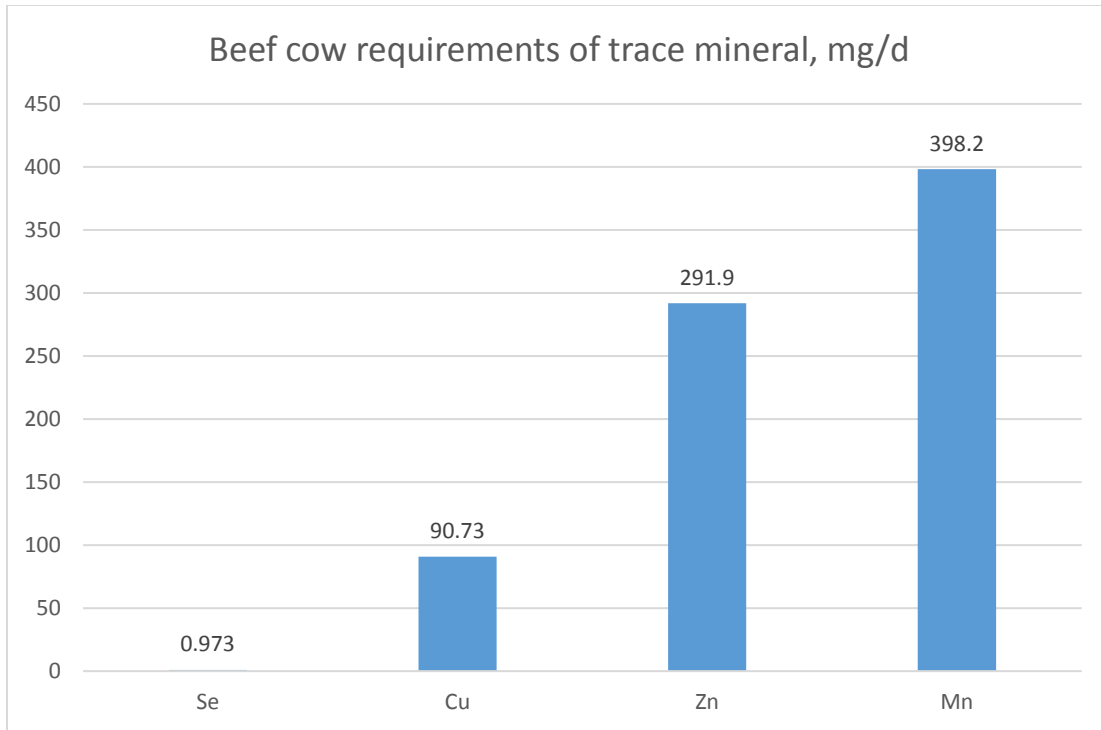
Figure 2.2. Trace mineral maintenance requirement for beef cows, mg/d.

Based on beef cattle requirements set forth by NASEM (2016) and dry matter intake.

      The amount of Se, Mn, Zn, and Cu provided at d of treatment for treated cows during the

second block was calculated based on mineral content in the feed, water, and Multimin®90 (Figure

2.3). Mineral water intake was based on mineral content in the water and water intake estimation

published by Ahlberg et al. (2018). It is important to point out that water intake is an estimation.
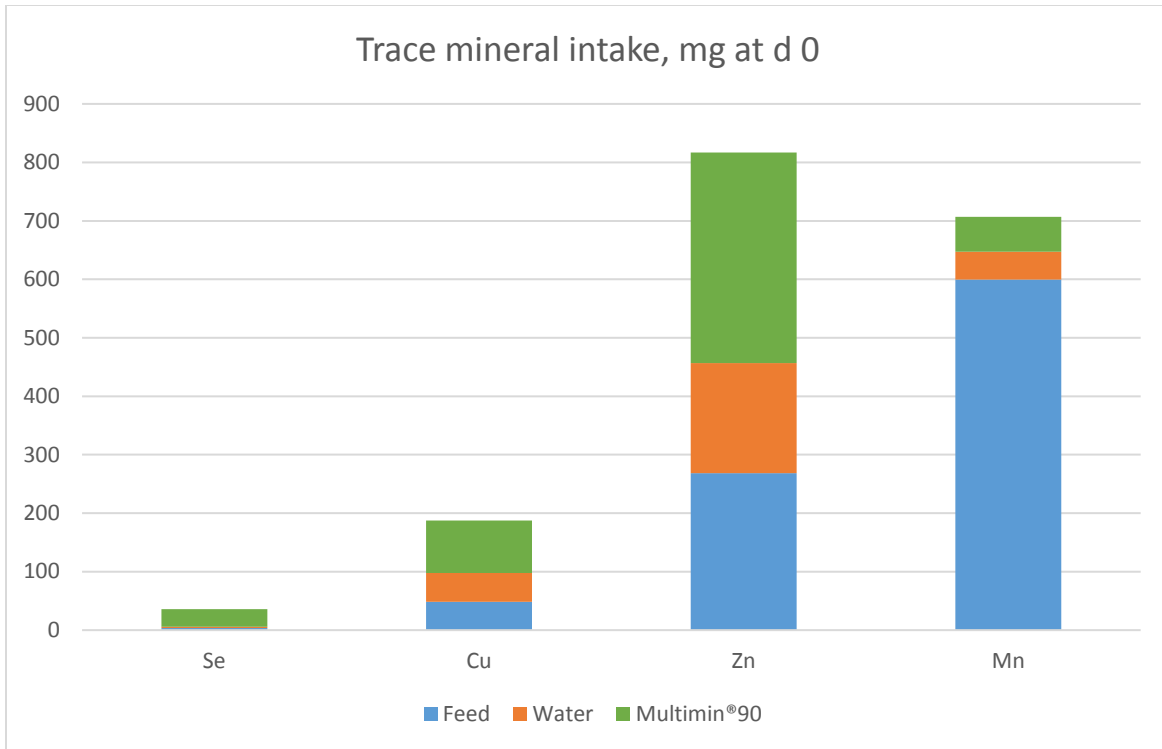
Figure 2.3. Trace mineral intake in treated cows at d 0 during second block.
Values calculated based on mineral content and intake (feed, water, and Multimin®90).

2.4.2. Concentrations of mineral in the liver

As the experimental design was a crossover with an average of 120 d in between periods, concentrations of trace mineral were tested for carryover effect, which means one flush interfering in another. There were no carryover effect on Cu ($P = 0.45$), Zn ($P = 0.47$), or Mn ($P = 0.38$) levels. However, Se presented a carryover ($P = 0.03$) effect. Carryover was removed from the model after tested. Even though Se presented a carryover effect, the TM was still removed from the model as by clearance rate stated by literature the mineral should not be affecting the following flushes. Following removal of that effect from the model, results were as follow.

There was a treatment $\times$ lactation status interaction ($P = 0.01$) in the change in the concentration of Se. However, there was no treatment $\times$ lactation status interaction in the change in the concentration of Cu ($P = 0.14$), Zn ($P = 0.37$), and Mn ($P = 0.47$).

The change in concentration of Se was greater ($P < 0.0001$) in ITM cows (1.09 ± 0.15 ug/g) compared with CON (0.04 ± 0.15 ug/g). In contrast, change in concentrations of Zn was greater ($P = 0.019$) in CON cows (30.27 ± 7.4 ug/g) compared with ITM cows (4.66 ± 7.4 ug/g). No difference, however, were observed ($P \geq 0.17$) in the change in concentrations of Cu (37.1 ± 18.9 and - 6.2 ± 18.9 ug/g, respectively) or Mn (- 0.10 ± 0.71 and 0.002 ± 0.71 ug/g, respectively) among cows in the ITM and CON treatments (Table 2.5).

Interestingly, cows in lactation presented greater change in the concentration of Se compared to non-lactating (0.74 ± 0.16 ug/g and 0.38 ± 0.16 ug/g, respectively; $P = 0.1$). In contrast, Zn and Cu levels were 38.21 ± 11.65 ug/g and 68.36 ± 23.62 ug/g greater in non-lactating cows compared to lactating, respectively, $P = 0.007$ and $P = 0.01$. Concentration of Mn were 0.60 ± 0.97 ug/g greater in lactating cows compared to non-lactating ($P = 0.54$).

Table 2.5. Change in hepatic trace mineral content at d 16 in cows receiving control (CON) or injectable trace mineral (ITM) at d 0 of superovulation.

		Period			
	Treatment	d 0	d 16	Change[1]	P – value[2]
		mean $\pm$ SE , ug/g DM			
Selenium	CON	2.72 ± 0.20	2.77 ± 0.17	0.04 ± 0.15^a	0.0001
	ITM	2.79 ± 0.18	3.89 ± 0.30	1.09 ± 0.15^b	
Copper	CON	193.86 ± 18.84	187.62 ± 17.44	$- 6.2 \pm 18.9^a$	0.17
	ITM	187.55 ± 17.15	224.66 ± 24.69	37.1 ± 18.9^a	
Manganese	CON	12.03 ± 0.40	12.03 ± 0.77	0.002 ± 0.71^a	0.97
	ITM	12.53 ± 0.49	12.43 ± 0.34	-0.10 ± 0.71^a	
Zinc	CON	109.31 ± 3.92	139.59 ± 7.66	30.27 ± 7.4^a	0.019
	ITM	127.99 ± 9.37	132.65 ± 6.07	4.66 ± 7.4^b	
Molybdenum	CON	4.04 ± 0.16	4.02 ± 0.25	-0.02 ± 0.23^a	0.74
	ITM	4.28 ± 0.13	4.35 ± 0.12	0.07 ± 0.23^a	
Iron	CON	224.55 ± 22.84	195.07 ± 10.91	$- 29.48 \pm 11.5^a$	0.42
	ITM	259.77 ± 28.01	209.9 ± 12.35	$- 40.87 \pm 11.5^a$	

[ab] Means not sharing a common superscript within a mineral in the change column are different ($P < 0.05$).

[1] Change in concentration reflect the concentration at d 16 minus d 0 in each group.

[2] Reflecting the comparison between the change in CON and ITM group.

Table 2.6. Hepatic trace mineral content at d 0 and d 16 in cows receiving control (CON) or injectable trace mineral (ITM) in each of 4 flushes[3].

	TRT	Flush 1		Flush 2		Flush 3		Flush 4		P-Value TRT	Flush[1]	TRT x Flush[2]
		d 0	d 16	d 0	d 16	d 0	d 16	d 0	d 16			
		mean ± SE , ug/g DM		mean ± SE , ug/g DM		mean ± SE , ug/g DM		mean ± SE , ug/g DM				
Selenium	CON	3.48 ± 0.26^a	3.44 ± 0.31^a	3.21 ± 0.26^a	3.19 ± 0.31^a	2.35 ± 0.26^a	2.7 ± 0.31^a	1.83 ± 0.26^b	1.75 ± 0.31^b	0.0001	0.29	0.06
	ITM	3.72 ± 0.26^a	5.44 ± 0.31^b	3.21 ± 0.26^a	4.64 ± 0.31^b	2.16 ± 0.26^a	2.74 ± 0.31^a	2.1 ± 0.26^a	2.71 ± 0.31^a			
Copper	CON	196.8 ± 37.4	175.2 ± 39.8	191.8 ± 37.4	178.2 ± 39.8	233.8 ± 37.4	253.6 ± 39.8	153.1 ± 37.4	143.5 ± 39.8	0.17	0.06	0.2
	ITM	177.3 ± 37.4	158.4 ± 39.8	200.1 ± 37.4	179.1 ± 39.8	181.3 ± 37.4	254.1 ± 39.8	191.5 ± 37.4	307 ± 39.8			
Manganese	CON	10.9 ± 0.9	12.8 ± 1.18	12.7 ± 0.9	12 ± 1.18	12.9 ± 0.9	12.6 ± 1.18	11.7 ± 0.9	10.7 ± 1.18	0.97	0.49	0.84
	ITM	13.9 ± 0.9	14.3 ± 1.18	11.9 ± 0.9	11.23 ± 1.18	12.4 ± 0.9	12.8 ± 1.18	11.9 ± 0.9	11.3 ± 1.18			
Zinc	CON	103.6 ± 13.9^a	102 ± 8.4^a	108 ± 13.9^a	117.3 ± 8.4^a	124 ± 13.9^a	159 ± 8.4^b	101.8 ± 13.9^a	180 ± 8.4^b	0.019	0.0001	0.27
	ITM	132.4 ± 13.9^a	96.2 ± 8.4^b	111 ± 13.9^a	132.7 ± 8.4^a	157.8 ± 13.9^c	146.5 ± 8.4^c	111 ± 13.9^a	155.1 ± 8.4^c			

[1]Difference in TM concentration between flushing periods.

[2]Interaction between flushing period and treatment group.

[3]Flushes were 121 ± 2 d apart.

[abc] Means not sharing a common superscript within each treatment are different ($P < 0.05$).

2.4.3. Impact of ITM treatment on embryo yield, developmental stage, and quality grade

There was no treatment × lactation status interaction ($P = 0.11$) on the number of viable embryos harvested, as well as degenerate ($P = 0.96$) and UFO ($P = 0.17$). However, there was a treatment × lactation status interaction on the number of total structures harvested ($P = 0.03$), where lactating cows produced 7.62 ± 1.12 total structures whereas non-lactating produced 6.02 ± 1.12. In addition, within lactating group CON cows produced 8.48 ± 1.3 while ITM produced 6.76 ± 1.31, in the non-lactating group CON cows produced 4.81 ± 1.33 whereas ITM produced 7.24 ± 1.33 structures.

There was no treatment effect ($P = 0.96$) on the number of viable embryos produced per cow when respective analysis compared all cows treated (CON = 3.37 ± 0.52 and ITM = 3.39 ± 0.52; Table 2.8), or when comparing only those cows responding to superovulation (CON = 4.46 ± 0.63 and ITM = 4.53 ± 0.63; Table 2.9). In addition, treatment with ITM did not influence the number of degenerate ($P = 0.75$) or unfertilized ($P = 0.54$) embryos. Also, number of embryos in each respective grade and developmental stage were similar ($P \geq 0.19$) among treatments (Table 2.8).

Cows in lactation produced 0.81 ± 0.73 more viable embryos ($P = 0.27$) and 0.73 ± 0.66 more UFO ($P = 0.27$) compared to non-lactating donors. The number of degenerate was not affected by lactation status ($P = 0.89$). In addition, lactating cows produced 1.59 ± 0.98 more structures than non-lactating cows ($P = 0.10$; Table 2.7).

Table 2.7. Embryo production in all treated donors sorted by lactation status.

	Block		
	Lactating	Non-lactating	*P* - value
No. cows[1]	70	69	
	(mean ± SE)		
Viable embryos, no.	3.8 ± 0.57	2.98 ± 0.57	0.27
Degenerate embryos, no.	0.67 ± 0.22	0.72 ± 0.22	0.89
Unfertilized oocytes, no.	3.19 ± 0.66	2.46 ± 0.66	0.27
Total structures, no.	7.76 ± 1.12	6.07 ± 1.12	0.10

[1]Females were lactating during uterine flushes 1 (n = 35) and 2 (n = 35). Females were non-lactating during uterine flushes 3 (n = 35) and 4 (n = 34).

Embryo stage of development was also not affected by treatment (*P* = 0.35), most of embryos collected were classified as Stage 4, CON cows had in average 2.0 ± 0.31 and ITM 1.66 ± 0.32 Stage 4 embryos per cow (Table 2.8).

Table 2.8. Embryo production in all treated donor cows receiving control (CON) or injectable trace mineral (ITM) at d 0 of superovulation.

	Treatment		
	Control (CON)	Multimin (ITM)	P - value
No. cows[1]	70	69	
	(mean ± SE)		
Viable embryos, no.	3.37± 0.52	3.39 ± 0.52	0.96
Degenerate embryos, no.	0.75 ± 0.22	0.65 ± 0.22	0.75
Unfertilized oocytes, no.	2.60 ± 0.60	3.07 ± 0.60	0.54
Total structures, no.	6.79 ± 1.13	7.04 ± 1.13	0.80
Grade 1	3.12 ± 0.50	3.10 ± 0.50	0.96
Grade 2	0.11 ± 0.04	0.11 ± 0.04	0.97
Grade 3	0.12 ± 0.07	0.17 ± 0.07	0.67
Morula[2]	1.92 ± 0.32	1.66 ± 0.32	0.52
Compact morula[3]	1.20 ± 0.26	1.15 ± 0.26	0.91
Blastocyst[4]	0.18 ± 0.14	0.31 ± 0.14	0.50
Expanded blastocyst[5]	0.01 ± 0.07	0.15 ± 0.07	0.19

[1]Con = Flush 1 (n = 18), Flush 2 (n = 17), Flush 3 (n = 18), and Flush 4 (n = 17). ITM = Flush 1 (n = 17), Flush 2 (n = 18), Flush 3 (n = 17), and Flush 4 (n = 17).

[2]Stage 4, [3] Stage 5, [4] Stage 6, [5] Stage 7.

We observed an increase in number of viable embryos recovered when only analyzing donors that responded to superovulation protocol (Table 2.9).

Table 2.9. Embryo production in donor cows responding to superovulation[1] that received control (CON) or injectable trace mineral (ITM) at d 0 of superovulation.

	Treatment		
	Control (CON)	Multimin (ITM)	*P* - value
No. cows[2]	51	50	
	(mean ± SE)		
Viable embryos, no.	4.46 ± 0.63	4.53 ± 0.63	0.94
Degenerate embryos, no.	1.03 ± 0.30	0.90 ± 0.30	0.74
Unfertilized oocytes, no.	3.48 ± 0.75	3.78 ± 0.75	0.67
Total structures, no.	8.83 ± 1.22	8.97 ± 1.24	0.91
Grade 1	3.91 ± 0.63	3.92 ± 0.63	0.98
Grade 2	0.16 ± 0.05	0.16 ± 0.05	0.96
Grade 3	0.17 ± 0.10	0.24 ± 0.10	0.61
Morula[3]	2.54 ± 0.40	2.20 ± 0.40	0.46
Compact morula[4]	1.55 ± 0.36	1.50 ± 0.37	0.90
Blastocyst[5]	0.24 ± 0.19	0.39 ± 0.19	0.58
Expanded blastocyst[6]	0.01 ± 0.11	0.20 ± 0.11	0.24

[1]Were considered to have superovulation response donors that produced at least 2 structures (viable, degenerate or unfertilized oocyte).

[2]Con = Flush 1 (n = 16), Flush 2 (n = 16), Flush 3 (n = 11), and Flush 4 (n = 8). ITM = Flush 1 (n = 16), Flush 2 (n = 11), Flush 3 (n = 14), and Flush 4 (n = 9).

[3]Stage 4, [4] Stage 5, [5] Stage 6, [6] Stage 7.

There was a fluctuation of embryo production throughout the experiment, number of viable embryos produced were influenced by flushing period (Table 2.10, Table 2.11). However, no effect of block was observed in embryo production (Table 2.7)

Table 2.10. Embryo production per flushing in donor cows that received control (CON) or injectable trace mineral (ITM) at d 0 of superovulation.

	Flush 1		Flush 2		Flush 3		Flush 4		P-Value		
	CON	ITM	CON	ITM	CON	ITM	CON	ITM	TRT	Flush	TRT × Flush
No. cows	18	17	17	18	18	17	17	17			
No. of viable	5.3 ± 1.0	4.5 ± 1.0	3.5 ± 1.0	2.2 ± 1.0	2.4 ± 1.0	5.0 ± 1.0	2.2 ± 1.0	1.6 ± 1.0	0.97	0.005	0.20
No. of degenerate	0.9 ± 0.4	0.2 ± 0.4	0.5 ± 0.4	1.0 ± 0.4	0.7 ± 0.4	1.2 ± 0.4	1 ± 0.5	0.1 ± 0.5	0.71	0.76	0.21
No. of unfertilized	2.8 ± 1.0	4.1 ± 1.0	3.7 ± 1.0	1.5 ± 1.0	1.6 ± 1.0	4.1 ± 1.0	2.1 ± 1.1	2.4 ± 1.1	0.18	0.16	0.30
Total no. of embryos/ova	9.1 ± 1.8	8.8 ± 1.8	7.8 ± 1.8	4.7 ± 1.8	4.4 ± 1.8	10.3 ± 1.8	5.1 ± 1.9	4 ± 1.9	0.73	0.01	0.06

Table 2.11. Embryo production per flushing in donor cows responding[1] to superovulation that received control (CON) or injectable trace mineral (ITM) at d 0 of superovulation.

	Flush 1		Flush 2		Flush 3		Flush 4		P-Value		
	CON	ITM	CON	ITM	CON	ITM	CON	ITM	TRT	Flush	TRT × Flush
No. cows	16	16	16	11	11	14	8	9			
No. of viable	6.0 ± 1.1	4.8 ± 1.1	3.75 ± 1.1	3.54 ± 1.32	3.45 ± 1.32	6.1 ± 1.17	4.4 ± 1.5	2.9 ± 1.46	0.32	0.06	0.83
No. of degenerate	1.0 ± 0.5	0.2 ± 0.5	0.5 ± 0.5	1.6 ± 0.6	1.0 ± 0.6	1.5 ± 0.5	1.9 ± 0.7	0.2 ± 0.7	0.60	0.64	0.11
No. of unfertilized	3.3 ± 1.4	5.0 ± 1.3	4.2 ± 1.3	1.5 ± 1.6	2.0 ± 1.6	4.3 ± 1.5	3.5 ± 2	4.5 ± 1.7	0.56	0.76	0.46
Total no. of embryos/ova	10.0 ± 1.9	9.3 ± 1.9	8.3 ± 1.9	6.6 ± 2.2	6.2 ± 2.2	12.5 ± 2	10.3 ± 2.5	7.0 ± 2.4	0.20	0.05	0.74

[1] Were considered to have superovulation response donors that produced at least 2 structures (viable, degenerate or unfertilized oocyte).

When only analyzing viable embryos and checking the proportion of developmental stages and quality grades within viable embryos, there was a tendency for CON donors to have 12 % ± 0.07 more grade 1 ($P = 0.06$) and 13 % ± 0.07 more stage 4 ($P = 0.06$) embryos compared to ITM donors.

We did not observe any treatment ($P = 0.57$) affect when analyzing number of CL per structures produced. Control donors presented an efficiency rate of 0.57 ± 0.04 while treated donors presented 0.53 ± 0.04.

2.4.4. Other factors impacting embryo yield, developmental stage, and quality grade

Number of viable embryos was affected ($P = 0.0002$) by whether donor cows displayed estrus by 0600 on the anticipated d of estrus that means they were in synchrony with the protocol, with cows displaying estrus by 0600 having 4.2 more viable per flush than cows that did not display estrus by 0600 (Table 2.12).

Table 2.12. Effect of estrus display by 0600 on embryo outcomes.

	Estrus by 0600		
	Yes	No	*P* - value
No. cows[1]	25	106	
	(mean ± SE)		
Viable	6.74 ± 0.80	2.60 ± 0.44	0.0001
Grade 1	6.19 ± 0.78	2.37 ± 0.42	0.0002
Grade 2	0.15 ± 0.07	0.11 ± 0.04	0.65
Grade 3	0.44 ± 0.13	0.09 ± 0.06	0.03
Morula[2]	3.25 ± 0.51	1.52 ± 0.27	0.006
Compact morula[3]	2.41 ± 0.44	0.91 ± 0.23	0.005
Blastocyst[4]	0.50 ± 0.24	0.19 ± 0.11	0.25
Expanded blastocyst[5]	0.49 ± 0.13	0.001 ± 0.06	0.0049

[1]Yes = Flush 1 (n = 1), Flush 2 (n = 6), Flush 3 (n = 7), Flush 4 (n = 11). No = Flush 1 (n = 30), Flush 2 (n = 26), Flush 3 (n = 27), Flush 4 (n = 23).

[2] Stage 4, [3] Stage 5, [4] Stage 6, [5] Stage 7.

Number of viable embryos was also affected ($P = 0.02$) by estrus display on the anticipated d of estrus. Cows that displayed estrus (n = 113) produced 3.74 ± 0.47 viable embryos whereas cows that did not display estrus (n = 22) produced 1.30 ± 0.91 (Table 2.12). In addition, number of grade 1 embryos was greater ($P = 0.02$) in cows that displayed estrus (3.44 ± 0.45) compared with cows that did not display estrus, but number of Grade 2 ($P = 0.29$) and Grade 3 ($P = 0.22$) embryos per flush were not affected by estrus display (Table 2.13).

Moreover, cows that displayed estrus tended ($P = 0.09$) to produce more Stage 4 embryos, 2.0 ± 0.28 versus 0.98 ± 0.54. However, no difference was observed in the number of Stage 5 ($P = 0.12$), Stage 6 ($P = 0.44$) and 7 ($P = 0.75$) embryos among cows that displayed estrus and those that did not (Table 2.13).

Table 2.13. Effect of estrus display on embryo outcomes.

	Estrus observed		
	Yes	No	*P* - value
No. cows[1]	113	22	
	(mean ± SE)		
Viable	3.74 ± 0.47	1.30 ± 0.91	0.02
Grade 1	3.44 ± 0.45	1.28 ± 0.83	0.02
Grade 2	0.10 ± 0.03	0.19 ± 0.08	0.29
Grade 3	0.18 ± 0.06	0.001 ± 0.13	0.22
Morula[2]	2.0 ± 0.28	0.98 ± 0.54	0.09
Compact morula[3]	1.32 ± 0.24	0.52 ± 0.47	0.12
Blastocyst[4]	0.28 ± 0.02	0.07 ± 0.25	0.44
Expanded blastocyst[5]	0.09 ± 0.06	0.04 ± 0.14	0.75

[1]Yes = Flush 1 (n = 31), Flush 2 (n = 25), Flush 3 (n = 27), Flush 4 (n = 30). No = Flush 1 (n = 4), Flush 2 (n = 7), Flush 3 (n = 7), Flush 4 (n = 4).

[2] Stage 4, [3] Stage 5, [4] Stage 6, [5] Stage 7.

There was a weak negative correlation between viable embryos harvested and donors BCS, $P = 0.04$ and r = - 0.18 (Figure 2.6).
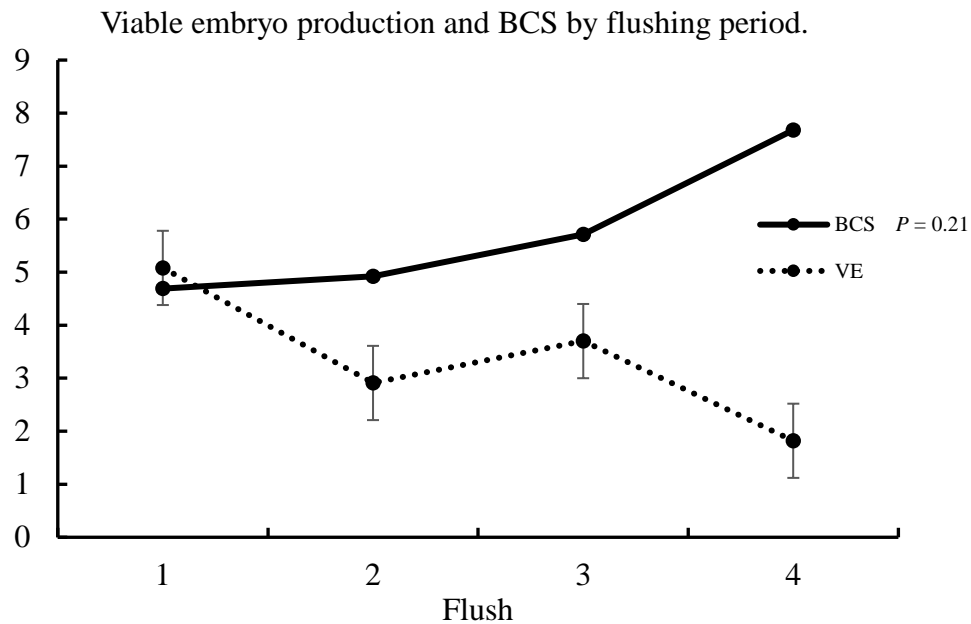
Viable embryo production and BCS by flushing period.

Figure 2.4. Number of viable embryos (VE) produced and donors body condition score (BCS) during each flushing period.

2.5. Discussion

An injectable TM would be beneficial to bypass antagonistic effects in the gastrointestinal tract, the TM supplement used in this study contained Se (as sodium selenite), Mn (as Mn disodium EDTA), Zn (as Zn disodium EDTA) and Cu (as Cu disodium EDTA). Those TM are known to have their absorption or retention compromised based on the concentrations of other minerals such as S, Ca, Mo, and Fe. Thus, our hypothesis was that an injectable TM would improve TM status within the body and affect superovulatory response and further embryo outcomes.

The injectable TM supplement was efficient in increasing Se hepatic status in treated donor cows 16 d after injections. In the present study, concentrations at d 16 were 2.76 and 3.89 µg/g DM, in control and treated animals, with both of these concentrations falling into the high adequate (> 2.5 µg/g DM) concentrations of Se (Kincaid, 2000). It is important to note that in this study

cow's diet were balanced according to NRC (2005) requirements, thus nutrients should not have been limiting. In addition, Purina® Wind and Rain® Storm® 7.5 CP Avalia® 4 Original XPC Altosid® (Land O'Lakes, Inc., Arden Hills, MN) were offered at manufacturer recommendation 113.4 g/d per head. Nevertheless, our results indicated that the supplement would probably be effective in increasing Se status in case cows were deficient. Similar results were achieved in a study where a group of steers was fed either an antagonist (Mo and Fe) diet, to simulate TM deficiency, or a diet supplemented with TM, followed by an administration of either an injectable TM or Saline (Genther and Hansen, 2013). The injectable TM was also effective in increasing Se status in treated steers in both control and antagonist diet groups, however both groups (treated versus non treated) were considerate having adequate status (Kincaid, 2000). Pogge et al. (2012) comparing the effects of an injectable TM between Simmental and Angus steers stated that Multimin®90 was efficient in increasing hepatic concentrations of Se for at least 15 d post injection. In addition, steers that received TM injection presented greater activity of erythrocyte glutathione peroxidase (GSH-Px) which Se is a cofactor indicating that Se was efficiently used in body processes (Pogge et al, 2012).

However, Multimin®90 was not efficient in increasing hepatic concentration of Cu, Zn, and Mn in the study herein. Interestingly, concentration of Zn was greater in CON cows compared to ITM. Zinc absorption and retention was shown to be impaired by calcium (Perry et., 1968; Pond and Wallace, 1986) and sulfur (Pogge et al., 2014) concentrations in ruminants. The concentrations of those two macrominerals within the body are unknown in the present study although ingested levels according to water (< 250 ppm, S) and feed analysis (2,889 ppm, Ca; 2,114 ppm, S) stated that cows ingestion were within adequate levels according to the Environmental Protection Agency (US EPA, 1973; reviewed by Socha et al., 2003) and NASEM (2016), respectively.

Although in the present study both groups (ITM and CON) were under the same nutritional management we could observe a disparity on the concentrations of molybdenum, TM antagonist, between the two groups (Table 2.5.). Cows in the ITM group presented greater hepatic concentration of the TM antagonist compared to CON, which could lead us to hypothesize the lack of treatment response in the present study regarding Cu concentrations. Although both groups were within the reference range (1.80 to 4.70 ug/g DM) considered as adequate, they were close to the upper top level, CON (4.02± 0.25) and ITM (4.35 ± 0.12).

Little is known regarding hepatic concentration range of molybdenum in beef cattle. The reference range used in the present study was set forth by DCPAH at Michigan State University where the samples were analyzed. Therefore, it is important to point out that reference ranges will present some variations throughout laboratories based on samples collected. Samples can be collected from different breed, age, sex, feeding system, and production purpose (i.e. dairy or beef). Perhaps the levels of Mo in the study herein were already high enough to affect Cu levels, leading to the increase of Cu urinary loss, decreasing the storage in the liver, and formation of Cu-Mo complex in the plasma (Kincaid and White, 1988). In addition, researchers reported dietary Mo as being the main negative effect on hepatic concentration of Cu (Kessler et al., 2012; Dias et al., 2013), affecting its absorption and therefore accumulation or mobilization (reviewed by Dias et al., 2013). There was a little discrepancy when comparing the reference range set forth by DCPAH with Kincaid (2000), as according to Kincaid the concentration of Mn and Se in the present study would be classified as marginal and high adequate, respectively; whereas, according to DCPAH both are classified as adequate.

Pogge et al. (2012) observed increased hepatic concentration of Cu for at least 15 d post injection of TM supplement in treated steers. In addition, there was a breed effect as Angus cattle

presented greater concentrations in plasma compared to Simmental. We did not see the same Cu pattern in the study herein.

The concentration of Mn in the current experiment did not differ between groups 16 d after ITM administration, whereas Pogge et al. (2012) noted a tendency for greater concentrations of Mn in treated steers 15 d post injection. Little is known regarding Mn deposition within body reserves, researchers in this area are limited because Mn is not known to cause deficiency in ruminants in practical settings (Spears, 2003).

The number of viable embryos was not affected by treatment. When we excluded the donors that did not respond to superovulation protocol we observed an increase in the number of viable embryos produced in CON and ITM. According to Velazquez (2008) 20 % of the donors will not respond to the superstimulation protocol. In our study 27.3% did not respond (38 out of 139) being 19 from ITM and 19 from CON group, which implies that the injectable TM did not influence the donor's response to superstimulation protocol. We considered as responding donors, cows that produced two or more structures at flushing (d 16). Lamb et al. (2008) did not see any difference in superovulation response among beef heifers when adding organically bound or inorganically bound trace mineral sources to their diet 23 d prior to embryo recovery. Hackbart et al. (2014) support ours and Lamb et al. (2008) findings as he reported no effect of TM supplementation in follicular wave and luteal measures in dairy heifers.

The yield of viable embryos was greater in our study compared to Lamb et al. (2008), where they investigated the influence of organically bound versus inorganically bound supplements, showing no treatment effect in the number of embryos produced. Similar results were observed by Hackbart et al. (2014) where the percentage of viable embryos did not differ when supplementing dairy cows with an inorganically bound source of Zn, Cu, Mn, and Co (control

group) and with a diet that had inorganically bound and organically bound sources (treatment group) for at least 90 d before superstimulation protocol. González-Maldonado et al. (2017) reported no effects of Multimin®90 in follicular and CL development, or pregnancy rate when supplementing dairy cows 25 d before expected estrus. Similar results were observed by Abdollahi et al. (2015) when administering TM ruminal bolus, containing Cu, Zn, Fe, Mn, Se, and I to ewes four weeks before CIDR insertion where no effects were observed in ovarian structures.

The number of viable embryos produced in the present study was below the average reported by the International Embryo Transfer Society (IETS) in 2016, North America reported 6.7 transferable embryos per collection. North America was responsible for producing 52.52 % of global transferable embryos labeled as in-vivo derived (IVD) embryos. It is noteworthy, that in a commercial embryo collections many donors are not flushed due to weak response to superstimulation protocol which did not happened in our research trial. The fact that donors were flushed independent of superstimulatory response could have led to the decreased average of embryos harvested compared to industry standards. Lower than industry average results were also observed by Lamb et al. (2008) and Boas et al. (2017) when looking at TM effects on embryo production.

When Boas et al. (2017) administered Multimin®90 at the initiation of a superovulation protocol in dairy heifers, they observed a tendency ($P > 0.08$) for increased number of quality 2, quality 3, stage 4, and stage 5 embryos compared to control heifers. This study used 18 heifers that were randomly assigned into treatment (n = 9) and control (n = 9) groups. In addition, heifers that received the injectable TM tended ($P = 0.15$) to have decreased proportion of UFO. In contrast, Hackbart et al. (2014) did not observe any difference in embryo quality and stage when supplementing organically bound and inorganically bound sources to dairy cows. In our

experiment, we did not observe any influence of treatment on embryo quality grade or developmental stage. However, when analyzing quality grades within viable embryo there was a tendency for CON cows to have a greater proportion of quality grade 1 and stage 4 embryos.

Embryo production was influenced by flushing period. The first flushing yielded more viable embryos compared to the other three. It is important to note that flushing 1 and 4 were in June, flushing 2 in October, and 3 in February. Studies have shown that reproductive efficiencies in cattle are impacted by climate conditions, potentially stress caused by heat or cold (de la Sota et al., 1998; Rensis and Scaramuzzi, 2003). In our study there was a discrepancy between flushes 1 and 4, while both occurred during the summer flush 1 yielded more viable embryos than flush 4. Flush 2 and 3 were intermediate regarding number of viable embryos produced per donor. Yet, there was no difference among flushes regarding UFO and degenerate numbers. It is noteworthy, there was a diet change after flushing 2 which could have an influence in the differences between number of viable embryos produced by flushing.

Interestingly, flushing 4 presented the lowest embryo yield within flushes while cows presented the highest BCS, increasing from 4.69 ± 0.07 in flush 1 to 7.68 ± 0.08 in flush 4. In addition, 17 of the 38 donors that did not respond to the superovulation protocol were from flush 4. Which is interesting, as the majority of the studies focus on the influence of undernutrition in reproductive outcomes, in general over feeding is overlooked. Siddiqui et al. (2002) showed that zebu cows kept on a high nutrition diet (59.12 MJ per cow/ME) had greater odds of developing ovarian cysts and did not respond properly to superovulation protocols, whereas cows in a good plane of nutrition (39.6 MJ per cow/ME) yielded more viable embryos and were more suitable to respond to superovulation protocol. These outcomes are normally due to a lower IGF-1 concentration which can be correlated either with high or low BCS over ideal BCS (Ryan et al.,

1994). Insulin-like growth factor-1 is known to stimulate oocyte maturation and the development of early stage embryos by increasing the sensitivity of granulosa cells to gonadotropins (Mariana et al., 1991; Kane et al., 1997). In addition, Nolan et al. (1998) stated that the donors plane of nutrition during or prior to superstimulation is a method to enhance the amount of viable embryos collected. The author reported that beef heifers in a less energetic diet (9.6 Mcal/kg/head/d) produced a greater number of 7 to 10 mm follicles, improved embryo quality, and had higher plasma progesterone concentration compared to high energetic diet (28.6 Mcal/kg head daily). In our study, body condition score had a negative weak correlation (r = - 0.18) with the number of viable embryos produced: however, no significance was noted in the model. It is still unknown what could have caused fluctuations among flushing in the experiment herein.

Bastidas and Randel (1987) reported decreased embryo recovery once repeated flushes were done in *Bos indicus* beef females. In addition, females subjected to more than three repeated recoveries presented the greatest embryo production in the first recovery. Bruno-Galarraga et al. (2014) reported that repeated embryo collections 5 d a part are possible using Merino ewes, however a decreased recovery rate was noted. Similar findings were reported by Saumande and Chupin (1977) when superovulating heifers every 7 to 9 weeks, where the number of ovulations decreased after the second protocol. Although our experiment used 120 d in between treatment, the fluctuation in the number of recovered embryo could be affected by repeated superovulation protocols.

Literature has shown conflicting results regarding the effects of TM supplementation in reproductive processes. Research have shown an increase in pregnancy rates when TM supplementation was given (Sales et al., 2011; Mundell at al., 2012; Ahola et al., 2014; Brasche et al., 2014; Kirchhoff, 2015; Stokes et al., 2017). Recently, Boas et al., (2017) reported an increase

in quality 2, quality 3, stage 4, and stage 5 embryos in heifers that were administered an injectable

TM over control heifers. However, little is known regarding the effects of TM supplementation in

reproductive processes especially during a superstimulation protocol. Thus, trace mineral

supplementation could potentially have a direct affect on the dam, on the conceptus, or on both.

Therefore, further research is still necessary to understand these relationships.

In our study, the injectable TM did not influence superovulatory response or embryo

outcomes, it is important to reiterate that donor cows used in the study were already receiving

adequate TM supplementation to meet NRC requirements. Our results were affected by donor

response and synchrony to superstimulation protocol.

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3. THE EFFECTS OF AN INJECTABLE TRACE MINERAL SUPPLEMENT ON EMBRYO DEVELOPMENT AND PREGNANCY RATES

3.1. Abstract

The objectives of this study were to investigate the impacts of treating embryo donors with injectable trace mineral supplements (Multimin®90) at initiation of the superovulation protocol on pregnancy outcomes in females receiving resultant frozen-thawed embryos. Three hundred and eighty cows (BCS = 6.03 ± 0.06, DPP = 85.6 ± 0.94) and one hundred and seventeen heifers (BCS = 5.74 ± 0.03) were selected from Central Grassland Research and Extension Center (CGREC) herd near Streeter, ND to serve as embryo recipients. Recipients were randomly assigned to one of two treatments; 1) females were transferred on the uterine horn ipsilateral to the CL an embryo originating from a donor that received 90 mg Cu (as Cu disodium EDTA), 60 mg Mn (as Mn disodium EDTA), 30 mg Se (as sodium selenite), and 360 mg Zn (as Zn disodium EDTA) as an injectable TM supplement (6 ml Multimin®90 s.q.; Multimin USA, Fort Collins, CO) 16 d before embryo recovery (ITM, n = 196) or 2) females were transferred on the uterine horn ipsilateral to the CL an embryo originating from untreated donor (CON, n = 212). Females were housed in different paddocks within the research station, and had free choice access to Purina® Wind and Rain® Storm® 7.5 CP Avalia® 4 Original XPC Altosid® (Land O'Lakes, Inc., Arden Hills, MN) located in a mineral feeder in the paddocks for at least 30 d before initiation of transfer protocol. At the time of embryo transfer blood was collected from recipient cows (n = 336) to determine concentrations of progesterone and liver samples were collected for trace mineral analysis from a subset of cows (n = 21). Transrectal ultrasonography (Aloka SSD-500V equipped with a 7.5 MHz linear array probe; Corometrics Medical Systems Inc., Wallingford, CT) was used to determine presence, side, and size of any CL at the time of embryo transfer. Recipient was defined as

responding to transfer protocol when a CL was present in the ovary at d of transfer. Pregnancy diagnosis was done by transrectal ultrasonography (Aloka SSD-500V with 5.0 MHz linear array probe, Corometrics Medical Systems Inc.) on 36, 48, and 101 d after embryo transfer. Pregnancy rates were analyzed by the GLIMMIX Procedure of SAS (SAS Institute Inc., Cary, NC) and recipient response by the GLM Procedure of SAS, significant levels were stated as ($P \leq 0.05$). No differences ($P = 0.52$) were detected in pregnancy rates among ITM (45.9 %) and CON (49.1 %) treatments. However, females that had an active patch at d 10 before receiving an embryo (2nd GnRH) had greater ($P = 0.04$) pregnancy rates (51.68 %, 123 of 238) compared with females not having an activated patch (41.66 %, 70 of 168). In addition, females with an active patch at d 10 had greater total luteal tissue volume, 7.43 ± 0.32 cm^3 versus 5.26 ± 0.38 cm^3 ($P < 0.0001$) and greater concentrations of P4 , 4.50 ± 0.12 ng/mL versus 2.91 ± 0.14 ng/mL ($P < 0.0001$) at the time of transfer compared with females that did not have an active patch at d 10. Concentrations of progesterone and total luteal tissue volume were weakly correlated, r = 0.29 and $P < 0.0001$. In addition, embryo treatment or developmental stage did not influence calf birth weight or gestation length ($P > 0.49$). In summary, pregnancy outcomes were not influenced by treatment of donors with ITM at the initiation of their superovulation protocol, but were influenced by estrus expression by the time of GnRH2 in recipient females.

3.2. Introduction

Embryo transfer (ET) was primarily introduced to maximize the number of potential offspring produced by a high merit female (Jones and Lamb, 2008). The technique has the potential to lower the cost of genetic trading among countries, boost genetic improvement within herds, and increase marketing with purebred cattle (Spell et al., 2001). Technologies and techniques that improve success rates and efficiencies in ET (i.e. reduce cost) have the potential to be widely

adopted by producers who currently use ET, and have the potential to expand the utilization of ET to a new audience of producers.

During embryo transfer protocols, embryos produced by a donor cow averaging 7 d of age are transferred into a recipient cow, which will be responsible to house the embryo until calving. Several factors can influence the success rate of the technique, and they can be divided into recipient and embryo factors. Recipient selection cannot be overlooked, as the greatest loss in the embryo transfer industry is due to recipient failure and maintenance (Looney et al., 2006). Nutrition and DPP are the primary management factors to be looked when selecting recipients, pregnancy rates are greater when females have a minimum 50 DPP interval and moderate body condition score (Looney et al., 2006).

From the embryo standpoint the effects of donor mineral supplementation in recipient pregnancy rates and further offspring performance are still poorly understood; however, with novel concepts of epigenetics and fetal programming those 7 d of early development could potentially have long lasting impact on the progeny performance (Du et al., 2010).

In summary, many variables can affect the outcomes of embryo transfer techniques. Thus, we hypothesized that embryos harvested from donors receiving an injectable TM as well as embryo stage and quality could lead to increased pregnancy rates. Our objectives were to determine how embryo characteristics and recipient response to synchronization protocol could impact pregnancy rates.

3.3. Materials and methods

All procedures involving animals were approved by the North Dakota State University Animal Care and Use Committee (#A16051).

3.3.1. Animals and treatments

Three hundred and eighty cows (BCS = 6.03 ± 0.06, DPP = 85.6 ± 0.94) and one hundred and seventeen heifers (BCS = 5.74 ± 0.03) were selected from Central Grassland Research and Extension Center (CGREC) herd near Streeter, ND to serve as embryo recipients. Recipients were randomly assigned to one of two treatments; 1) females were transferred on the uterine horn ipsilateral to the CL an embryo originating from a donor that received 90 mg Cu (as Cu disodium EDTA), 60 mg Mn (as Mn disodium EDTA), 30 mg Se (as sodium selenite), and 360 mg Zn (as Zn disodium EDTA) as an injectable TM supplement (6 mL Multimin®90 s.q.; Multimin USA, Fort Collins, CO) 16 d before embryo recovery (ITM, n = 196) or 2) females were transferred on the uterine horn ipsilateral to the CL an embryo originating from untreated donor (CON, n = 212).

3.3.2. Study area

Recipient females were also housed at the CGREC; however, animals were located in different paddocks within the station. The research station is located in south-central North Dakota in the Missouri Coteau ecoregion (USDA-SCS, 1981). The mixed-grass prairie area is part of the North American Great Plains and mostly composed by fine-loamy mollisols (Bluemle, 1991). According to Limb (2018), prevalent plant communities in the area are composed by western wheatgrass (*Pascopyrum smithii* [Rydb.] À. Löve), green needlegrass (*Nassella viridula* [Trin.] Barkworth), blue grama (*Bouteloua gracilis* [Willd. ex Kunth] Lag. ex Griffiths), sedges (*Carex* spp.), prairie junegrass (*Koeleria macrantha* [Ledeb.] Schult.), sages (*Artemisia* spp.), goldenrods (*Solidago* spp.), Kentucky bluegrass (*Poa pratensis* L.), and western snowberry (*Symphoricarpos occidentalis* Hook.). Recipient females were stocked in different pastures, based on parity (i.e. cows or heifers) and paddock (i.e. soil type, grass DM production) characteristics. Stocking rate averaged 2.25 AUM/hectare.

3.3.3. Recipient's synchronization and embryo transfer

Females were synchronized for fixed-time embryo transfer (FTET) using the 7 d Co-Synch and CIDR estrus synchronization protocol (Fig 3.1). All females received a CIDR insert (Eazi–Breed[TM] CIDR 1.38 g of progesterone, Zoetis Inc., Kalamazoo, MI) and 100 µg gonadotropin-releasing hormone (GnRH, as 2 mL Factrel, Zoetis, Inc), followed in 7 d at 1800 by CIDR removal and 25 mg PGF$_{2\alpha}$ (as 2 mL of Lutalyse® HighCon, Zoetis Inc) followed in 36 (heifers) or 60 h (cows) by GnRH (GnRH2), then followed in 7 d by embryo transfer. At the time of CIDR removal all females received an estrus detection patch placed on the tailhead (ESTROTECT[TM] Inc., USA), and patches were evaluated for activation status ($\geq$ 50% colored or missing = Activated; <50% colored = Non-Activated) at the time of GnRH2 and again when each female was presented for FTET.

Liver samples were collected on the d of CIDR extraction as described for Exp. 1 from a subset of recipient females (n = 21) to document the baseline mineral status of the herd. Recipients were divided in 7 groups varying from 34 to 114 females, in order to facilitate animal handling and all females had equal access to native range pastures and free choice access to Purina® Wind and Rain® Storm® 7.5 CP Avalia® 4 Original XPC Altosid® (Land O'Lakes, Inc., Arden Hills, MN) in mineral feeder located at the paddocks for at least 30 d before transfer protocol started. Pastures had their own or a common handling facility where cows were brought to be synchronized and to receive embryos.

On d 7 after GnRH2 females were presented as candidates to receive embryos. Ovaries of each female were scanned via transrectal ultrasonography (Aloka SSD-500V equipped with a 7.5 MHz linear array probe; Corometrics Medical Systems Inc., Wallingford, CT) to determine presence, side, and size of any CL present. Vertical and horizontal measurements of each CL were

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recorded and luteal volume was calculated using the formula $V = 4/3\pi r^3$, where r was calculated as half of the average of vertical and horizontal measurements. Any lumen present within a CL was measured and its volume was calculated and subtracted from CL volume to calculate a total volume of luteal tissue present.

Females having a CL were considered as responsive to synchronization protocol and were randomly assigned to receive either 1) an embryo originating from a donor that received 90 mg Cu, 60 mg Mn, 30 mg Se, and 360 mg Zn as an injectable TM supplement (6 mL Multimin®90 s.q.; Multimin USA, Fort Collins, CO) 16 d before embryo recovery (ITM, n = 196) or 2) an embryo originating from untreated donor (CON, n = 212). After the confirmation of a CL on the ovary, an embryo straw was thawed and transferred according to the technique of the International Embryo Transfer Society (Savoy, IL) into the uterine horn ipsilateral to the CL. Each transfer was assigned a transfer score of 1 to 3 with 1 being an easy transfer placed deep into the uterine horn, whereas 3 was a very difficult transfer and/or a transfer where blood was present on the transfer gun when removed from the uterus.
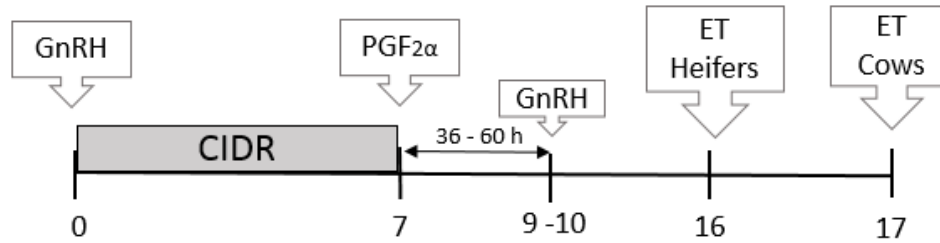
Figure 3.1. Schematic of Co-Synch and CIDR estrus synchronization protocol for recipient females.

Indicating that on d 0 females were treated with 100 µg GnRH (Factrel, Zoetis, Inc) and CIDR (Eazi–Breed™ CIDR 1.38 g of progesterone, Zoetis Inc., Kalamazoo, MI) insert. On d 7 at 1800, CIDR removal, 25 mg PGF$_{2\alpha}$ (Lutalyse® HighCon, Zoetis Inc), followed 36 h later by 100 µg of GnRH in heifers and by 60 h in cows. All females had their BCS scored on d 0. At CIDR removal females received estrus detection patches (ESTROTECT™ Inc., USA) that were evaluated at GnRH2 dose and transfer. Blood was collected from a subsample of cows at d 17.

3.3.4. Blood samples and immulite

On the d of transfer, blood samples were collected via coccygeal venipuncture using a Vacutainer® (serum, BD, Franklin Lakes, NJ) from recipient cows (n = 336) for determination of concentrations of progesterone. Samples were kept in a cooler with ice during collection, by the end of the d samples were placed at room temperature for 20 minutes before being centrifuged. Samples were centrifuged at 1500 × g for 20 minutes at 4°C, serum was collected and frozen until analyzed for concentrations of progesterone using an Immulite 1000 (Siemens, DPC Cirrus Inc., Flanders, NJ).

Interassay CV was 10% and pooled sample was 5.49, assay sensitivity was 0.5 ng/mL.

3.3.5. Pregnancy diagnosis, gestation length and calf body weight

Pregnancy rates to embryo transfer were determined by transrectal ultrasonography (Aloka SSD-500V with 5.0 MHz linear array probe, Corometrics Medical Systems Inc.) 36 d after transfer in cow recipients and 48 d after transfer in heifer recipients by identifying embryo heartbeat. A second ultrasound was done in cows 101 d after transfer to determine pregnancy losses.

Gestation length was calculated subtracting d of transfer from calving d, within 24 h from calving all calves were weighted using a scale.

3.3.6. Statistical analyses

Pregnancy data were analyzed by the GLIMMIX Procedure of SAS (SAS Institute Inc., Cary, NC), significant levels were stated as ($P \leq 0.05$). Cows were considered the experimental unit. The model included embryo treatment (CON or ITM), age class of recipient (heifer or cow), BCS, technician, flush number, transfer score and their appropriate interactions. The GLM Procedure of SAS (SAS Institute Inc., Cary, NC) was used to analyze recipient response to protocol, significant levels ($P \leq 0.05$) were separated by LSMEANS statement. The model included treatment, pregnancy rate, luteal tissue volume, concentration of P4, patch score at GnRH, and transfer score. Calf birth weight and gestation length were analyzed by the GLM Procedure of SAS (SAS Institute Inc., Cary, NC), the model included fixed effects of treatment and random effect of calf sex and embryo developmental stage. The Proc Means statement was used to summarize the trace mineral baseline of the herd. The Proc Corr statement of SAS (SAS Institute Inc., Cary, NC) was used to analyze the relationship between recipient variables.

3.4. Results

3.4.1. Trace mineral concentration baseline

A subsample (n = 21) of cows had their liver biopsied to assess TM baseline of the herd. Baseline mineral concentration for Se, Mn, Cu, and Zn were respectively, 2.15 ± 0.12, 12.49 ± 0.28, 98.34 ± 13.13, 126.19 ± 9.91 ug/g tissue DM (Table 3.1).

Table 3.1. Concentrations of liver mineral in recipient females at d 7.

Mineral	Mean Value	SE	Reference Range[1]
Cobalt, µg/g	0.23	0.01	0.10-0.40
Copper, µg/g	98.34	13.13	40.0-650.0
Iron, µg/g	205.54	14.50	170.0-750.0
Manganese, µg/g	12.49	0.28	5.50-15.0
Molybdenum, µg/g	4.15	0.07	1.80-4.70
Selenium, µg/g	2.15	0.12	0.60-3.30
Zinc, µg/g	126.29	9.91	90.0-500.0

[1]Normal ranges are based on MSU-DCPAH. Levels are determined to be adequate if within listed ranges (ug/g tissue DM).

3.4.2. Pregnancy rate

Pregnancy rates to embryo transfer were similar ($P = 0.52$) among recipient females that received embryos from donors in CON (49.1 %) and ITM (45.9 %) treatments, respectively (Table 3.2).

Embryo stage did not affect ($P = 0.78$) pregnancy rates with 49.53 % ± 0.03 of females that received a stage 4 embryo being pregnant (106 of 214). Moreover, pregnancies were similar ($P = 0.98$) among CON (49.57 % ± 0.05, 58 of 106) and ITM (49.48 % ± 0.05, 48 of 106) within stage 4 embryos.

In addition, pregnancy rates were not influenced ($P = 0.84$) by embryo quality with 47.79 % ± 0.02 of females that received a grade 1 embryo were pregnant (184 of 385). Pregnancies were similar ($P = 0.62$) among CON (49 % ± 0.03, 99 of 184) and ITM (46.44 % ± 0.04, 85 of 184) within grade 1 embryos.

Table 3.2. Pregnancy rates in beef females after transfer of embryos from dams receiving control or injectable trace mineral treatments at the initiation of superovulation protocol.

	Treatment		
	Control (CON)	Multimin (ITM)	P - value
		%	
Heifer recipients	43.4	49.01	0.56
Cow Recipients	50.9	44.8	0.28
All recipients	49.1	45.9	0.52

Transfer score played a role in pregnancy rates (P = 0.05), as transfer score 1 resulted in 50.62 % ± 0.027 of recipients pregnant while transfer score 2 and 3 resulted in 36.04 % ± 0.05 and 50.0 % ± 0.24, respectively (Table 3.3). In addition, pregnancy rates tended (P = 0.07) to be influenced by technician (Table 3.3).

Table 3.3. Pregnancy rates in beef females influenced by technician and transfer score.

	Pregnancy rates
Technician	%
1	54[a]
2	51[ab]
3	42[b]
Transfer score	
1	51[a]
2	36[b]
3	50[a]

[abc] Means not sharing a common superscript within each category are different ($P < 0.05$).

3.4.3. Recipient response to protocol

Females that had activated estrus detection patches on d 10 of the synchronization protocol (GnRH 2; 51.68 % ± 0.03) had greater pregnancy rates (P = 0.04) compared with those that did not have activated patches (41.66 % ± 0.04; Table 3.4). Total luteal tissue volume was also affected ($P < 0.0001$) by patch score at d 10 with females with an active patch presented greater tissue volume compared to ones that did not have an active patch (7.43 ± 0.32 cm^3 and 5.26 ± 0.38 cm^3,

respectively; Table 3.4). Moreover, females with an active patch had greater ($P < 0.0001$) concentrations of progesterone compared with females that did not have an activated patch (4.50 versus 2.91 ± 0.13 ng/mL, respectively; Table 3.4).

Table 3.4. Effects of patch score at d 10 on reproductive characteristics.

Reproductive characteristics	Patch at d 10		
	Activated	Not activated	*P* - Value
Pregnancy rate (%)	51.68 ± 0.03	41.66 ± 0.04	0.04
Total luteal tissue volume (cm^3)	7.43 ± 0.32	5.26 ± 0.38	0.0001
Concentration of progesterone (ng/mL)	4.50 ± 0.12	2.91 ± 0.14	0.0001

Pregnancy rates were similar ($P = 0.53$) between groups that had an activated estrus patch ($48.31 \% \pm 0.02$) at the time of transfer compared with those that did not have an activated patch ($44.44 \% \pm 0.05$; Table 3.5).

However, patch score at transfer influenced ($P = 0.009$) volume of luteal tissue, where females with an active patch had greater total volume compared to inactive patch females, 6.86 ± 0.28 cm^3 and 5.23 ± 0.55 cm^3, respectively (Table 3.5).

Moreover, concentration of P4 were also affected ($P < 0.0001$) by patch score at transfer. Females with an active patch at transfer presented greater concentration of P4 compared to inactive patch females, 4.07 ± 0.11 ng/mL versus 3.04 ± 0.22 ng/mL, respectively (Table 3.5).

Table 3.5. Effects of patch score at d 17 on reproductive characteristics.

Reproductive characteristics	Patch at d 17		
	Activated	Not activated	*P* - Value
Pregnancy rate (%)	48.31 ± 0.02	44.44 ± 0.05	0.53
Total luteal tissue volume (cm^3)	6.86 ± 0.28	5.23 ± 0.55	0.009
Concentration of progesterone (ng/mL)	4.07 ± 0.11	3.04 ± 0.22	0.0001

3.4.4. Concentration of progesterone and luteal tissue volume

There was a weak positive correlation between concentrations of progesterone and total luteal tissue volume, r = 0.29 and $P < 0.0001$ (Table 3.6). Days post-partum and concentration of progesterone ($P = 0.1$) tended to be correlated. However, no correlation was observed between total luteal volume and BCS ($P = 0.55$) as well as BCS and DPP ($P = 0.93$; Table 3.6.).

Table 3.6. Correlation between recipient variables.

	Recipient variables	
	Concentration of progesterone	Body condition score
Total luteal tissue volume	0.29[a]	0.02
Days post-partum	0.09[b]	- 0.04

[a] Correlation exists ($P < 0.0001$).

[b] Tendency for correlation ($P = 0.1$).

3.4.5. Calf birth weight and gestation length

There was no effects of embryo treatment in calf birth weight ($P = 0.58$) and gestation length ($P = 0.49$). In addition, developmental stage did not affect calf birth weight ($P = 0.66$) and gestation length ($P = 0.55$).

3.5. Discussion

In the present study, pregnancy rates were not affected by injectable trace mineral supplements provided to the donor females at the initiation of superovulation. Embryos were collected 7 d after AI, and the majority of them were classified as Morula, which are developmental stage 4. Meaning that the influence of treatment could have a direct effect just until that time point, we did not see any effect of treatment on quality grades and developmental stage in that certain point in time (d 16). However, some studies have correlated the importance of TM adequacy for fetus development as the concentration of them (Zn, Cu, and Mn) were higher in the conceptus compared to other reproductive tissues (Hostetler el at., 2003). Therefore, we were speculating if

treatment within those first 7 d of development could influence further development of the embryos even though they were transferred to recipient females that did not receive the injectable TM tested in Exp. 1.

The conceptus will rely on the dam for nutrient supply. In our study, donors were already receiving TM according to NRC requirements, apparently TM balance during that phase was already meeting the requirements to provide proper embryonic development and the extra dose of TM (via ITM administration) did not have an observable impact on embryo development. However, concepts of fetal programming states that maternal nutrition during gestation can lead to alteration in offspring metabolism and physiology which can affect conceptus further development (Barker, 1997). Also uterine environment can alter gene expression in the conceptus (Holland and Rakyan, 2013). In addition, early embryonic stages cannot be overlooked, as during the initial phases of development the genome is programmed and will impact performance of conceptus throughout its life (Barker, 1997). Even though in the current study we did not see any effect of the injectable TM in pregnancy rates, we could potentially have an effect of maternal nutrition on the conceptus further during development through histone modification and DNA methylation. This area of research is both a current and future area of fetal programming and DNA sequencing.

Several studies have shown that trace mineral supplementation can increase pregnancy outcomes (Sales et al., 2011; Mundell at al., 2012; Ahola et al., 2014; Brasche et al., 2014; Kirchhoff, 2015; Stokes et al., 2017); however, little is known if the effects on pregnancy outcomes are due to an effect on the embryo, the uterine environment, or both. We did not observe any effect of TM status in embryo characteristics (i.e. stage and quality) in Experiment 1, and embryo characteristics did not play a role in pregnancy rates either. However, studies have shown

conflicting data relating the impacts of embryo quality grade and developmental stage to pregnancy outcomes (Hasler, 2001; Spell et al., 2001; Kubisch et al., 2004; Rodrigues et al., 2018). As mentioned before, the majority of embryos harvested in our study were classified as stage 4 and quality 1. Potentially, the low number of embryos in other grades and stages may have suppressed any potential observable differences. Spell et al. (2001) corroborate our findings, showing no influence of embryo grading in pregnancy rates. In addition, the author stated the importance of recipient response to protocol over embryo characteristics.

In contrast, Rodrigues et al. (2018) reported a drop in pregnancy rates from 57% when grade 1 embryo were transferred to 43% using grade 3. Moreover, the author reported pregnancy rates averaging approximately 44% using frozen–thawed embryos, which support our pregnancy rate findings. Similar results were observed by Hasler (2001) when transferring fresh and frozen-thawed embryos where grade 1 embryos resulted in increased pregnancy rates compared to grades 2, 3, and 4. However, no difference was observed regarding developmental stage. In addition, Kubisch et al. (2004) showed increased odds of pregnancy success when transferring expanded blastocyst over blastocyst.

In the current experiment, recipient response to synchronization protocol was the major factor influencing pregnancy rates. According to McMillan et al. (1998), the success of the technique in the first 60 d relies more on the ability of the recipient in carrying the pregnancy than in the embryo to develop itself, assuming donors are adequately selected. In our experiment, females that had an activated patch on d 10 presented greater pregnancy rates than females that did not show estrus. In addition, those females also presented greater levels of progesterone and volume of luteal tissue. Hasler (2001) showed that pregnancy rates were affected by recipient d of estrus, stating that an early (d 9) or delayed (d 11) estrous display had a negative effect on

pregnancy rates. This fact is correlated with a mismatch between embryo age with stage of recipient estrous cycle (Hasler, 2001). Synchronization protocols aim to synchronize recipient cycle to embryo age, thus having an activated patch at d 10 means that cow is in synchrony with the protocol and should be prepared to receive a 7 d embryo at transfer d (d 17). In contrast, Aoki et al (2004) showed an increased pregnancy rate when dairy cows and heifers where on d 8 of estrous cycle at transfer compared to d 6 and d 7. Similarly, Lamb (2005) reported better results with d 8 recipients instead of d 6 when transferring more developed embryos (expanded blastocyst). In the current study, we also observed an effect of patch activation at d 10 and estrus display in pregnancy rates. However, no difference in pregnancy rates were observed because of patch activation at transfer (d 17) which could be due to delayed estrus display and a mismatch between recipient cycle and embryo age.

At d of transfer, optimum progesterone circulating in the blood ranges from 2 to 5 ng/mL (Remsen and Roussel, 1982; Niemann et al., 1985). However, Spell et al. (2001) reported pregnant females with levels lower than 0.5 ng/mL. We did not observe any correlation between pregnancy rates to circulating progesterone levels and luteal tissue volume; however, as expected progesterone levels increased with total luteal volume.

It is noteworthy the effect of transfer score and technician in pregnancy rates in our study, showing the importance they play in the technique outcome (i.e. pregnancy rate). The technician is responsible to determine not only the location that the embryo will be released but also to rate the degree of trauma during the technique. Schneider et al. (1980) reported a big fluctuation on pregnancy rates from 28 to 53% depending on technician skill level. Location were the embryo is transferred within the uterine horn have also shown to influence pregnancy rates, embryos transferred in an adjacent point to the external uterine bifurcation (shallow) resulted in 25% of

recipient pregnant while embryos transferred two-thirds from the bifurcation (deep) to the oviductal end resulted in 72%.

Our results showed that embryo characteristics as well as CL characteristics and progesterone levels did not influence pregnancy outcomes. Recipient response to synchronization protocol and transfer score were the major factors affecting pregnancy outcomes in our study.

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4. GENERAL DISCUSSION AND FUTURE DIRECTIONS

We conducted two experiments to investigate the effects of injectable trace mineral on embryo outcomes and pregnancy rates, using Multimin®90 which contains 15 mg Cu/mL (as Cu disodium EDTA), 60 mg Zn/mL (as Zn disodium EDTA), 10 mg Mn/mL (as Mn disodium EDTA), and 5 mg Se/mL (as sodium selenite). Our findings showed that the injectable TM was effective in increasing hepatic status of Se; however, no treatment effect was observed in response to Cu, Zn, and Mn.

Studies have shown effectiveness of the product in increasing Se and Cu status for at least 15 d, which reinforce our results regarding Se but contradicts Cu levels. In the present study, donor cows presented great concentration of Mo known as Cu antagonist, and that could be of the reasons we did not see any treatment effect in the experiment herein. In similar studies, Zn and Mn change due to treatment were noted but only when analyzing mineral concentration in the plasma.

Treated donors received an injection of the TM on d 0 and embryos were harvested on d 16. We did not see any effect of the TM in that window of time. Embryo production as well as quality grades and developmental stages were similar among treatments. So far, there is only one report done by Iowa State University researching this topic, where they looked into embryo outcomes in dairy donor cows receiving Multimin®90, showing a tendency to reduce the amount of unfertilized oocytes (UFO) produced.

It is important to reiterate that donors in our study were receiving TM incorporated to TMR according to NRC requirements. Moreover, the majority of the embryos harvested were classified as stage 4 and quality 1. The fact that we did not have many embryos in other stage or categories could have an influence our results. Lastly, there were no differences in pregnancy rates when

viable CON or ITM embryos were transferred. In our study, pregnancy rates were mainly affected by recipient response to protocol.

In summary, based on our findings Multimin®90 can be an efficient method to improve Se status in beef cattle herd. Thus, the present study strengthens the need for further investigation of the impact of the product in herds presenting TM deficiency. In addition, further investigations can be done to investigate if the product could have an effect during that short window in embryo development, as based on epigenetics modifications, the maternal diet can further influence the offspring development.