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POSGRADO EN PRODUCCIÓN ANIMAL

**IMPACTS OF THE INJECTIONS OF VITAMINS C AND E ON
REPRODUCTIVE EVENTS IN HOLSTEIN DAIRY CATTLE DURING A
SYNCHRONIZED ESTRUS**

THESIS

Submitted in partial fulfillment of the requirements for the degree of:

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Presented by:

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**IMPACTS OF THE INJECTIONS OF VITAMINS C AND E ON
REPRODUCTIVE EVENTS IN HOLSTEIN DAIRY CATTLE
DURING A SYNCHRONIZED ESTRUS**

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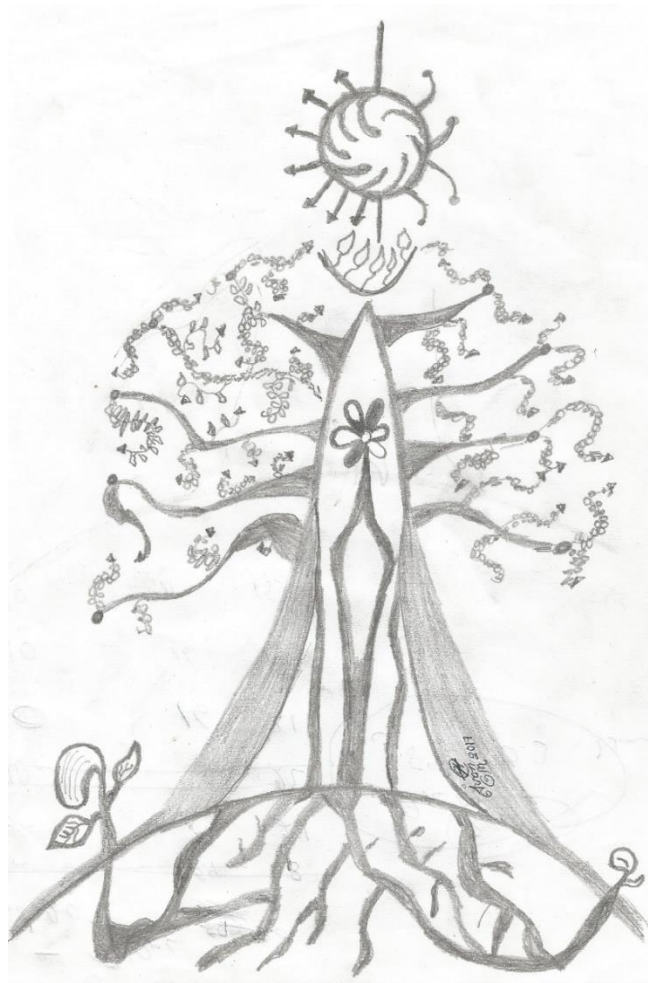
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LIST OF ABBREVIATIONS

AOPP	Advance oxidation protein products
Camp	Cyclic 3',5'-adenosine monophosphate
CIDR	Controlled internal drug release
CL	Corpus luteum
DPF0	Diameter of the largest follicle at CIDR removal
ERK	Extracellular signal-regulated kinases
HNE	4-hydroxy-trans-2-nonenal
JNK	C-jun n-terminal kinases
Keap1	Kelch-like ECH protein 1
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
NEFA	Non-esterified fatty acids
NF- κ B	Nuclear factor- κ B
Nrf2	Nuclear factor E2-related factor
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
sMaf	Maf proteins
THI	Temperature humidity index
VC	Vitamin C
VE	Vitamin E

DEDICATION

I like to dedicate my dissertation thesis to you..... that never doubt me.



"When I was a child, I spoke as a child, I understood as a child, I thought as a child, but when I became a man, I put childish things away " -I Corinthians 13:11-

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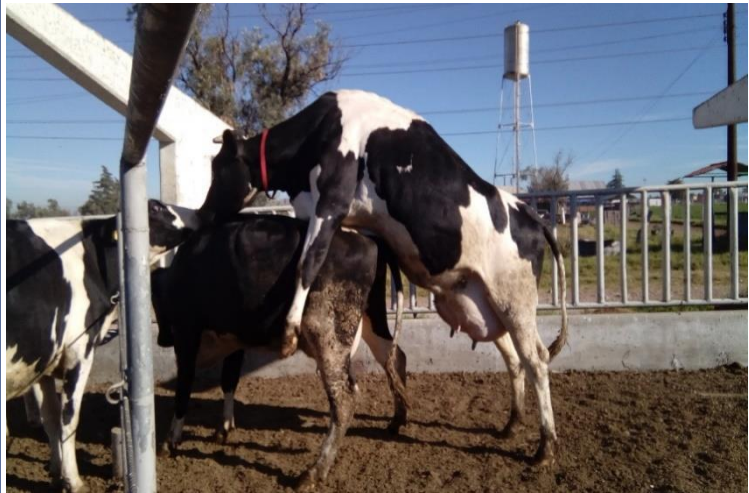
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GENERAL PREFACE



The “Impacts of the injections of vitamins C and E on reproductive events in Holstein dairy cattle during a synchronized estrus” is a thesis submitted as partial fulfillment of the requirements for the degree of Doctor in Sciences at the Animal Production Postgraduate Program, Chapingo Autonomous University, Mexico (Posgrado en Producción Animal, Universidad Autónoma Chapingo, México). This thesis represents the end product of the doctoral program carried out from 2014 to 2017. During this period a project was set up to develop a strategy to improve reproductive performance in dairy cattle during a synchronized estrus. The supplementation of vitamins C and E was part of this strategy. The former with base in previous experiences supporting a beneficial effect of injecting vitamin E on pregnancy rate in dairy cattle. However, at the beginning of this project we did not have much knowledge about the effects of vitamin C on cattle reproductive performance. Therefore, in order to establish the doses and times at which vitamins supplementation could improve fertility in dairy cattle, a literature review was done. The gathered information was used to write three review articles:

- **Review article I:** Situations leading to oxidative stress in dairy cattle. Submitted to Archivos de Zootecnia
- **Review article II:** Antioxidant supplementation in female ruminants during the periconceptual period: a review. Submitted to Revista Colombiana de Ciencias Pecuarias
- **Review article III:** Supplementation of ascorbic acid to improve fertility in dairy cattle- a review. Accepted by the Revista Mexicana de Ciencias Pecuarias

The information analyzed in the three review articles served as foundation to undertake a trial (research article I) testing the effects of injecting vitamins C and E separately and together on reproductive events during a synchronized estrus in

dairy cattle under heat stress conditions. The results from this trial revealed that injecting both vitamins increases the number of pregnancies in dairy cattle than giving them separately. Thereafter, a series of experiments evaluating the impacts of injecting both vitamins on ovarian structures development and functionality were carried out (results are shown in review article III), using increased doses of vitamins (research article II) and during a fixed time artificial insemination protocol (results are shown in review article III). The research articles I and II were send to scientific journals for publication:

- **Research article I:** Gonzalez-Maldonado J., Rangel-Santos R., Rodriguez-De Lara R., and Ramirez-Valverde G. 2017. Impacts of vitamin C and E injections on ovarian structures and fertility in Holstein cows under heat stress conditions. *Turkish Journal of Veterinary and Animal Sciences* 41: 345-350
- **Research article II:** Gonzalez-Maldonado J., Rangel-Santos R., Rodriguez-De Lara R., Ramirez-Valverde G., Ramírez-Bribiesca J.E., Vigil-Vigil J.M., and García-Espinosa MF., 2017. Effects of injecting increased doses of vitamins C and E on reproductive events in Holstein dairy cattle. Under revision by the *Revista Mexicana de Ciencias Pecuarias*

All articles (reviews and research) formatted following specific guides to authors of the scientific journal the article was submitted to.

GENERAL ABSTRACT

IMPACTS OF THE INJECTION OF VITAMINS C AND E ON REPRODUCTIVE EVENTS IN HOLSTEIN DAIRY CATTLE DURING A SYNCHRONIZED ESTRUS

High milk production and stressing environmental factors as heat stress, reduce reproductive performance in dairy cattle and decrease blood concentrations of vitamins C and E, which are necessary to sustain fertility in mammals. The objective of this thesis was to develop a reproductive strategy to improve pregnancy rate in Holstein dairy cows after a synchronized estrus by supplementing vitamins C and E. To accomplish this objective, Holstein dairy cows were assigned to one of two groups: 1, Control (n=80) cows were not injected with vitamins. 2, vitamins C and E (n=125), the cows were injected with vitamin C (3,000-6,000 mg) and E (3,000-6000 IU) before and after a synchronized estrus during four experiments (I-IV). The impacts of vitamins injections on reproductive performance in dairy cattle were measured on size of the preovulatory follicle and corpus luteum, blood concentrations of estradiol and progesterone, as well as pregnancy rate at 30-45 days after artificial insemination. In general, the supplementation with vitamins did not affect ($p>0.05$) size of preovulatory follicle or corpus luteum. Similarly, blood concentrations of estradiol and progesterone were not affected ($p>0.05$) by vitamins supplementation. Regarding pregnancy rate, more cows were found pregnant after artificial insemination in the group injected with vitamins than those in control group. However, significant differences were not obtained between groups in any of the experiments ($p>0.05$), but a tendency was found in experiment II ($p=0.06$). In conclusion, injections of vitamins C and E before and after a synchronized estrus did not affect preovulatory follicle and corpus luteum development, blood concentrations of estradiol and progesterone, but tend to improve pregnancy rate in dairy cattle.

Key words: ascorbic acid, bovine, corpus luteum, fertility, follicle, tocopherol

Doctor in Sciences Thesis, Innovación Ganadera, Universidad Autónoma Chapingo

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RESUMEN GENERAL

IMPACTOS DE LA INYECCIÓN DE VITAMINAS C Y E EN EVENTOS REPRODUCTIVOS EN GANADO LECHERO HOLSTEIN DURANTE UN CELO SINCRONIZADO

La elevada producción de leche y factores ambientales, como el estrés calórico, reducen el comportamiento reproductivo del ganado lechero y disminuyen las concentraciones sanguíneas de vitaminas C y E, las cuales son necesarias para mantener la fertilidad en mamíferos. El objetivo de esta tesis fue desarrollar una estrategia reproductiva que permitiera incrementar la tasa de gestaciones en vacas lecheras Holstein después de un celo sincronizado, mediante la suplementación de vitaminas C y E. Para lograr este objetivo, vacas lecheras Holstein fueron asignadas a uno de dos grupos: 1, Control (n=80), las vacas no fueron inyectadas con vitaminas. 2, vitaminas C y E (n=125), las vacas fueron inyectadas con vitaminas C (3,000-6,000 mg) y E (3,000-6,000 UI) antes y después de un celo sincronizado durante cuatro experimentos (I-IV). Los impactos de las inyecciones de vitaminas en el comportamiento reproductivo de vacas lecheras fueron medidos en el tamaño del folículo preovulatorio y cuerpo lúteo, concentraciones sanguíneas de estradiol y progesterona, así como en la tasa de gestaciones a los 30-45 días después de la inseminación artificial. En general, la suplementación con vitaminas no afectó ($p>0.05$) el tamaño del folículo preovulatorio y cuerpo lúteo. De manera similar, las concentraciones sanguíneas de estradiol y progesterona no fueron afectadas ($p>0.05$) por la suplementación con vitaminas. En relación a la tasa de gestaciones, más vacas fueron diagnosticadas gestantes después de la inseminación artificial en el grupo inyectado con vitaminas que en el control. Sin embargo, las diferencias no fueron significativas ($p>0.05$), pero se obtuvo una tendencia en el experimento II ($p=0.06$). En conclusión, las inyecciones de vitaminas C y E antes y después de un celo sincronizado no afectan el desarrollo del folículo preovulatorio y cuerpo lúteo, las concentraciones sanguíneas de estradiol y progesterona, pero tienden a incrementar la tasa de gestaciones en vacas lecheras Holstein.

Palabras clave: ácido ascórbico, bovinos, cuerpo lúteo, fertilidad, folículo, tocoferol

Tesis de Doctor en Ciencias, Innovación Ganadera, Universidad Autónoma Chapingo
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**CHAPTER 1. GENERAL
INTRODUCTION**



The improvement in genetic merit to milk production and heat stress predispose to low fertility in dairy cattle (De Rensis et al., 2015; Dobson et al., 2007). They are also known to cause oxidative stress in cattle (Bernabucci et al., 2002; Castillo et al. 2003). Oxidative stress results from an imbalance between antioxidants and prooxidants (Yoshikawa & Naito, 2002), which compromise cattle fertility (Rizzo et al., 2012). Other situations leading to oxidative stress includes reproductive practices such as palpation, insemination and administration of progesterone release devices (Cingi et al., 2012; Sönmez et al., 2009). Oxidative stress is counteracted by antioxidants such as vitamin C and E. Vitamin C affects fertility by serving as antioxidant, cofactor to hormone and collagen synthesis (Luck et al., 1995). In addition, it is necessary to re-activate vitamin E after fulfilling its antioxidant activity (Chauhan et al., 2015).

Vitamin E is essential for follicle development, oocyte quality and embryo survival. According to Martin and Moore (1939), disruption of follicle development, estrous cycle abnormalities and pregnancy loss are some of the outcomes in females with vitamin E deficiency. Abnormalities in gonadotropin secretion (Das & Chowdhury, 1999; Umeda et al., 1982) and suppression of gonadal function (Akazawa et al., 1987) may be responsible, at least in part, for these outcomes. On the other hand, supplementation of vitamin E has been effective for improving fertility (Baldi et al., 2000) and prolificacy (Mahan, 1991; Mahan, 1994), likely due to a higher fertilization rate (Marques et al., 2010), better oocyte quality (Miclea et al., 2011) or increased embryo quality and survival (Olson & Seidel, 2000; Sales et al., 2008).

The information regarding vitamin C supplementation on reproductive events in dairy cattle is scarce, most likely because it is believed that the endogenous production of this vitamin is enough to meet nutritional requirements in ruminants (NRC, 2001). However, if situations such as elevated milk production, heat and oxidative stress reduce blood concentrations of vitamin C and E (Calamari et al., 1999; Joźwik et al., 2012; Padilla et al., 2006), and considering its reproductive relevance, it is logical to suspect that supplementation of these vitamins might be

advantageous to improve fertility in dairy cattle under those situations. The objective of the present thesis was to develop a reproductive strategy to increase pregnancy rate in dairy cattle by supplementing vitamins C and E before and after a synchronized estrus.

The present thesis comprises two main chapters, a literature review and research articles. The literature review chapter is integrated by three review articles. The objective of this chapter was to gain knowledge of the relationship between fertility, oxidative stress and vitamins C and E supplementation, which was then used to design the experimental procedures to test the effects of these vitamins on reproductive events in dairy cattle. The research articles tested the effects of vitamins C and E injections on ovarian structures development, steroidogenic activity and pregnancy rate in dairy cattle.

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CHAPTER 2. REVIEW ARTICLES



2.1 Preface to literature review

This chapter consist of three review articles. The goal of the first article was to review the physiology and conditions leading to oxidative stress in dairy cattle. Oxidative stress was reviewed because the vitamins under study in the present thesis are antioxidants, which prevent oxidative damage. After recognizing that dairy cattle are susceptible to oxidative stress damage, the effects of oxidative stress on reproductive events during the periconceptual period were revised in the second review. This period was chosen because supplementation of antioxidants in the present thesis was made at this time. Therefore, it will be advantageous to know the effects of antioxidants supplementation during this period. The third article focuses on the effects of vitamins C and E on reproduction.

Submitted to the Journal of Archivos de Zootecnia

2.2 Situations leading to oxidative stress in dairy cattle

Causas de estrés oxidativo en vacas lecheras

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Antioxidants.

Bovine.

Cellular response.

Oxidants.

Antioxidantes.

Bovinos.

Respuesta celular.

Oxidantes.

2.2.1 Summary

Summary. Oxidative stress results from the inability of the antioxidant system to prevent free radicals damage. Free radical such as reactive oxygen species are normally produced by living organism, at controlled production rate they perform physiological functions as signal transduction molecules. However, situations leading to an overproduction that surpasses antioxidant capacity creates oxidative stress. As a consequence, damage to cell membrane, protein, DNA and cell death are observed. Dairy cattle are susceptible to oxidative stress, which can compromise their health and productivity. Situations such as infections, metabolic disorders and heat stress are known to cause oxidative stress in cattle either by depleting body antioxidants concentrations or by increasing endogenous free radical production. The organism response to oxidative stress by activating cell factors that after evaluating the damage to cell, a repair or death signal will be programmed. The objective of this review is to empower the reader with knowledge related to oxidative stress and to provide information on the situations leading to this type of stress and the cellular response to it in dairy cattle.

2.2.2 Resumen

Resumen. El estrés oxidativo resulta de la incapacidad del sistema antioxidante para prevenir el daño causado por radicales libres. Los radicales libres, como las especies reactivas de oxígeno, son normalmente producidos por organismo vivos, bajo condiciones normales sirven como señales de transducción. Sin embargo, situaciones que conlleven a una sobreproducción que sobrepase la capacidad del sistema antioxidante causan estrés oxidativo. Como consecuencia, se presenta daño en membranas celulares, proteínas, ADN y muerte celular. Las vacas lecheras son susceptibles a estrés oxidativo, lo cual compromete su salud y productividad. Factores como infecciones, desordenes metabólicos y estrés calórico causan estrés oxidativo en bovinos al disminuir las concentraciones corporales de antioxidantes o al incrementar la producción endógena de radicales libres. El organismo enfrenta al estrés oxidativo por medio de la activación de factores celulares, los cuales después de evaluar el daño causado a la célula, programan una señal de reparación o muerte. El objetivo de esta revisión es empoderar al lector con conocimiento relacionado con estrés oxidativo y proveer información de las causas que conllevan a este tipo de estrés y la respuesta celular en vacas lecheras.

2.2.3 Introduction

Oxidative stress is “the state at which oxidative forces exceed the antioxidant system due to loss of balance between them” (Yoshikawa and Naito, 2002). An imbalance of this nature may result in reduced concentrations of antioxidants or increased production of free radicals. Free radicals are oxidative forces; they are atoms, molecules or compounds with a short life and an unpaired electron that makes them unstable and highly reactive (Bhattacharya, 2014), capable of threatening life in high and uncontrolled concentrations. Fortunately, living organisms counteract free radicals with antioxidants, which significantly delay, prevent or inhibit damage of a substrate by free radicals (Halliwell and Gutteridge, 1995). The term “substrate” includes everything found in a live organism (Halliwell *et al.*, 1995).

There are several types of free radicals (Halliwell and Whiteman, 2004), but the most relevant in biological systems are reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Aprioku, 2013). A regulated amount of free radicals is normally produced in living organisms; these are not harmful, but they are used as signal transduction molecules. Common endogenous sources of free radicals include mitochondrial production of ATP (Jastroch *et al.*, 2010), degradation of purines by xanthine oxidase (Kuppusamy and Zweier, 1989), phagocytosis of microorganisms (Knight, 2000), inflammation (Mittal *et al.*, 2014) and oxidation of very long-chain fatty acids (Reddy, 2001). Situations leading to additional production of ROS and consequently to oxidative stress in farm animals includes heat stress, inflammation, dietary imbalances, respiratory diseases, and parasites (Celi and Gabai, 2015). Once produced, additional free radicals will damage cells by extracting an electron from a substrate (lipids, proteins or DNA), making them more stable and less reactive.

Oxidative stress opens a novel field of research in ruminant medicine (Celi, 2011). However, there is evidence of oxidative stress involved in events that compromise dairy cattle welfare. The objective of this paper is to review existing knowledge related to oxidative stress and to provide information on the situations leading to this type of stress.

2.2.4 Oxidation of lipids

Damage by ROS or lipid oxidation, also termed peroxidation, occurs when a free radical steals an electron (hydrogen) from a lipid. Polyunsaturated fatty acids (PUFA) from the cell membrane are highly susceptible to oxidative damage due to the presence of methylene groups, which are characterized by a weak carbon-hydrogen bond (Bochkov *et al.*, 2010). Basically, an ROS will generate a lipid radical by abducting a hydrogen molecule from PUFA. The lipid radical that reacts with oxygen will generate a lipid peroxy radical, which will abstract another electron from the nearest PUFA creating a new lipid radical and a lipid hydroperoxide. The new lipid radical will react with oxygen as indicated above. This is a self-sustained process that could convert all the cell membrane

PUFA into lipid hydroperoxides. Additionally, hydroperoxides can react with free transitional metals such as Fe^{2+} and Cu^+ to produce a lipid alkoxy radical, which can abstract an electron from another PUFA, creating a new lipid radical (Esterbauer *et al.*, 1990).

Peroxidation results in structural changes in the cell membrane. According to Greenberg *et al.* (2008), fatty acid peroxidation will change the normal fluid mosaic of the cell membrane to one with protruded fatty acids known as the whisker model, which may serve to elicit cell phagocytosis from macrophages.

In addition to hydroperoxides, peroxidation will render endoperoxides (Valko *et al.*, 2006) that can be fragmented to generate reactive carbonyl species such as malondialdehyde (MDA) and 4-hydroxy α -trans-2-nonenal (HNE), depending on the type of fatty acid oxidized. Oxidation of lipids containing ω -6 PUFAS will give rise to HNE, while those containing three or more methylene interrupted double bonds will produce MDA (Esterbauer *et al.*, 1991). Due to its short life, ROS will normally react with the nearest substrate, but reactive carbonyl species have a longer half-life and are able to migrate through membranes and cytosol, extending damage to other cell constituents as well as the membrane (Pamplona, 2008). MDA is mutagenic (Niedernhofer *et al.*, 2003); it impedes the mechanism of DNA repair after damage (Feng *et al.*, 2006) and alters physiological functions of proteins (Rittié *et al.*, 2002) by forming adducts with DNA and proteins.

Elevated concentrations of MDA have been reported in cows affected with mastitis (Ranjan *et al.*, 2005). In fact, a positive correlation between MDA and somatic cells counts was reported by Suriyasathaporn *et al.* (2006), indicating that antioxidant capacity of cows is compromised during mastitis. Interestingly, Weiss *et al.* (2004) found that udder infection leads to decreased concentrations of the antioxidant vitamin C, evidence that oxidative stress is involved in the pathology of mastitis (Atakisi *et al.*, 2010). A source of ROS leading to lipid peroxidation and formation of MDA could be bacterial lipopolysaccharides. Lipopolysaccharides are components of the outer cell membranes of bacteria and are considered endotoxins, capable of inducing inflammatory response in animal cells (Raetz and Whitfield, 2002). They are released during bacterial growth, division and lysis. Clinical mastitis has been induced in cows challenged with intramammary infusion of lipopolysaccharides (Zimov *et al.*, 2011), and increased MDA concentrations have been measured in bovine mammary epithelial cells cultured with lipopolysaccharides (Shi *et al.*, 2016).

Another source of lipid peroxidation in cattle is parasitic infection (Ellah, 2013). Cows infected with lungworms and fasciolosis have decreased antioxidant capacity and augmented lipid peroxidation, while infected animals have higher concentrations of MDA than those not infected (Bahrami *et al.*, 2014; Silva *et al.*, 2016). Lipid peroxidation could result from over production of ROS during host defense by phagocytes. Phagocytes use NADPH-oxidase system in their cell membrane to generate superoxide during phagocytosis (Roos, 1991). Nitric oxide also generated during parasite infection by

phagocytes can react with superoxide to form peroxynitrite. Both nitric oxide and peroxynitrite are used as weapons to kill parasites (Brunet, 2001; Linares *et al.*, 2001), but peroxynitrite produces MDA on the side (Radi *et al.*, 1991).

2.2.5 Protein oxidation

Protein oxidation is defined as “covalent modification of a protein induced by direct reaction with reactive oxygen species or indirect reaction with secondary by-products of oxidative stress” (Zhang *et al.*, 2013), which leads to oxidation of amino acid residue side chains, peptide bond cleavage, formation of cross-like aggregates and ultimately to alterations in protein structure and functionality (Stadtman and Levine, 2003). All the amino acid residues of a protein are susceptible to oxidative damage, but cysteine and methionine are considered the most sensitive (Berlett and Stadtman, 1997). However, only with these two did reversible oxidation occur, giving them antioxidant properties. According to Yan (2014), irreversible oxidation of amino acid residues may result in loss of protein function. However, on the contrary, reversible oxidation serves as a protein function regulator. According to Levine *et al.* (1996), under oxidative stress conditions, preferential oxidation of methionine allows the protein to maintain its biological function.

Abstraction of hydrogen from the α -carbon of the amino acid generates a carbon-centered radical that can also abstract hydrogen from thiols or react with iron to render peroxy radicals. Peroxy radicals are converted to alkyl peroxides by reaction with superoxide or by abstraction of a hydrogen molecule from other molecules. Alkyl peroxide produces an alkoxy radical by reacting with a hydroperoxy radical, which can undergo protein peptide bond cleavage by α -amidation or diamide pathway. Other radicals resulting from protein oxidation include thiyl radicals generated by hydrogen abstraction from free thiol groups or cleavage of disulfide linkages, chloramines produced by hypochlorous acid reacting with proteins and carbonyl groups by oxidation of lysine, proline, arginine and threonine (Hawkins and Davies, 2001; Stadtman and Levine, 2003).

Protein oxidation has been evaluated in dairy cattle by assessing the status of advanced oxidation protein products (AOPP). AOPP are produced by hypochlorous acid that reacts with proteins during neutrophil activation (Bordignon *et al.*, 2014). Cows with high concentrations of AOPP had a higher embryo mortality incidence after artificial insemination, probably due to a pathogen eliciting an inflammatory response in the uterus (Celi *et al.*, 2011; Celi *et al.*, 2012). Moreover, parasitic infection has also increased AOPP blood serum concentrations.

2.2.6 DNA oxidation

Oxidative damage to DNA occurs at abasic sites, purine and pyrimidine bases. Abasic sites are produced by hydrolysis of the N-glycosylic bond, while free radical attack at positions 1, 2 or 4 of the sugar residues gives rise to oxidized abasic sites (Häring *et al.*,

1994). Oxidative damage can also be inflicted on purine and pyrimidine bases. The oxidative damage products of purines and pyrimidines are discussed by Cadet and Wagner (2013) and, according to David *et al.* (2007), due to the high susceptibility of guanine to oxidative damage; 7,8-dihydro-8-oxo-2'-deoxyguanosin is the most studied product of oxidative DNA damage.

Oxidative damage to DNA in dairy cattle was reported by Ellah *et al.* (2014). According to these authors, dairy cows during the dry period experience greater oxidative DNA damage than during other phases of lactation. In a different study, Ellah *et al.* (2016) found that during the transition period, the concentration of DNA oxidative damage products was greater during pre-partum than after parturition. These studies suggest that oxidative stress is more severe during the dry period. However, Sharma *et al.* (2011) and Gong and Xiao (2016) agree that the first weeks post-partum are the most stressful for dairy cattle. They reported increased lipid peroxidation and reduced antioxidant capacity in cows during the first days of lactation compared with cows 30 days after calving. In concordance with these findings, Omid *et al.* (2016) reported higher antioxidant capacity in cows at the end of lactation. Thus, it seems that oxidative stress is established before and after parturition.

The degree of oxidative damage is expected to be greater after parturition, when a negative energy balance predisposing lipid mobilization will favor free radical formation (Piccione *et al.*, 2007; Pedernera *et al.*, 2010). In addition, cows in early lactation that experience a negative energy balance have lower concentrations of antioxidants such as vitamin C, tocopherol and glutathione (Cigliano *et al.*, 2014; De Bie *et al.*, 2016). On the other hand, Mandebvu *et al.* (2003) suggest that antioxidant capacity in dry cows may be compromised due to the low content of antioxidants in rations formulated at this stage of lactation. In addition, low activity of the hepatic antioxidant paraoxonase-1 has been found in cows during the transition period (Turk *et al.*, 2008).

Reactive oxygen species can also induce DNA strand break. The mechanism leading to strand break begins with hydrogen being stolen from sugar (2-deoxyribose), causing the formation of a carbon base radical, which in the presence of oxygen is converted to a peroxy radical. This can abstract hydrogen molecules from neighboring sugars and lead to strand break (Kryston *et al.*, 2011). In dairy cattle, increased hepatic DNA strand break has been identified as the transition period progresses, resulting in hepatocytes apoptosis (Tharwat *et al.*, 2012). The latter indicates that during this period liver functionality is compromised, explaining the reduction in the previously mentioned hepatic antioxidant production.

2.2.7 Cellular responses to oxidative stress

The increase in production of reactive oxygen species is counterattacked by well-organized response elements. One of them is the nuclear factor E2-related factor (Nrf2). Under non-oxidative stress conditions Nrf2 is found in the cell cytoplasm bound to its regulator, the kelch-like ECH protein 1 (Keap1), but an increase in reactive oxygen

production causes oxidation of Keap1 and the release of Nrf2. This can be translocated to the cell nucleus where it associates with Maf proteins (sMaf) and bind to antioxidant responsive elements (ARE), which in turn control the expression of several antioxidants, including glutathione, thioredoxin and NADPH (Gorrini *et al.*, 2013; Hayes and Dinkova-Kostova, 2014).

Activation of Nrf2 helps to ameliorate oxidative stress. In dairy cattle, activation of Nrf2 in mammary epithelial cells exposed to heat shock stress reduces the production of reactive oxygen species and improves cell survival (Jin *et al.*, 2016). Like heat stress, high concentrations of NEFA and ketosis are known to induce oxidative stress damage. NEFA produces oxidative stress damage and apoptosis in hepatocytes by activating p38 mitogen-activated protein kinase (MAPK) and the subsequent increased expression of transcription factor p53 and the down regulation of Nrf2 (Song *et al.*, 2014). The guardian of the genome, p53, protects the cell from oxidative damage under low level of oxidative stress, but induces cell death when oxidative stress increases (Liu and Xu, 2011).

As the result of incomplete metabolism of NEFA in liver, the cows develop ketosis, which is characterized by a high blood concentration of ketone bodies during periods of negative energy balance. Ketone bodies such as β -hydroxybutyrate produce similar damage to hepatocytes such as NEFA (Song *et al.*, 2016). In addition, Gessner *et al.* (2013) found that after calving the mRNA for genes controlled by Nrf2 declined. Thus, the cell antioxidant response to oxidative insult is compromised in cows with negative energy balance after parturition.

The nuclear factor- κ B (NF- κ B) is also activated during oxidative stress events. Similar to Nrf2, NF- κ B is sequestered in the cell cytoplasm bound to a protein I κ B. However, increased production of reactive oxygen species breaks the bond between NF- κ B and I κ B, allowing translocation of NF- κ B to the cell nucleus, where it stimulates the expression of pro-inflammatory factors (Gloire *et al.*, 2006). In dairy cattle, oxidative stress and activation of NF- κ B has been observed in cows with acidosis, together with a down regulation of Nrf2 (Abaker *et al.*, 2017). Another situation leading to NF- κ B turn on is ketosis. Shi *et al.* (2014) reported that hepatocyte damage induced by β -hydroxybutyrate was mediated by increased activation of NF- κ B and expression of pro-inflammatory factors (tumor necrosis factor- α and interleukin-6). Interestingly, the expression of these pro-inflammatory factors was reduced by antioxidant treatment.

The mitogen activated protein kinase (MAPK) is turned on by increased reactive oxygen species concentrations. It is integrated by three subfamilies, the extracellular signal—regulated kinases (ERK), C-Jun N-terminal kinases (JNK) and p38. The first subfamily is normally involved in the cell survival process and the last two are implicated in apoptotic cell death, but not always (Wada and Penninger, 2004). Cell treatment with hydrogen peroxide resulted in cell apoptosis and activation of the three subfamilies of MAPK, but cell death was decreased by inhibition of ERK and the contrary occurred when JNK was inhibited (Wang *et al.*, 1998). In dairy cattle, Tian *et al.* (2014) suggest that the oxidative stress damage and apoptotic cell death induced by β -hydroxybutyrate in abomasum smooth muscle cells is responsible for abomasum displacement. The latter occurred under the upregulation of p38 and JNK, but downregulation of ERK.

2.2.8 Conclusions

Oxidative stress occurs in dairy cattle. Knowledge of the physiology of oxidative stress in disrupting cell homeostasis and dairy cattle health can support the incorporation of antioxidants in treatments of diseases, parasitic infections and metabolic disorders.

2.2.9 Bibliography

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2.3 Antioxidant supplementation in female ruminants during the periconceptional period:

A review

Suplementación de antioxidantes en hembras rumiantes durante el periodo periconcepcional:

Revisión de literatura

Suplementação de antioxidantes em fêmeas de ruminantes durante o período

periconcepcional: Revisão de literatura

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2.3.1 Abstract

Oxidative stress is the result of an imbalance between free radicals and antioxidants. Under normal physiological conditions, free radicals are involved in reproductive events such as cell cycle activation, ovulation and luteolysis. However, when an overproduction of free radicals surpasses antioxidant capacity, oxidative damage, reproductive anomalies and diminished fertility occur. Supplementation with antioxidants prevents oxidative damage and can be incorporated into reproductive management to improve fertility in females. Selection of the preovulatory follicle, ovulation, fertilization, embryo development and formation of the corpus luteum occur during the periconceptual period. This is a dynamic period and the events are susceptible to oxidative stress damage. Therefore, the objective of this review article is to share with the reader knowledge related to the effect of oxidative stress on reproductive events during the periconceptual period, as well as to address the topic of antioxidant supplementation during this period.

Key words: *embryo, fertility, oocytes, ovulation, stress.*

2.3.2 Resumen

El estrés oxidativo es generado por un desbalance entre los radicales libres y antioxidantes. En condiciones fisiológicas normales, los radicales libres participan en eventos reproductivos tales como activación del ciclo celular, ovulación y luteólisis. Sin embargo, cuando estos son producidos en cantidades que sobrepasan la capacidad antioxidante del organismo producen daño oxidativo y trastornos reproductivos que disminuyen la fertilidad de la hembra. La suplementación con antioxidantes previene el daño oxidativo y su incorporación a programas de manejo reproductivo puede ser una opción para mejorar la fertilidad de la hembra. La selección del folículo preovulatorio, ovulación, fecundación, desarrollo embrionario y formación del cuerpo lúteo ocurren durante el periodo periconcepcional. Este es un periodo dinámico y los eventos que ocurren dentro de él son susceptibles a daño por estrés oxidativo. Por tanto, el objetivo de este trabajo de revisión es proveer al lector con conocimiento sobre el efecto del estrés oxidativo en eventos reproductivos durante el periodo periconcepcional, así como discutir la suplementación de antioxidantes en rumiantes durante este periodo.

Palabras clave: *embrión, estrés, fertilidad, ovocito, ovulación.*

2.3.3 Resumo

O stress oxidativo é gerado por um desequilíbrio entre radicais livres e antioxidantes. Sob condições fisiológicas normais, os radicais livres participam de eventos reprodutivos, como ativação do ciclo estral, ovulação e luteólise. No entanto, quando é produzido em quantidades que excedem a capacidade antioxidante do organismo, produzem danos oxidativos e distúrbios reprodutivos que diminuem a fertilidade da fêmea. A suplementação com antioxidantes previne o dano oxidativo e sua incorporação em programas de gerenciamento reprodutivo pode ser uma opção para melhorar a fertilidade da fêmea. A seleção do folículo pré-ovulatório, ovulação, fecundação, desenvolvimento embrionário e formação do corpo lúteo ocorrem durante o período periconcepcional. Este é um período dinâmico e os eventos que ocorrem são suscetíveis ao dano por estresse oxidativo. Portanto, o objetivo dessa revisão é fornecer ao leitor conhecimento sobre o efeito do estresse oxidativo em eventos reprodutivos durante o período periconcepcional, e também discutir a suplementação com antioxidantes em ruminantes durante este período.

Palavras-chave: *embrião, fertilidade, ovócito, ovulação, stress.*

2.3.4 Introduction

Oxidative stress is defined as an imbalance between free radicals and antioxidants caused by an increased production of the former or decreased concentrations of the latter (Halliwell and Whiteman, 2004). A free radical is a highly reactive atom or molecule that contains one or more unpaired electrons in its outer orbit, and abstract an electron from other compounds to gain stability (Phaniendra *et al.*, 2015). Reactive oxygen species (ROS), and reactive nitrogen species are the main types of free radicals (Agarwal *et al.*, 2005). They are normally produced in the mitochondria, peroxisomes, and during inflammation and phagocytosis (Lobo *et al.*, 2010). Free radicals participate in diverse physiological processes without causing any harm, but high concentrations can cause oxidative stress and damage to lipids, protein and DNA (Silva and Coutinho, 2010). In farm animals, heat stress, dietary imbalances and bacterial infections cause an increase in free radical production and oxidative stress (Celi and Gabai, 2015).

Antioxidants prevent or inhibit oxidation of the substrate (lipids, protein or DNA) by donating electrons (Halliwell and Gutteridge, 1995). Antioxidants can be classified as enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase and glutathione oxidase, while the non-enzymatic group includes vitamins A, C and E, beta-carotene, and trace elements such as copper, manganese, selenium and zinc, which are cofactors of antioxidant enzymes (Leung, 1998; Agarwal *et al.*, 2012; Pisoschi and Pop, 2015).

Free radicals are normally involved in reproductive events such as follicular development, ovulation, corpus luteum development, luteolysis and early embryo development (Rizzo *et al.*, 2012). However, an imbalance between free radicals and antioxidants causes failure to conceive. Reactive oxygen species are part of the mechanism controlling ovulation (see next section of this manuscript), but failure to achieve enough intra-follicular concentrations to produce preovulatory follicle rupture causes follicular cyst in cattle (Rizzo *et al.*, 2009; Talukder *et al.*, 2014a). Similarly, Talukder *et al.* (2015) reported that cows with ovulatory oestrous cycles have greater concentrations of the antioxidant superoxide dismutase and lower concentrations of oxidative damage to lipid products than cows that did not ovulate. In addition, repeat breeder cows that fail to conceive after artificial insemination have higher serum concentrations of oxidative stress markers than pregnant cows (Rizzo *et al.*, 2007). In this regard, Celi (2011) mentioned that

oxidative stress indicators are higher in cows experiencing late embryo mortality. In buffaloes, blood concentrations of antioxidants vitamin E and β -carotene were lower and the intra-follicular concentrations of ROS were higher in animals in anestrus than those with normal oestrous cycles (Kahlon and Singh, 2004; Jan *et al.*, 2014).

Oxidative stress occurs at different periods of time in animal systems, and antioxidants may be supplemented to improve reproductive performance (Nayyar and Jindal, 2010). In this review, we are focusing our attention on the periconceptual period. The periconceptual period comprises the events preceding, including and immediately occurring after conception (Louis *et al.*, 2008). In this review, these events are restricted to follicle wave emergence, preovulatory follicle selection, ovulation, fecundation, early embryo development and corpus luteum formation. The reason for this restriction is that available hormone treatments allow control of these events and the time of occurrence can be predicted (Lucy *et al.*, 2004; Rahman *et al.*, 2008; Menchaca *et al.*, 2017). Therefore, if oxidative stress is suspected during the occurrence of events in this period, antioxidants can be supplemented to specifically protect/improve such events. The objective of this review article is to provide evidence of the impacts of oxidative stress during the periconceptual period. In addition, the effect of antioxidant supplementation during hormonal treatments in female ruminants will be addressed.

2.3.5 Effects of oxidative stress on preovulatory follicle development and oocyte competence

The follicles undergo primordial, primary, small preantral, large preantral and small antral stages before reaching the preovulatory stage (Braw-Tal and Yossefi, 1997). Mammalian females are born with a fixed number of primordial follicles from which a preovulatory follicle will be recruited. There are two types of follicular recruitment: initial and cyclic. Initial recruitment is a continuous process in primordial follicles that occurs before puberty, while cyclic recruitment starts after puberty under the control of increased gonadotropin production (McGee and Hsueh, 2000). After puberty, the antral follicles are recruited in a predictable wave fashion between estrus (Adams *et al.*, 2008). The follicle wave is preceded by a surge of FSH, which allows cyclic antral follicle recruitment (Fortune *et al.*, 2001). After recruitment takes place, selection and growth of the dominant follicle occurs under the influence of LH until the ovulatory stage occurs (Ginther,

2000). For a follicle to accomplish the dominant status, it must be capable of performing three fundamental tasks: expressing gonadotropin receptors, performing steroidogenesis and having access to IGF-I (Quirk *et al.*, 2004). Under conditions of declining progesterone production such as luteolysis, the dominant follicle will reach the ovulatory stage. The time required for a primordial follicle to reach the ovulatory stage is at least 80 days (Britt, 2008). During this period, the follicle and its enclosed oocyte are exposed to harmful conditions such as oxidative stress.

The oocyte from the primordial follicle to the preovulatory stage is arrested at the diplotene stage of prophase I. The preovulatory surge of LH breaks diplotene arrest by reducing the intra-oocyte concentration of cyclic 3',5'-adenosine monophosphate (cAMP) (Tripathy *et al.*, 2010), which is associated with a rise in the intra-oocyte concentration of hydrogen peroxide (H₂O₂) (Pandey and Chaube, 2014). The intra-oocyte generation of a tonic level of H₂O₂ is necessary to exit diplotene stage arrest, but high concentrations of H₂O₂ produce oocyte apoptosis (Chaube *et al.*, 2005; Tripathi *et al.*, 2009). The H₂O₂ is part of the ROS family. It is generated after the dismutation of superoxide radical by superoxide dismutase and can then be scavenged by glutathione peroxidase or catalase (Valko *et al.*, 2007). Potential intra-follicular sources of reactive oxygen species include steroidogenesis (Hanukoglu *et al.*, 1993), leukocytes (Brännström and Enskog, 2002) and ATP generation by mitochondrial electron transport (Kala *et al.*, 2016). In addition, the preovulatory peak of LH induces an increase in ROS production (Yacobi *et al.*, 2007), which is necessary to accomplish ovulation (Shkolnik *et al.*, 2011).

Embryo implantation was reduced when the oocyte came from a follicle with a high percentage of granulosa cells producing ROS (Jancar *et al.*, 2007). Apoptosis induced by oxidative stress to granulosa cells (Shen *et al.*, 2012) implies a reduction in the supply of nutrients and signal molecules to the oocyte (Kidder and Vanderhyden, 2010), which may disrupt oocyte meiotic maturation (Ratchford *et al.*, 2008). Additionally, ROS causes DNA fragmentation, cell cycle arrest, and apoptosis (Chaube *et al.*, 2005; Prasad *et al.*, 2016). Thus, on one hand, a moderate increase in ROS production is used as a signal to break cell cycle arrest, but on the other, high concentrations disrupt oocyte quality. It is likely that the required amount of ROS within the follicle and oocyte is regulated, at least in part, by the intra-follicular antioxidant system.

The presence of antioxidants such as catalase, glutathione peroxidase, superoxide dismutase, ascorbic acid, vitamin E, and β -carotene has been demonstrated in ruminant follicle compartments (Schweigert and Zucker, 1988; Behl and Pandey, 2002; Combelles *et al.*, 2010; Gupta *et al.*, 2011; Hennem *et al.*, 2013; Hozyen *et al.*, 2014). Antioxidants prevent oxidative damage by preventing ROS concentrations from reaching levels that may harm the follicle and oocyte. However, situations leading to increased ROS production or to a reduction in antioxidant concentrations are likely to affect fertility by causing oxidative damage. Examples of these situations are mastitis, negative energy balance and heat stress.

Mastitis causes a 26 to 28% reduction in conception rates when it occurs within 10 days before or 30 days after artificial insemination (Lavon *et al.*, 2011). The reduced pregnancy rate after mammary gland infection can be explained by alterations in ovarian steroid production and gonadotropin secretion (Wolfenson *et al.*, 2015). In addition, mastitis produces an imbalance between antioxidants and oxidants, leading to oxidative stress (Sharma *et al.*, 2016; Shahid *et al.*, 2017). Recently, it was found that cytoplasmic maturation and embryo viability is compromised when oocytes are cultured with follicular fluid from cows with mastitis (Roth *et al.*, 2015). Mastitis is experimentally induced by injection of gram negative toxins, the lipopolysaccharides (Asaf *et al.*, 2014). Lipopolysaccharides disrupt follicular steroidogenesis by reducing the expression of gonadotropin receptors and steroidogenic enzymes (Magata *et al.*, 2014), inducing apoptosis and oxidative stress in oocytes (Zhao *et al.* 2017). Moreover, mastitis decreases antioxidant blood concentrations and increases oxidant capacity in milk (Kizil *et al.* 2007; Atakisi *et al.*, 2010). Thus, oxidant/antioxidant balance is compromised during mastitis infection.

The increase in milk production after parturition demands a great amount of nutrients, but the inability to consume the required quantity produces a negative energy balance. Thus, the body removes nutrients from internal reserves to sustain vital functions and milk production. The state of negative energy balance is characterized by weight loss, and an increase in blood concentrations of non-esterified fatty acids (NEFA), and ketone bodies such as beta-hydroxybutyrate (Adewuyi *et al.*, 2005).

A negative energy balance may last for 10-12 weeks after calving, during which fertility is compromised (Butler, 2003). After analyzing the NEFA profile and β -hydroxybutyrate in blood serum and follicular fluid of cows after parturition (Leroy *et al.*, 2004), Leroy *et al.* (2005; 2006) found that administration of these two metabolites to culture medium reduces oocyte competence. Moreover, Van Hoeck *et al.* (2013) reported that oocyte redox status is affected by NEFA, suggesting that oxidative stress induced by NEFA is responsible for lowering oocyte competence in dairy cattle with negative energy balance. In this regard, Song *et al.* (2014; 2016) reported that NEFA and β -hydroxybutyrate cause hepatocyte apoptosis by means of oxidative stress. In addition, cows showing signs of negative energy balance, such as body weight lost, increased concentrations of NEFA and β -hydroxybutyrate, suffer oxidative stress (Bernabucci *et al.*, 2005; Pedernera *et al.*, 2010), which can be explained by diminished blood and follicular concentrations of antioxidants such as β -carotene, vitamins C and E, superoxide dismutase, and glutathione peroxidase (Cigliano *et al.*, 2014; De Bie *et al.*, 2016).

Oxidative stress induced by heat stress disrupts fertility in dairy cattle (Roth, 2015). Heat stress increases ROS production and reduces the proportion of oocytes that attain nuclear maturation and the blastocyst formation rate (Nabenishi *et al.*, 2012). In addition, Takahashi (2012) suggests that oxidative stress induced by high temperatures creates adverse intraoviductal conditions for oocytes, sperm and embryo. Even though antioxidant supplementation seems to be a feasible way of ameliorating the negative effect of heat stress in fertility (Hansen, 2013), De Rensis *et al.* (2017) concluded that more research is needed to identify an antioxidant regimen that can effectively protect oocytes from heat stress.

2.3.6 Oxidative stress effects on sperm, corpus luteum development and embryo viability

Fertilization of the ovulated oocyte, formation of the corpus luteum and embryo cleavage occur after ovulation. As with the oocyte, reactive oxygen species are necessary for the sperm to achieve capacitation, but high concentrations are detrimental (Aitken *et al.*, 2012). Sources of ROS in the sperm include mitochondria, cytosolic L-amino acid oxidases, and plasma membrane nicotinamide adenine dinucleotide phosphate oxidases (Aitken, 2017). Infertile men have been found to have higher concentrations of reactive oxygen species, lower concentrations of vitamin E and lower total antioxidant capacity than fertile men (Benedetti *et al.*, 2012).

In cattle, oxidative stress induced by heat stress reduces the fertilization rate in dairy cattle during summer season relative to winter (Sartori *et al.* 2002).

An antioxidant system supports corpus luteum functionality. Luteal superoxide dismutase and vitamin C concentrations increase as the corpus luteum develops (Luck and Zhao, 1993; Vu *et al.*, 2013), and an increase in luteal antioxidant activity has been reported during pregnancy (Al-Gubory *et al.*, 2004). An antioxidant system is necessary to counteract the ROS generated by steroidogenic cells and mononuclear phagocytes (Kato *et al.*, 1997) or by the inner environment in the corpus luteum. This is relevant since the mechanism used during luteolytic events by PGF_{2α} implies the production of ROS and a decrease in antioxidant activity (Hayashi *et al.*, 2003; Vu *et al.*, 2012; Vu *et al.*, 2013), resulting in low progesterone production.

A changing antioxidant status is detectable throughout the estrous cycle. According to Aydilek *et al.* (2014), total antioxidant activity is lower in the luteal than in the follicular phase of the estrus cycle in cows. Repeat breeder cows have higher concentrations of ROS during the crucial period of corpus luteum survival (day 12 and 16 of the estrous cycle), causing failure to conceive (Rizzo *et al.*, 2007). However, a failure to generate enough ROS after induced luteolysis by PGF_{2α} results in anovulation (Talukder *et al.*, 2014b). This suggests that increased antioxidant activity during the luteal phase may be detrimental to reproductive performance in empty cows, but not for pregnant cows.

Oxidative stress is detrimental to embryo survival either by blocking progesterone supply or by a direct effect on embryo cells. The source of reactive oxygen species may come from metabolic activity of the embryo itself (Gupta *et al.*, 2006) or from the maternal environment (Poston *et al.*, 2011). Regarding the maternal environment, it is known that maternal heat stress is responsible for induced embryonic death by increasing ROS production and antioxidant depletion, but not by heat stress itself (Ozawa *et al.*, 2002). When ROS become uncontrolled, they cause morphological and functional alterations, which may block embryo development or apoptosis (Guérin *et al.*, 2001), supporting the suggested implication of ROS in bovine embryo mortality (Celi *et al.*, 2011).

The early embryo may be capable of developing some degree of resistance to adverse effects of ROS as it grows. Ealy *et al.* (1993) reported that after one day of pregnancy Holstein cow embryos became less susceptible to maternal heat stress. Similarly, Morales *et al.* (1999) reported that nine

to 16 cell embryos are more resistant to ROS insults than zygotes and blastocysts. Bain *et al.* (2011) suggested that susceptibility of the bovine embryo to ROS increases during the first 72 hours of embryonic life. In addition, differences in ROS resistance related to embryo sex have been reported (Pérez-Crespo *et al.*, 2005); the female embryo is more resistant than the male embryo. These findings suggest that severity of ROS-induced embryo damage is developmental and sex dependent.

2.3.7 Antioxidant supplementation during the periconceptual period in female ruminants

Oocyte and embryo well-being may be compromised by oxidative damage generated as the result of common reproductive practices around the periconceptual period. Rectal palpation and artificial insemination are stressful to dairy cattle (Nakao *et al.*, 1994), and cause oxidative stress (Cingi *et al.*, 2012). These practices are commonly and repeatedly performed during estrus synchronization. A common practice among reproductive technicians, before artificial insemination, is to stimulate the reproductive tract via rectal massage for visualization of cervical mucus in order to reveal any infection. However, unexperienced technicians may cause rectal irritation, evidenced by the presence of blood on the palpating hand. This situation is also observed after multiple, prolonged or rough palpations, but it is unknown how much this affects fertility.

Estrus synchronizations using a progesterone release device is common in small ruminants and cattle. However, several studies have reported an inflammatory response as well as changes in normal flora and vaginal histology after the use of estrus synchronization devices in ewes and cattle (Manes *et al.*, 2010; Suárez *et al.*, 2006; Walsh *et al.*, 2007; Manes *et al.*, 2015). In addition, Sönmez *et al.* (2009) reported a steady increase in ROS after intravaginal sponge insertion in goats, suggesting sponge insertion was causing oxidative stress. The inflammatory response and oxidative stress induced by insertion of progesterone release devices may be responsible for the reduced fertility in ewes carrying intravaginal sponges (Manes *et al.*, 2014).

The ovarian superstimulation of small and large ruminants is a procedure used to increase the number of oocytes and embryos that a female would normally produce during a normal estrous cycle. However, superstimulation is known to upregulate genes related to oxidative stress in cattle (Dias *et al.*, 2013). In mice, an increase in oxidative damage in the uterus and oviduct (Park *et al.*, 2015), as well as a reduction in oocyte mitochondria number and function, have been reported

after repeated and single superstimulation. In addition, low quality oocytes, resulting in embryos with mitochondrial functional defects, which are more susceptible to oxidative damage, have been found during superstimulation (Komatsu *et al.*, 2014). Since an oxidative insult is present during superstimulation, antioxidant supplementation may help to overcome some of the detrimental effects on oocyte and embryo quality. According to Liu *et al.* (2013) and Ben-Meir *et al.* (2015), antioxidant supplementation not only counteracts oxidative damage in the oocyte but also restores mitochondrial function.

The evidence suggests that antioxidant supplementation may be beneficial improving fertility during the periconceptual period. Evidence that validates the effectiveness of antioxidant supplementation during hormonal treatments in ruminant female around this period is given in Table 1. Parenteral antioxidant supplementation is probably the best option during the periconceptual period. As shown in Table 1, antioxidants were given via injection in all cases, probably because higher concentrations in blood and other body compartments can be reached faster than through feed supplementation. In the case of vitamin E, for example, parenteral supplementation is a more effective way to improve antioxidant status in the short term compared with in-feed supplementation (Bourne *et al.*, 2007; Mokhber-Dezfouli *et al.*, 2008). This is relevant because a rapid effect is desired.

Table 1. Effect of antioxidant injection during the periconceptual period on ruminant fertility

Animal model and antioxidant supplemented	Time of supplementation	Effect	Authors
Dairy cattle injected with 3,000 IU of vitamin E and 3,000 mg of vitamin C	Three days after intravaginal device insertion (at estrus), and two days after artificial insemination	Increased number of cows pregnant	Gonzalez-Maldonado <i>et al.</i> , 2017
Dairy Holstein cows injected with 840 mg of vitamin E and 8 mg of sodium selenite	Just before ovisynch protocol began	Increased antioxidant activity and progesterone production	Yildiz <i>et al.</i> , 2015
Goats injected with 200 mg of vitamin E	At intravaginal sponge removal and at artificial insemination	Increased multiple births and prolificacy	Sönmez <i>et al.</i> , 2009
Superovulated Holstein cows injected with 1200 mg of β -	At the time of norgestomet ear implant insertion for estrus synchronization	Increased total viable embryos	Sales <i>et al.</i> , 2008

carotene and 750 mg of
tocopherol

and at first
superovulation
injection

Superovulated ewes injected
with 500,000 IU of all-trans
retinol

On the first and last day
of FSH injections

Increased the percentage of hatched
blastocyst

Eberhardt *et al.*, 1999

Ewes injected with 2100 IU
of vitamin E

-12, -2, 10, 24 and 48 h
with respect to PGF_{2α}
injection

Protection of corpus luteum from PGF_{2α}
induced luteolysis

Vierk *et al.*, 1998

2.3.8 Conclusion

Free radicals participate in reproductive events occurring during the periconceptual period. However, situations leading to overproduction that surpasses antioxidant capacity cause oxidative stress and compromise fertility. Antioxidant supplementation during hormonal treatments carried out during this period can be an advantageous tool for improving fertility in female ruminants.

2.3.9 Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

2.3.10 References

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**2.4 Supplementation of ascorbic acid to improve fertility in dairy cattle- a
review**

**Suplementación de ácido ascórbico para mejorar la fertilidad del ganado
lechero: una revisión**

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2.4.1 Resumen

Ácido ascórbico (Vitamina C: VC) es un antioxidante que participa en procesos regulatorios del desarrollo de estructuras ováricas y fertilidad. La suplementación con VC ha ganado lechero para mejorar su fertilidad ha recibido poca atención. Sin embargo, una baja fertilidad en este ganado es asociada con un aumento en el mérito genético para producción de leche y estrés calórico, los cuales también disminuyen las concentraciones sanguíneas de VC, esto sugieren un efecto benéfico potencial de la suplementación de VC en la fertilidad del bovino. Los objetivos de esta revisión son contribuir al actual conocimiento de la relación entre VC y fertilidad, así como compartir nuestras experiencias suplementando VC para mejorar el comportamiento reproductivo del ganado lechero.

Palabras clave: antioxidantes, ácido ascórbico, reproducción

Supplementation of ascorbic acid to improve fertility in dairy cattle- a review

2.4.2. Abstract

Ascorbic acid (Vitamin C: VC) is an antioxidant that participates in the regulatory processes involved in development of ovarian structures and fertility. Supplementation of VC to dairy cattle to improve fertility has received little attention. However, reduced fertility in dairy cattle associated with high genetic merit for milk production and heat stress, which also diminish blood VC concentrations, suggest a potentially beneficial role for VC supplementation. The objectives of this review are to contribute the current knowledge regarding the relationship between VC and fertility and to share our experiences that support the relevance of VC supplementation to improve dairy cattle reproductive performance.

Key words: antioxidants, ascorbic acid, reproduction

2.4.3 INTRODUCTION

The economic gains of a dairy farm increase as cattle reproductive efficiency improves. However, the historical decline in fertility of Holstein dairy cows hampers profitability, but at the same time offers a challenge to develop strategies to enhance reproductive performance. The cause of low fertility in modern Holstein dairy cattle is multifactorial. The leading factors are considered to be improvement in genetic merit to milk production, inability to meet nutritional requirements and adverse environmental conditions such as heat stress, causing stress and susceptibility to diseases that compromise oocyte and embryo viability (1).

The exact cause of low fertility is unknown, but oxidative stress could be implicated. Oxidative stress results in free radicals that exceed the organism's antioxidant capacity (2). Free radicals are molecules with an unpaired electron, highly reactive and normally produced in living aerobic organisms (3). At a controlled production rate, they serve as molecular signals, but over production may result in a pathological process (4). Sources of free radicals that may surpass the cow's antioxidant capacity include milk production yield and heat stress. High milk producers have higher blood concentrations of oxidative stress markers than those that produce less milk (5), and are also more susceptible to heat stress (6). This is relevant because heat stress produces oxidative stress in dairy cattle (7). Oxidative stress creates unfavorable intraoviductal conditions (8) that result in embryo death (9).

Oxidative stress is counteracted by antioxidants, which suppress the deleterious effect of free radicals by giving them one electron. One antioxidant that is relevant to mammalian reproduction is water soluble ascorbic acid (vitamin C, here after referred to as VC) (10). The chemistry and biological functions of VC in cattle have been reviewed by others (11), and will therefore be not further addressed here. However, the impact of VC supplementation on dairy cattle fertility has been poorly studied, probably because bovines can synthesize their own VC in the liver from glucose (11), and thus have not need for external supplementation (12). Nevertheless, the same factors that are blamed for disrupting fertility (high milk yield and heat stress) decreased blood VC concentration in dairy cattle (13,14). It might be suspected that if VC is necessary for reproduction, a diminished supply could affect fertility. Previous research has shown that supplementation of VC improves reproductive performance of repeat breeder cows (15) and dairy cattle under heat stress conditions (16). VC supplementation and impacts on dairy cattle fertility deserve more attention. The objective of the present article was to review the current knowledge regarding the relationship between VC and fertility.

2.4.4 OVARIAN FOLLICLE DEVELOPMENT

Vitamin C deficiency increases the number of atretic follicles (17). However, supplementation attenuates follicular cell apoptosis (18), promotes primordial follicle activation (19), increases the population of growing follicles (20) and reduces those in atretic state (21). These findings suggest that VC supports the development of healthy ovarian follicles.

The ovarian follicle is under constant structural remodeling. Its diameter increases up to 475 times from the primordial to the ovulatory size (22,23). This increase in size implies a constant remodeling of the follicular basal lamina (24) and changes in the intrafollicular concentrations of VC, which are higher in smaller follicles (25). The follicular basal lamina gives the follicle stability and serves as a molecule filter (24), but it needs increasing amounts of collagen as it increases in size (26). Since VC is a cofactor in collagen synthesis (27), it is logical to assume that VC would be required in higher quantities in developing follicles. In fact, supplementation of VC improves follicle survival and increases the odds of a follicle reaching preovulatory size (28). This could be explained by VC preventing follicular cell death and maintaining base membrane integrity as the follicle grows (18,29).

Under an environment with a regressing corpus luteum, the dominant follicle will reach the preovulatory state. At this stage, VC is needed for normal follicular steroidogenesis (30), which is accomplished by promoting the expression of key enzymes involved in steroidogenesis such as aromatase and P450 cholesterol side-chain cleavage (31). However, as the follicle grows there is a reduction in the

concentration of VC. Preovulatory follicle has lower intrafollicular concentrations of VC than large follicles from other stages of the estrus cycle (32). This reduction may result from a higher intrafollicular concentration of IGF-I, which induces the uptake of VC by granulosa cells (33). The LH surge also causes a reduction in VC concentrations (34), probably by increasing intrafollicular ROS concentrations (35).

The reduced intrafollicular concentrations of VC at the preovulatory stage may be part of the mechanism controlling ovulation. The collagen in the follicular basal lamina is reduced as the follicle grows, which makes it more expandable and easier to remodel (36). The reduced intrafollicular concentrations of VC, together with degradation of collagen in preovulatory follicles, results in the weakening and rupture of the basal lamina, which are crucial events that can lead to preovulatory follicle rupture (37,38).

2.4.5 VITAMIN C AND FERTILITY

The number of pregnant women with luteal phase defects increases after supplementing VC, which likely worked by increasing corpus luteum progesterone (39). Corpus luteum diameter (32) and concentration of progesterone (40) has been related to VC concentration. In addition, the content of VC is higher during the early stages of corpus luteum development (41), reaching the highest concentration, at least in bovines, on day 12 of the estrous cycle (42). Furthermore, one key element in the relationship between VC and the corpus

luteum is that this vitamin is required, as mentioned previously, for the synthesis of collagen, which is essential for corpus luteum development (43).

Vitamin C improved fertility (44). The improvement in oocyte and embryo quality could explain these results. Unfortunately, the limited information available on this topic has been obtained mostly under in vitro conditions. VC supplementation enhances oocyte and embryo development (45,46). In contrary, high doses of VC might harm both the oocyte and embryo development (47,48), possibly resulting from a pro-oxidant effect of VC. The VC at low concentrations can act as an antioxidant while the opposite occurs at high concentrations, which may depend on the concentration of metal ions (iron) (49). A pro-oxidant effect of VC could be expected as the concentration of metal ions increases (50). The latter may be true under in vitro conditions, but it is unlikely to occur in living organisms (51).

2.4.6 RELATIONSHIP BETWEEN VITAMIN C AND VITAMIN E

Vitamin C may control follicular development by interacting with others elements known to affect fertility. It is well accepted that after vitamin E fulfills its antioxidant activity, it can be reactivated by VC (52), which increases its availability (53). Vitamin E deficiency disrupt follicle development, produces estrous cycle abnormalities and pregnancy loss (54).

The relationship between vitamins C and E in reproductive issues has received little attention. An antioxidant system, that includes vitamins C and E, is activated during ovarian steroidogenesis (42). The supplementation with vitamin C and E to rats increases blood concentrations of testosterone, FSH and LH (55), but not

preovulatory follicle diameter, area of the corpus luteum, estradiol or progesterone concentrations in cows (Table 2). These higher concentrations of gonadotropins are in agreement with the fact that VC stimulates its secretion from pituitary (56). Studies *in vitro* have shown a positive effect of vitamin C and E on oocyte quality and embryo development when supplemented separately, but not together (53,57,58). Addition of vitamin C and E to the maturation medium impairs blastocyst occurrence rate by preventing the formation of the amount of ROS necessary for oocyte developmental competence (53). This is acceptable because a tonic supply of ROS has proved to interrupt oocyte meiotic arrest (59). However, it is unlikely that the situation described by Dalvit and colleagues (53) also occur *in vivo* because supplementation of both vitamins has resulted in more pregnancies in dairy cattle (16, Figure 1 and 2). In addition, an improvement in embryo quality after injecting superovulated cattle donors before estrus with two antioxidants, β -carotene and vitamin E, has been reported (60).

2.4.7 CONCLUSION

In conclusion, VC is an antioxidant that supports structural and functional development of ovarian structures, as well as fertility. Contrary to current thought, scientific evidence suggests the need of supplementing vitamin C to dairy cattle to improve reproductive performance. Supplementation of vitamin C and E can be incorporated to estrous synchronization protocols to improve pregnancy rate in dairy cattle.

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Table 2 Least square means (\pm SE) for the effect of injecting vitamin C and E on ovarian structures size, estrus presentation and hormone concentrations in Holstein dairy cows

Variable	Treatment		<i>P</i> -value
	Control (n=28)	Vitamin C and E* (n=32)	
Time to estrus (h)	57.1 \pm 4.89	58.4 \pm 4.57	0.67
Diameter of the preovulatory follicle (mm)	18.3 \pm 0.57	17.2 \pm 0.60	0.21
Plasma estradiol concentration (μ g mL ⁻¹)	45.1 \pm 3.12	46.8 \pm 3.26	0.71
Area of the corpus luteum (cm ²)	6.9 \pm 0.39	6.7 \pm 0.37	0.74
Plasma progesterone concentration (ng mL ⁻¹)	10.8 \pm 1.60	12.5 \pm 1.60	0.26

* The general procedure, as well as justification of the doses and time of vitamins injections is explained in detail elsewhere (16).

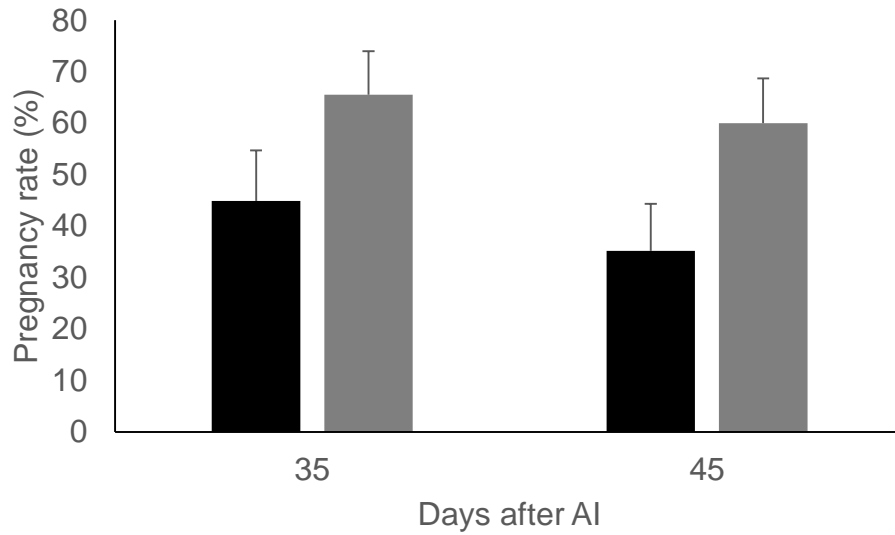


Figure 1 Pregnancy rate 35 and 45 days after AI in control group (black bars, n=28) and Holstein dairy cows injected with vitamins C and E (grey bars, n=32). The general procedure, as well as justification of the doses and time of vitamins injections is explained in detail elsewhere (16).

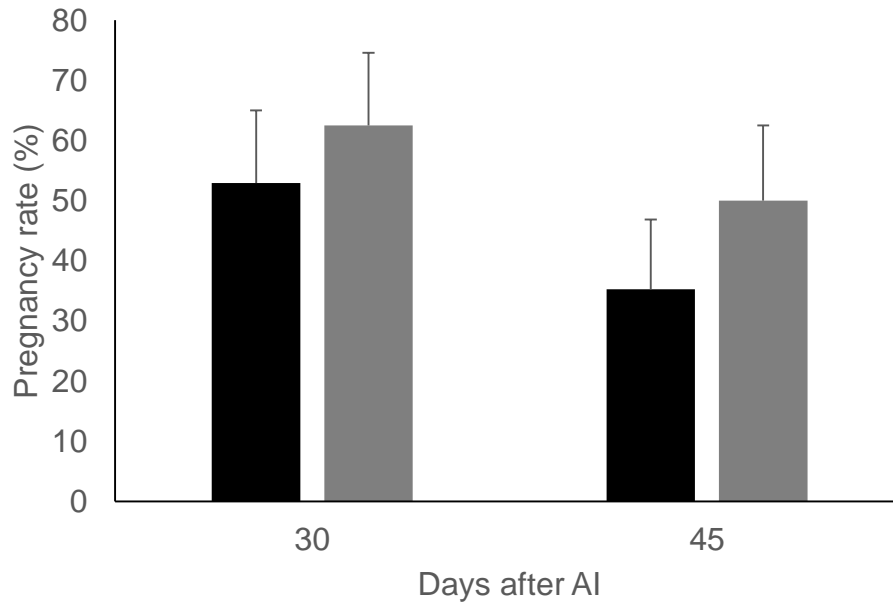


Figure 2 Pregnancy rate 30 and 45 days after fixed time AI (ovsynch protocol) in control group (black bars, n=17) and Holstein dairy cows injected with 3000 mg of vitamins C and 3000 IU of vitamin E (grey bars, n=16). First injections of vitamin C and E were given three days after first injection of GnRH. The second and third injections of vitamin C were administered just after the second injection of GnRH and two days after artificial insemination.

CHAPTER 3. RESEARCH ARTICLES



3.1 Preface to research articles

The research article I in the present thesis evaluated the effect of vitamins C and E injections on reproductive performance in dairy cattle under heat stress conditions. These conditions were chosen because the known negative effect of heat stress on dairy cattle fertility and on blood concentrations of vitamins C and E. The results of this article showed that the combined injection of two vitamins is more advantageous to improve fertility in dairy cattle than giving them separately. These results served as foundation for the second experiment (research article II), where the effect of injecting increased doses of both vitamins on ovarian structures development, steroidogenic hormone production and fertility were evaluated.

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**3.2 Impacts of vitamins C and E on ovarian structures and fertility in Holstein cows
under heat stress conditions**

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3.2.1 Abstract

This study evaluated the effect of injecting vitamin C (VC) and E (VE) on size of the preovulatory follicle, volume of the corpus luteum, and pregnancy rates in Holstein cows under heat stress conditions (temperature humidity index > 74). Sixty-two cows were randomly assigned to one of four treatments: 1, control, n =15: cows were not supplemented with vitamins; 2, VCG, n = 15: cows were simultaneously injected i.v. with 500 and s.c. with 2,500 mg of VC before and after estrus; 3, VEG, n = 15: cows received an i.m. injection of 3,000 IU of VE before estrus; 4, VCEG, n = 17: cows were injected with VC and VE in the same way and doses as in treatments 2 and 3. Treatment did not significantly affected any of the measured variables, despite of a numeric increase in pregnancy rates in cows injected with vitamins (7.5 ± 7.3 , 9.6 ± 9.4 , 15.1 ± 10.0 , and $18.34 \pm 9.9\%$ for control, VCG, VEG, and VCEG, respectively). In conclusion, injections of vitamin C and E did not affect either the development of the preovulatory follicle and the corpus luteum or pregnancy rates in Holstein dairy cattle under heat stress conditions.

Key words: corpus luteum, dairy cattle, heat stress, pregnancy rate, vitamin C, vitamin E

3.2.2 Introduction

Heat stress induces behavioral, metabolic and hormonal changes in dairy cattle, resulting in poor reproductive performance (1). To date, there is no treatment that can fully restore the fertility of dairy cattle under heat stress. However, supplementation of antioxidants could be a feasible way to improve fertility in cows under such conditions (2). This seems logical, since blood concentrations of antioxidants, such as vitamins C and E, are diminished by heat stress (3, 4).

Vitamin C and E are necessary for normal reproduction in cattle (5, 6) and required for follicle and corpus luteum (CL) development (7, 8). Low fertility in dairy cattle may be a consequence of smaller preovulatory follicle and CL size compared to animals without heat stress (9, 10, 11). We speculated that cows exposed to heat stress conditions and supplemented with vitamin C and E have larger preovulatory follicles and CL, resulting in higher pregnancy rates compared to cows which did not receive vitamin supplementation.

3.2.3 Materials and methods

3.2.3.1 Animal welfare

All technical and management procedures were performed based on the guidelines set by the Canadian Council on Animal Care (12).

3.2.3.2 Location

The experiment was conducted at the dairy farm “18 de Julio” of the Universidad Autónoma Chapingo. The farm is near Tlahualilo, Durango, México, located at 25° 54′ N and 103° 35′ W, 1,137 m above sea level. The climate of the region is semiarid, with a mean annual temperature of 21.1°C and 239 mm of rainfall per year (13). The experiment was conducted during the third week of August and the first week of September 2014.

3.2.3.3 Animal, treatments, and experimental design

Multiparous Holstein dairy cows (n = 62,) with an average of 188.75 ± 15.90 days in milk and producing 37.50 ± 1.13 liters of milk day⁻¹, were randomly assigned to one of four treatments: 1, Control, n = 15: cows were not injected with vitamins; 2, VCG, n = 15: cows received a total dose of 3,000 mg of vitamin C (ascorbic acid, Q.P., Reasol; 500 mg via i.v. and 2,500 mg via s.c.) at night on day -5 (day 0 was the day of progesterone release device (CIDR) removal), immediately after estrus detection and two days after artificial insemination; 3, VEG, n = 15: cows received a single i.m. injection of 3,000 IU of vitamin E ((±)α-tocopherol, Sigma-Aldrich) at night on day -5; 4, VCEG, n = 17: cows were injected with both vitamins on the same days and doses as mentioned in treatments 2 and 3. The experimental design was completely randomized, with one cow as experimental unit.

3.2.3.4 Estrus synchronization and breeding

The follicular wave of the cows was synchronized with a CIDR containing 1.9 g of progesterone (CIDR 1900 CATTLE INSERT®, Zoetis), inserted intravaginally for eight

days, and an i.m. injection of 250 µg of GnRH analogue (GnRH®, Sanfer). Estrus behavior was induced by i.m. injection of 500 µg of cloprostenol (Celosil®, MSD Animal Health) at CIDR removal. Once the CIDR was withdrawn, the animals were constantly monitored by direct observation for signs of standing estrus. The cows were artificially inseminated 12 h after standing estrus with a single dose (approximately 2×10^6 spermatozoa) of semen from a single bull of proven fertility. To induce ovulation, an injection of 250 µg of GnRH analogue was given to each cow immediately after AI.

3.2.3.5 Nutrition and feeding

The animals received a diet formulated to provide 1,600 IU of vitamin E (56.5 kg day^{-1} cow⁻¹) of a total mixed ratio: alfalfa, 15.0; corn silage, 26.5; steam corn, 6.0, and concentrate, 9.0 kg as fed. The food composition of the concentrate mixture was as follows: cottonseed, 1.099; walnut, 0.895; soybean, 2.049; wheat bran, 1.911; molasses, 1.431; soy plus (cooker-expeller soybean, Soyplus®), 1.135; magnesium oxide, 0.0162, sodium bicarbonate, 0.135; lactomil (bypass fat, Lactomil®), 0.054; calcium carbonate, 0.189, micro minerals, 0.030; vitamins, 0.030; Ganadero plus (*Saccharomyces cerevisiae*, Biotecap), 0.019 and maxifolipol (Flavofosfolipol, Pisa), 0.002 kg as fed).

3.2.3.6 Response variables

To assess the effects of vitamin C and E injections on follicle and CL development and, subsequently, on the fertility of Holstein dairy cattle, the following variables were evaluated: diameter of the largest follicle at CIDR removal (DPF0, mm) and at estrus detection (DFP1, mm), time of estrus after CIDR removal (h), growth rate of the largest follicle (mm day^{-1}), volume of the CL (cm^3), and pregnancy rate (%). Temperature and

relative humidity in the stable were recorded each day from day -4 to four days after AI. Daily temperature humidity index (THI) values were calculated using the equation by Mader et al. (14): $THI = 0.8 \times \text{ambient temperature} + [(\% \text{ relative humidity} \div 100) \times (\text{ambient temperature} - 14.4)] + 46.4$.

The cows were considered to be exposed to heat stress when THI values exceeded 74. In addition, body temperature was recorded in the morning and afternoon for the same period of time as in THI. The parameters DPF0, DFP1, and volume of CL were measured by real-time ultrasonography (Medison SonoVet 2000, 7.5 MHz, linear-array transducer; Universal Medical Systems Inc. Bedford Hills, NY, USA). The diameter of the largest follicle was calculated by the average of horizontal and vertical measurements, while the volume of CL was calculated directly via ultrasound. The location of the largest follicle at CIDR removal was recorded and its diameter was measured once again at estrus detection. The growth rate of the largest follicle was calculated taking into consideration the difference in size between DFP0 and DFP1 and the time from CIDR removal to estrus detection. The pregnancy test was performed 33 days after AI by ultrasonography.

3.2.3.7 Statistical analysis

Of the total initial 62 cows, only 52 showed estrus and were therefore considered in the analysis. The number of cows which completed the study for each of the treatments was control = 13, VCG = 10, VCE = 12, and VCEG = 17. All measured variables were subjected to analysis of variance under the following mixed linear model:

$$Y_{ijk} = \mu + T_i + C_j + b_1(X_{ijk} - \bar{X}_{ijk}) + e_{ijk},$$

were μ is the overall mean, T_i is the effect of the i th treatment ($i = \text{Control, VCG, VEG, VCEG}$); C_j is the random effect of the j th cow ($j = 1, \dots, 52$) $\sim \text{NI}(0, \sigma_e^2)$, b_1 is the respective coefficient of linear effect of milk production level (X); $< 35, 35\text{-}40$ and $> 40 \text{ L day}^{-1}$ for low, medium and high level, respectively, and e_{ijk} is the residual $\sim \text{NI}(0, \sigma_e^2)$.

Covariates which did not show significant effects ($P > 0.05$) were deleted from the final model. Means for the main effect were calculated by least squares. The variables were analyzed using the Glimmix procedure of the statistical package SAS (Statistical Analysis System, version 9.3). In the case of variables considered with normal distribution, such as DFPO, DFP1, time to estrus after CIDR removal, growth rate of the largest follicle, and CL volume, the function link used was identity. The variable pregnancy rate was analyzed considering a binary distribution and using logit as the function link. In all cases, $P \leq 0.05$ was considered significant. Means of all variables were compared using Tukey's test.

3.2.4 Results

The study evaluated the effect vitamin C and E injections on ovarian follicle structure development during a synchronized estrus as well as pregnancy rates in Holstein cows under heat stress conditions. Table 3 shows calculated THI and measured body temperature values. In general, the parameters DFPO, DFP1, time of estrus after CIDR removal, growth rate of the largest follicle, and CL volume were $14.6 \pm 0.57 \text{ mm}$, $17.8 \pm 0.54 \text{ mm}$, $52.2 \pm 2.59 \text{ h}$, $1.8 \pm 0.24 \text{ mm day}^{-1}$, and $9.3 \pm 0.79 \text{ cm}^3$, respectively. Overall pregnancy rate was $12.8 \pm 0.04\%$. The effects of the different treatments on each of these variables are shown in Table 4 and Figure 3.

The experimental units (cows) behaved similar in terms of the evaluated variables, regardless of the experimental group. Although cows injected with both vitamins showed estrus 10 hours earlier and 10% higher pregnancy rates compared to animals in the control group, these differences were not statistically significant ($P > 0.05$).

3.2.5 Discussion

Heat stress, as a result of high THI values, causes an increase in normal body temperature. The body temperatures of the cows used in this study were similar to those reported by Srikandakumar and Johnson (15), who studied cows under heat stress conditions. We therefore assume that the animals in our study were subject to heat stress.

The mechanism by which heat stress diminishes fertility in dairy cattle is not fully understood, but abnormal follicle and CL development, low quality oocytes, and high embryo mortality could be considered as the leading factors. To our knowledge, there is no information regarding the effects of supplementing vitamin C and E on preovulatory follicle development in cows under heat stress. However, the early onset of estrus in cows receiving vitamins has also been reported in ewes supplemented with vitamin E (16), indicating that vitamin supplementation positively affects some endocrine processes controlling the onset of estrus.

The low fertility observed in Holstein dairy cattle under heat stress may be a consequence of ovaries carrying a preovulatory follicle with reduced oxidative status (17). However, this scenario could be reverted by vitamin C and E. On one hand, vitamin C is necessary for normal follicle development (18), and supplementation of this vitamin increases the follicle diameter in a dose-dependent manner (19). However, in this study, vitamin C

supplementation did not significantly affect the diameter of the preovulatory follicle. A likely explanation for this may be the small sample size; in addition, the dose of supplemented vitamin C was probably not sufficient to alter the preovulatory follicle diameter.

On the other hand, the effect of vitamin E on fertility is mediated by a direct antioxidant effect on the follicle (20). This is noteworthy, since heat stress impairs fertility by follicle and oocyte disruption (17), probably through inducing oxidative damage (21). Since vitamin C is necessary for reactivating vitamin E functionality (22), these vitamins may act together or separately to improve follicle functionality and oocyte quality.

The suppressed luteal function (low progesterone) caused by heat stress may be responsible for the low fertility in dairy cattle (9). In order to improve fertility in dairy cattle under heat stress, an increase in progesterone production by increasing CL size seems logical. Previous researches have shown a positive correlation between vitamin C supplementation and CL diameter, progesterone concentration (23), and pregnancy rate (24). With regard to vitamin E, there is little information about its effect on CL functionality, but Vierk et al. (25) demonstrated that vitamin E supplementation protects the CL from apoptosis. In this study, the supplementation of vitamin C and E did not improve the volume of the CL, but it is possible that progesterone production could be affected. However, since we did not measure progesterone concentrations, we cannot confirm such asseveration.

To our knowledge, there is no previous evidence of the impacts of vitamin C or E supplementation on dairy cattle under heat stress conditions. However, in chickens (26),

rabbits (27), and rats (20) under heat stress, supplementation of these vitamins improved reproductive performance.

The results show that vitamin C and E supplementation did not significantly improve pregnancy rates in cows suffering heat stress. The reason for this could be the small sample size used in this study. According to McIntosh (28), several injections of vitamin C are required to improve fertility in cattle. On the other hand, blood concentrations of vitamin E are increased for up to seven days after parenteral injection (29), and we consider that this period of time is sufficient to affect fertility. The reason for supplementing 3,000 IU of vitamin E originates from previous field experience. On the other hand, the dose of vitamin C was chosen based on previous research (30).

The first injections of vitamin C and E were carried out with the objective of protecting both follicle development and oocyte quality. The decision of injecting vitamin C at estrus detection was based on results from past research that reported an increase in vitamin C concentrations at this time (30). The third injection of vitamin C was performed two days after AI and intended to protect the embryo from heat stress as well as to support CL development.

3.2.6 Conclusion

The supplementation of vitamin C and E before and after a synchronized estrus under heat stress conditions did not affect preovulatory follicle and corpus luteum development or pregnancy rates in Holstein cows. However, the reliability of these results is limited by the small sample size. Further studies evaluating the effects of vitamin C and E supplementation on reproductive performance in cows under heat stress conditions should therefore include larger numbers of animals to obtain reliable results.

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Table 3. Microclimate conditions in the stable and mean body temperatures (\pm SE) of cows during the experimental period.

Days	Daily temperature °C	Relative humidity %	THI*	Morning body temperature °C	Afternoon body temperature °C	Mean body temperature °C
-4	34.1	26.7	79.0	38.7 \pm 0.09	39.7 \pm 0.10	39.2 \pm 0.09
-3	38.5	23.2	82.8	38.9 \pm 0.11	39.8 \pm 0.10	39.5 \pm 0.10
-2	36.6	23.5	80.9	38.9 \pm 0.09	39.8 \pm 0.10	39.3 \pm 0.09
-1	36.0	25.0	80.6	38.9 \pm 0.09	39.9 \pm 0.10	39.4 \pm 0.09
0	41.3	25.7	86.3	39.2 \pm 0.09	39.9 \pm 0.10	39.5 \pm 0.09
1	38.5	25.0	83.2	38.9 \pm 0.09	39.9 \pm 0.10	39.4 \pm 0.09
2	38.0	25.5	82.8	39.3 \pm 0.09	39.8 \pm 0.10	39.6 \pm 0.09
3	41.5	23.5	85.9	39.0 \pm 0.09	39.8 \pm 0.10	39.4 \pm 0.09
4	41.5	23.25	85.9	39.1 \pm 0.09	40.0 \pm 0.10	39.5 \pm 0.09
5	40.2	23.0	84.4	39.0 \pm 0.09	40.0 \pm 0.10	39.5 \pm 0.09
6	38.7	28.7	84.4	38.9 \pm 0.09	39.7 \pm 0.10	39.3 \pm 0.09
7	28.8	47.7	76.3	38.8 \pm 0.09	39.4 \pm 0.10	39.1 \pm 0.09

*Temperature humidity index. $THI = 0.8 \times \text{ambient temperature} + [(\% \text{ relative humidity} \div 100) \times (\text{ambient temperature} - 14.4)] + 46.4$.

Table 4. Least square means (\pm SE) for the effects of injecting vitamin C (VCG), E (VCE), and its combination (VCEG) on follicular and corpus luteum development and estrus presentation in Holstein dairy cows under heat stress conditions.

Variables	Treatment				P-value
	Control	VCG	VEG	VCEG	
Follicular and corpus luteum development					
Diameter of the largest follicle at CIDR removal (mm)	13.0 \pm 1.0	15.2 \pm 1.1	15.6 \pm 1.0	15.0 \pm 0.9	0.22
Diameter of the largest follicle at estrus detection (mm)	17.5 \pm 1.1	18.0 \pm 1.2	18.0 \pm 1.1	17.9 \pm 1.0	0.99
Growth rate of the largest follicle (mm day ⁻¹)	2.3 \pm 0.4	1.5 \pm 0.5	1.6 \pm 0.5	1.6 \pm 0.4	0.61
Volume of the corpus luteum (cm ³)	10.4 \pm 1.6	8.1 \pm 1.7	9.6 \pm 1.7	9.1 \pm 1.4	0.81
Estrus detection					
Time of estrus after CIDR removal (h)	59.2 \pm 5.1	50.7 \pm 5.6	49.5 \pm 5.3	49.3 \pm 4.8	0.47

Within rows, means with different superscripts are significantly different ($P < 0.05$).

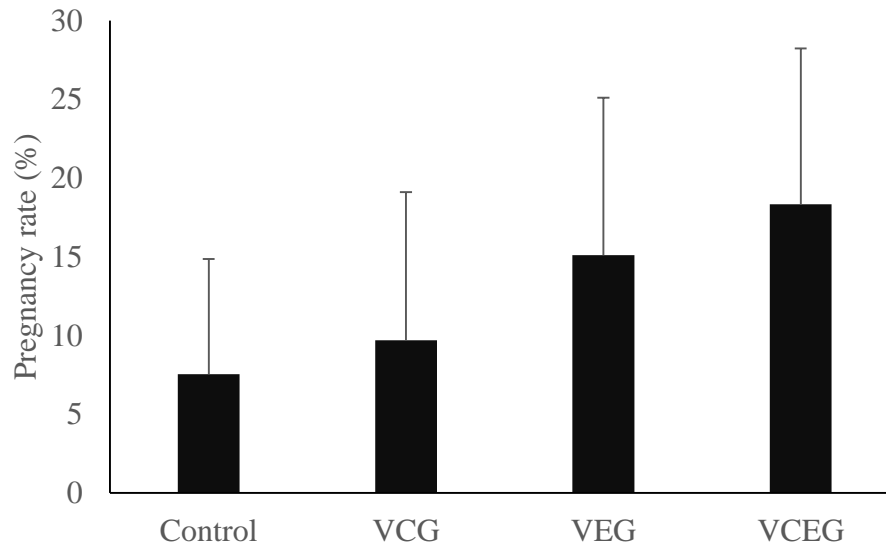


Figure 3. Influence of injecting vitamin C (VCG), E (VCE), and its combination (VCEG) on pregnancy rates (33 days after AI) in Holstein dairy cows under heat stress conditions.

Under revisión by the Revista Mexicana de Ciencias Pecuarias

**3.3 Effects of injecting increased doses of vitamins C and E on
reproductive events in Holstein dairy cattle**

**Efectos de dosis crecientes de vitamina C y E en eventos reproductivos de
vacas Holstein**

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3.3.1 Resumen

Las vitaminas C y E son suplementadas por separado para mejorar la fertilidad en bovinos. El objetivo de este estudio fue evaluar el efecto de la inyección con dosis crecientes de ambas vitaminas en eventos reproductivos de vacas lecheras. Se utilizaron 44 vacas Holstein, las cuales fueron asignadas aleatoriamente a uno de tres tratamientos: 1, Control: n=15, las vacas no recibieron vitaminas; 2, VCE3: n=15, las vacas recibieron una inyección por vía intramuscular de 3,000 UI de vitamina E y múltiples inyecciones por vía subcutánea de 3,000 mg de vitamina C antes del celo; 3, VCE6: n=14, las vacas fueron tratadas como se indica en VCE3, pero las dosis de ambas vitaminas fueron incrementadas al doble. Las variables respuesta fueron diámetro del folículo preovulatorio, tiempo al celo, área del cuerpo lúteo, porcentaje de gestaciones a 35 y 45 días después de la inseminación artificial y concentraciones sanguíneas de estradiol y progesterona. El efecto de tratamiento en las variables evaluadas no fue significativo ($p > 0.05$), excepto que las vacas que recibieron la dosis más baja de ambas vitaminas tuvieron porcentajes de gestaciones similares a los otros grupos evaluados, a pesar de haber presentado concentraciones sanguíneas de progesterona más bajas ($p \leq 0.05$) (19.4 ± 2.66 vs. 10.1 ± 2.55 vs. 19.2 ± 2.44 ng mL⁻¹ para los grupos Control, VCE3 y VCE6, respectivamente). En conclusión, las vacas inyectadas con 3,000 mg de vitamina C y 3,000 UI de vitamina E mantienen gestaciones en un medio uterino con bajas concentraciones de progesterona.

Palabras clave: antioxidantes, bovinos, fertilidad

Effects of injecting increased doses of vitamins C and E on reproductive events in Holstein dairy cattle

3.3.2 Abstract

Vitamins C and E have been supplemented separately to improve fertility in cattle. The objective of this study was to evaluate the effect of combined injections of increased doses of vitamins C and E on fertility traits in dairy cattle. Lactating Holstein cows ($n = 44$) were randomly assigned to one of three treatments: 1, Control: $n = 15$, cows were not injected with vitamins; 2, VCE3: $n = 15$, cows received a single intramuscular injection of 3,000 IU of vitamin E before estrus and multiple subcutaneous injections of vitamin C with a total dose of 3,000 mg before and after estrus; 3, VCE6: $n = 14$, cows were treated as in VCE3, but doses of vitamins C and E were doubled. The response variables were diameter of the preovulatory follicle, time of estrus, area of the corpus luteum, pregnancy rate 35 and 45 days after AI and plasma concentrations of estradiol and progesterone. There was no effect of treatment on any of the evaluated variables ($p > 0.05$), except that the lowest dose of vitamins sustained similar pregnancy rates among treatments, even though they had lower progesterone concentrations ($p \leq 0.05$) (19.4 ± 2.66 vs. 10.1 ± 2.55 vs. 19.2 ± 2.44 ng mL⁻¹ for Control, VCE3 and VCE6, respectively). In conclusion, cows injected with 3,000 mg of vitamin C and 3,000 IU of vitamin E became pregnant with low progesterone concentrations.

Key words: antioxidants, bovines, fertility

3.3.3 INTRODUCTION

Studies have suggested a physiological role of vitamins C and E in cattle reproduction (1, 2). From field experience, we found that cows injected with 3,000 IU of vitamin E approximately seven days before estrus increased pregnancy rates. Others have also reported an improvement in cattle fertility after vitamin E supplementation (3, 4). This vitamin may improve fertility by a direct antioxidant effect on follicle and embryo development (5) or by influencing follicular cell apoptosis and proliferation (6). In addition, cows had elevated plasma concentrations of this vitamin for up to seven days after injection (7). Therefore, we suspect that the increased pregnancy rate obtained in cows injected with vitamin E before estrus may have been mediated by its effect on follicle or oocyte competence.

Vitamin C is necessary to re-activate antioxidant activity of vitamin E (8, 9). The effect of vitamin C on reproductive function is mediated by its participation in collagen synthesis, hormone secretion and its antioxidant property (10). It has been suggested that several injections of vitamin C before and after estrus can improve fertility in repeat breeder cows (11). Unfortunately, there is little research that evaluates the effect of this vitamin on dairy cattle reproductive performance. Recent studies analyzing the impacts of vitamin C on fertility are lacking, researchers may have lost interest in evaluating reproductive responses of cattle to this vitamin because it is thought that bovines do not need vitamin C supplementation (12).

Based on the physiological relationship between vitamin C and E and their relevance in reproduction, we hypothesized that injecting both vitamins could have a beneficial effect on dairy cattle fertility. From unpublished results, we know that pregnancy rate in cows increases when vitamin C and E are injected at the same time on the expected day of preovulatory follicle emergence, combined with subsequent injections of vitamin C at estrus detection and two days after AI. The first injection of these vitamins aimed to affect follicle development (13, 6) and possibly oocyte quality. The second injection of vitamin C was administered to emulate the natural rise of this vitamin during estrus in cattle (14). The third dose of vitamin C was injected to influence corpus luteum functionality (15, 16). Thus, based on previous field experience, the hypothesis tested in this study was that injections of increased doses of vitamins C and E before and after synchronized estrus to Holstein dairy cattle improves pregnancy rate.

3.3.4 MATERIALS AND METHODS

3.3.4.1 Animal welfare

All the technical and animal management procedures in this study were performed following the guidelines of the Canadian Council on Animal Care (17).

3.3.4.2 Location

The experiment was performed at the dairy cattle research farm of the Universidad Autónoma Chapingo, México. The farm is located at 19° 29' N and 98° 52' W, 2250 m above sea level. The climate is temperate sub-humid, with a mean annual temperature of 15 °C and 645 mm mean annual rainfall (18).

3.3.4.3 Animals, treatments and experimental design

Lactating 4.6±0.35-year-old Holstein dairy cows (n = 44) with an average of 163.4±20.0 days in milk and in a herd with historical record of 22 L day⁻¹ cow⁻¹ were assigned randomly to one of three treatments: 1, Control: n = 15, cows were not injected with vitamins; 2, VCE3: n = 15, cows received a single i.m. injection of 3,000 IU of vitamin E ((±)α-tocopherol[®], Sigma-Aldrich) on day -5 (day 0 is the day of intravaginal device removal) and multiple s.c. injections with a total dose of 3,000 mg of vitamin C (Ácido Ascórbico[®], Q.P., Reasol) on day -5, immediately after estrus detection and two days after artificial insemination; 3, VCE6: n = 14, cows were treated as in VCE3, but the doses of vitamins E and C were increased to 6,000 IU and 6,000 mg. The experimental design was completely random, and the experimental unit was one cow.

3.3.4.4 Estrus synchronization and breeding

The follicular wave of the cows was synchronized with an intravaginal device containing 1.0 g of progesterone (Sincrogest[®], Ourofino Agronegocio, Sao Paulo, Brazil), inserted intravaginally for eight days, and an i.m. injection of 250 µg of GnRH analogue (GnRH[®], Sanfer) at intravaginal device insertion. Estrus behavior was induced by i.m. injection of 500 µg of cloprostenol (Celosil[®], MSD Animal Health) at intravaginal device removal. Once the intravaginal device was removed, the animals were monitored constantly by direct observation for signs of standing estrus. The cows were artificially inseminated 12 h after estrus

detection with a single dose (approximately 20×10^6 spermatozoa) of semen from a single bull of proven fertility.

3.3.4.5 Nutrition and feeding

The animals received a diet providing 1117 IU of vitamin E ($51.5 \text{ kg day}^{-1} \text{ cow}^{-1}$) of a total mixed ratio: fresh alfalfa, 21.9; corn silage, 21.9 and commercial concentrate (Ganadero 18, Productos Agropecuarios Tepexpan, S.A. de C.V. (protein 18%, fat 4%, fiber 12%)) 7.7 Kg as fed.

3.3.4.6 Response variables

The response variables were diameter of the preovulatory follicle, time of estrus after intravaginal device removal, area of the CL, pregnancy rate and blood concentrations of estradiol and progesterone.

The diameter of the preovulatory follicle and area of CL were measured by real time ultrasonography (Aloka Prosund 2, equipped with 7.5 MHz linear-array transducer, Hitachi Aloka Medical, Ltd Japan). Diameter of the preovulatory follicle was calculated by averaging horizontal and vertical measurements immediately after estrus detection, while the area of CL was calculated directly in the ultrasound nine days after AI. The pregnancy test was performed by ultrasonography 30 and 45 days after AI. Blood samples were collected from the coccygeal vein, using vacutainer tubes containing sodium heparin as anticoagulant, immediately after estrus detection and nine days after AI. The blood samples were centrifuged at 3,000 rpm for ten minutes and plasma was

separated and stored at -20 °C until the day of analysis for estradiol and progesterone concentrations (Estradiol- and Progesterone-Elisa, DRG Instruments, GmbH, Germany).

3.3.4.7 Statistical analysis

The statistical analysis was performed on variables collected only from cows that exhibited estrus. The number of cows that exhibited estrus for each of the treatments were Control=14, VCE3=13 and VCE6=14.

A normality test was run in PROC CAPABILITY on the residuals of the final model in each variable. When residuals did not satisfy the normality test, the data was subjected to logarithmic transformation. The statistical model included the effect of treatment. In addition, days in milk and age of the cow were included in the final model only when statistical significance was found. The results are presented as mean±SE. In all cases, $p \leq .05$ was considered significant. The data were analysed by PROC GLM, and the means, except for pregnancy rate, were compared by the Tukey Test. The pregnancy rate at 30 and 45 days were analysed by PROC GLIMMIX considering a binary distribution and using the link function logit. The SAS statistical package was used for all analyses.

3.3.5 RESULTS

The impacts of injecting increased doses of vitamins C and E on ovarian structure development and hormone concentrations in dairy cattle are shown in Table 5. Overall, cows injected with the highest doses of vitamins C and E tended to have

a smaller ($p \leq 0.06$) preovulatory follicle, but the corpus luteum of cows receiving the lowest dose of vitamins produced less ($p \leq 0.05$) progesterone than those of the control group and those that received the highest dose of vitamins. Moreover, pregnancy rate 30 and 45 days after AI of cows in the control group was not different ($p > 0.05$) from that of those that received vitamins (Figure 4).

3.3.6 DISCUSSION

A relationship between size of preovulatory follicle and probability of a cow diagnosed as pregnant after a fixed-time AI has been reported in dairy cattle (19). Cows with preovulatory follicles between 13.5 to 17.5 mm are more likely to become pregnant after a fixed-time AI (20). A possible explanation for the effect of preovulatory follicle size on pregnancy rate could rely on the degree of oocyte competence. According to results of an *in vitro* study (21), as follicle increases in size from 3 to 15 mm, the oocyte diameter also increases, and larger oocytes have been reported to have greater developmental competence (22). Another possibility is that young corpora lutea from larger follicles produce more progesterone than those from small preovulatory follicles (23). Supporting the above findings, donor cows with preovulatory follicles larger than 12.5 mm had a higher probability of yielding good quality embryos (24), but those with preovulatory follicles larger than 20 mm in size are at risk of pregnancy loss (25).

The cows injected with vitamins C and E had preovulatory follicles falling under the threshold at which the probability of pregnancy after AI increases (20). Since cows injected with the highest dose of vitamins tend to have a small preovulatory

follicles, a similar tendency was expected in estradiol concentrations. However, concentrations of this hormone and pregnancy rate among experimental groups was not different. Results from *in vitro* studies indicate that vitamin C does not affect follicular estradiol production, but it does affect follicle structure (13), and vitamin E improves follicle cell survival (6). Results of our study support those obtained from *in vitro* studies regarding estradiol production. In addition, previous research found that estradiol concentrations and pregnancy rate are not influenced by preovulatory follicle size in cows showing estrus and ovulating spontaneously (26), as in the case of the present study.

The progesterone produced by the corpus luteum after AI is responsible for pregnancy maintenance. Dairy cows with good genetic merit for fertility traits had a larger corpus luteum and produce more progesterone than those with poor genetic merit (27). Thus, increasing corpus luteum size and progesterone production might be targeted to improve fertility in dairy cattle. Based on its physiological relevance, vitamin C may be an important asset to influence corpus luteum development. It has been reported that ascorbic acid supports collagen biosynthesis during tissue formation and maturation of corpus luteum (15), reaching the highest concentration at mid luteal phase (28). In addition, vitamin C concentrations correlate positively with corpus luteum size and progesterone level (16). However, corpus luteum size was not affected by vitamin supplementation in this study and progesterone concentrations were minor in cows injected with the lowest doses of vitamins C and E.

With ultrasound we observed and measured the corpus luteum in real time, and a positive correlation between its size and functionality is assumed (29). However, results from this and other studies are in disagreement. The cows injected with the reduced dose of vitamins regardless of having a similar corpus luteum size, produced less progesterone than the other groups. Similarly previous research did not find a correlation between size of the CL in regression phase and progesterone concentrations in cows (30). In addition, others found that after day eight of the oestrus cycle, the size of the corpus luteum does not determine progesterone concentrations (31). This finding supports our results when we measured corpus luteum on day nine of the oestrus cycle. We do not know the reasons for the discrepancies among studies, but three points should be considered when CL measurements and progesterone concentrations are to be analysed at the same time. First, from field experience, we know that sometimes ultrasound practitioners fail to find the transducer position that gives the largest view of the CL. This can produce confounding effects when a relationship with progesterone concentrations is sought. Second, the corpus luteum is a dynamic ovarian structure, which is more easily identified and measured during the mid-luteal stage of the oestrus cycle, but measuring this structure at a very early stage (day two to three after estrus) of development requires a great deal of experience. Third, when diagnosing the status of the corpus luteum, not only its size but also its echographic appearance should be taken into consideration (32).

We are unaware of previous studies that attempt to evaluate the effect of increasing doses of vitamin E and C on dairy cattle fertility. Other studies have

demonstrated a positive effect on pregnancy rate when supplementing vitamins C (14) and E (33) separately. We believe that the effect is mediated by enhancing follicle cell survival (6), oocyte competence, corpus luteum functionality (15, 34,16) or embryo survival (35, 36). Despite our previous field experiences showing an increase in pregnancy rate in cows injected with vitamins, the results of the present study do not support such findings. However, it is worth noting that cows injected with the lowest dose of vitamins, despite having lower concentrations of progesterone, were capable of sustaining similar and even numerically higher pregnancy rates 30 and 45 days after AI compared with the other evaluated groups.

Progesterone stimulates changes within the uterine environment allowing embryo receptivity and survival (37). The concentrations of progesterone required to increase the probability of pregnancy occurrence is not well established. One may argue that higher, rather than lower, concentrations of progesterone are better for getting a cow pregnant. However, researches have suggested a range of milk progesterone concentrations within which embryo survival was maximal (38). The existence of a range of progesterone concentrations suitable for pregnancy success is acceptable because a large concentration of progesterone could affect fertility by creating an asynchrony between the uterine environment and the embryo (39), while a uterine environment with low progesterone concentrations will fail to induce the changes necessary for hosting the embryo (40). Besides progesterone concentration, it is well known that embryo quality affects probability of pregnancy, and good quality embryos are better at achieving not only

pregnancy, but also live birth in a uterine environment with variable progesterone concentrations, than those embryos of lower quality (41). Therefore, it is possible that cows injected with the lowest dose of vitamins may have had good quality embryos capable of surviving and establishing pregnancy in a uterine environment with low progesterone concentrations.

3.3.7 CONCLUSION

In conclusion, injection of increased doses of vitamins C and E does not affect preovulatory follicle and corpus luteum size, estradiol production on the day of estrus, or pregnancy rate 30 and 45 days after AI. However, cows injected with 3,000 mg of vitamin C and 3,000 IU of vitamin E are able to establish a pregnancy under low progesterone concentration conditions.

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Table 5 Mean (\pm SE) for the effect of injecting 3,000 to 6,000 mg/IU of vitamins C and E on ovarian structure size, estrus presentation and hormone concentrations in Holstein dairy cows

Variable	Treatment			<i>p</i> -value
	Control	Vitamin C and E-3,000	Vitamin C and E-6,000	
Time to estrus (h)	48.1 \pm 5.17	55.2 \pm 5.36	62.1 \pm 5.10	0.17
Diameter of the preovulatory follicle (mm)	18.9 \pm 0.71	17.1 \pm 0.73	16.5 \pm 0.69	0.06
Plasma estradiol concentrations (pg mL ⁻¹)	37.8 \pm 4.19	40.1 \pm 4.00	38.8 \pm 3.85	0.92
Area of the corpus luteum (cm ²)	6.7 \pm 0.52	7.3 \pm 0.54	6.0 \pm 0.52	0.25
Plasma progesterone concentrations (ng mL ⁻¹)	19.4 \pm 2.66	10.1 \pm 2.55*	19.2 \pm 2.44	0.02

* Significantly different from other groups ($p \leq 0.05$).

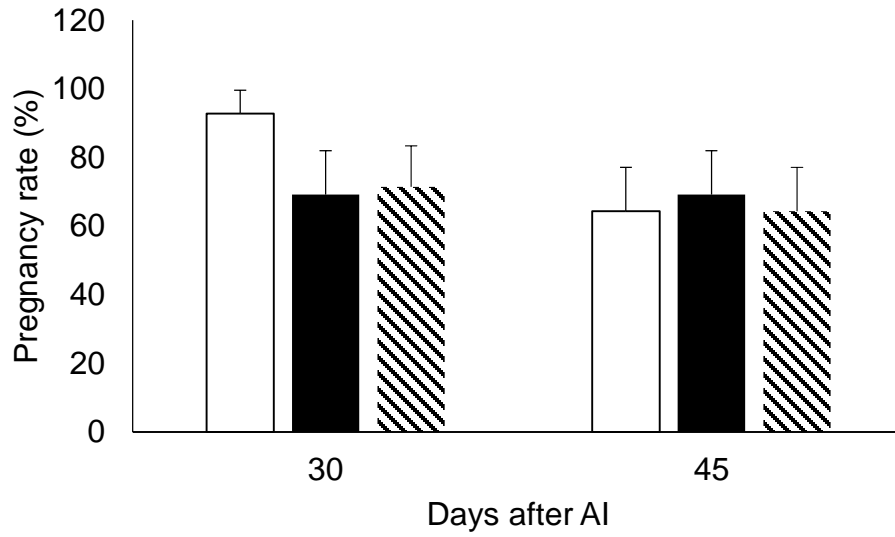


Figure 4 Pregnancy rate 30 and 45 days after AI in control group (white bars) and Holstein dairy cows injected with 3,000 (black bars) or 6,000 (hatched bars) mg/IU of vitamins C and E

CHAPTER 4. GENERAL CONCLUSION



The administration of vitamins C and E injections before and after a synchronized estrus is a feasible way to increase pregnancy rate in Holstein dairy cattle. However, this cannot be explained by changes on preovulatory follicle and corpus luteum size, nor by affecting blood concentrations of estradiol and progesterone.