The short term effect of intramuscular tetracycline injection on blood, fecal and bone mineral homeostasis in goats in Mafikeng area.

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DECLARATION

I, **RAMAKIDIANA PATRICK RANTLHWATLHWA (23833688)**, declare that the dissertation entitled "The short-term effects of intramuscular tetracycline injection on blood, faecal and bone mineral homeostasis in goats in the Mafikeng Area", hereby submitted for the degree of Master's in Animal Health has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein, have been duly acknowledged.

Signature: …… Date: …….…….

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ABSTRACT

The aim of this study was to examine the short-term effect of intramuscular Tetracycline injection on blood, faecal and bone mineral homeostasis in boar goats in Mafikeng, South Africa. In order to achieve this objective, 10 goats (aged about 2 years) were placed into 5 different groups and used for the experiment. The treated groups were injected with Tetracycline at 10mg/kg dose, according to individual goat weight intramuscular while the control group was not injected. Blood and faecal samples were collected before 0 hour and at 3, 6, 24, 48 and 72 hours intervals while bone samples were collected at 0 hour before the injection of Tetracycline and also at 3, 24, 48 hours post-treatment.

Samples collected were analysed for Ca, Mg and P and measured in both groups of goats on serum, bone and faecal samples using the biochemical method / ICP MS/MS analysis machine.

Results obtained showed an increase of calcium and magnesium concentrations in the serum and faeces of treated goats (over time) with significant differences ($p<0.05$) of concentrations compared to the control group. The results also showed no significant effect of Calcium in bones. However, there was an increase of Phosphorus (197.51mg/dl) and a decrease of Mg in bones.

In addition, an increase in concentrations of Phosphorus was revealed in the study while Calcium and Magnesium showed a slight increase for the first 24 hours before decreasing (which could be explained by the fact that the injection of Tetracycline, triggered the mobilisation of cations such as Calcium and Magnesium to form the adduct or chelation, thus resulting in an increase in concentration in the serum. However, due to the low concentration of Tetracycline in the system, due to the single injection, free cations were excreted in the faeces and after 24 hours, the homeostasis regulated the system, thus controlling excretion to maintain the balance.

It is, therefore, concluded that in the short-term, there is mobilisation of Calcium and Magnesium, especially from tissues to form adducts with Tetracycline, however, this requires high concentrations of Tetracycline in the system or repeated treatment and long periods of observations to effectively assess the impact on the system.

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Tetracycline was first discovered in 1948 by Benjamin Duggar (Duggar, 1948) as a natural product produced by species belonging to Streptomyces. Since then, it has proved to be an economically valuable drug for over six decades (Karthikegan, 2005). Tetracyclines are used worldwide as antibiotics and they are cheap and affordable. The antibacterial effects of tetracycline have been explained by its binding mechanism to the 30s ribosomes, leading to inhibition of protein synthesis, thus causing the break in codon-anticodon interactions between the tRNA and mRNA (Bijan and Gerald, 2008).

Tetracycline is considered as the main antimicrobial agent to which the term broad spectrum has been applied. It acts against a broad range of Gram-negative and Gram-positive bacteria, including obligatory anaerobic ones (Chopra *et al.,* 1992). Tetracycline also acts against intracellular pathogens such as chlamydiae, rickettsiae and mycoplasmae, not forgetting eukaryotic protozoan parasites (Roberts, 2003). Tetracycline has some adverse effects on both farm animals and humans as follows: gastro intestinal effects (distress, diarrhoea, vomiting and hypersensitivity); phototoxicity; asthma; urticarial contact dermatitis; and photosensitisation (Edward, 2005). Other side-effects of tetracycline in humans and animals are: teratogenic effects; deposition in the bone of tetracycline and pigmentation of teeth occurs during calcification in animals. Accumulation of tetracycline may cause aggravation of pre-existing renal failure (Ginsberg and Tager, 1980).

An incredible aspect of tetracycline structure is the presence of keto-enol functional groups on one face of the scaffold, giving it the ability to chelate metal ions present in biological fluids (Bijan and Gerald, 2008). The drug is transported as calcium complexes in the blood plasma (Lambs *et al.,* 1983). Chelation of metal ions usually decreases the bioavailability and antibacterial effects of tetracycline (Bijan and Gerald, 2008). Complexation is an important factor for accessing their environmental fate and effects, as divalent metals are often present in high concentrations (Bijan and Gerald, 2008). In the intracellular medium, magnesium complexes appear to be more important, possibly playing a part in binding to the ribosome (Brion *et al.,* 1981).

Lambs *et al.* (1984 and 1988) state that in blood plasma concentrations, di-nuclear complexes of calcium and magnesium outweigh (dominate) mononuclear species. The effects of tetracyclines on body mineral homeostasis in farm animals have not been elaborated in the literature. The purpose of this study was to assess the possible effect(s) of tetracycline on Ca, P and Mg chelation.

1.2 Aim and objectives of the study

1.2.1 Aim of the study

The aim of this study was to evaluate the concentration of phosphorus, calcium and magnesium in faeces, blood and bones of Boer goats treated with tetracycline in Mafikeng, South Africa.

1.2.2 Objectives of the study

The objectives of the study were to:

- Assess bone, blood and faecal phosphorus, calcium and magnesium homeostasis in goats after intravenous injection of tetracycline; and
- To assess how calcium, magnesium and phosphorus interact in bones, blood, faecal samples and after intravenous injection of Tetracycline in goats in different time intervals.

1.3 Problem statement

Tetracycline is normally used as an antibiotic in farm animals. Unfortunately, it chelates minerals. The effect of tetracycline chelation on body minerals have not yet been assessed in farm animals in the literature reviewed. Also, not much has been discussed in the literature with regard to the interaction of calcium, magnesium and phosphorus in tissues relative to calcium chelation by tetracycline. The aim of this study, therefore, was to assess what happens to calcium, phosphorus and magnesium in the blood, bones and faeces after intravenous tetracycline injection (i.e. calcium, magnesium and phosphorus homeostasis due to calcium chelation).

1.4 Hypothesis

Intravenous Tetracycline injection has an effect on the blood, bone and faecal calcium, phosphorus and magnesium concentrations.

CHAPTER 2

Literature review

2.1 The Tetracyclines

2.1.1 Introduction

Tetracyclines were first discovered in the 1940s (Duggar, 1948) and considered as a family of antibiotics that act on the inhibition of protein synthesis by blocking the attachment of aminoacyl-tRNA to the ribosomal acceptor site (Chopra and Roberts, 2001). These antibiotics are broad spectrum agents, which fight against a large range of gram-negative and gram-positive bacteria, rickettsiae, chlamydia, mycoplasmas and parasites (Chopra *et al.*, 1992; Roberts, 1996). The chemical scaffold of tetracycline is a highly modified chemical that allows them to interact with different biological targets (Bijan and Wright, 2007). The antibiotic has an incredible feature of being able to chelate divalent cations (Chopra and Roberts, 2001). Chloramphenicol, tetracycline and oxtetracycline are the most extensively used drugs in veterinary practice as additives to feed and the promotion of growth due to their low cost (Wang *et al.*, 2008). Due to the satisfactory antimicrobial properties of these agents and lack of major adverse side-effects, it has led to the drug being used in the treatment of animal infections and human beings (Chopra and Roberts, 2001).

Excellent reviews have been conducted on the different stages of tetracycline inhibition and its resistance mechanisms (Roberts, 1996; Chopra and Roberts, 2001; Connel *et al.*, 2003b; Thaka *et al.* 2010; Nelson and Levy, 2011). The extensive use of tetracycline has become a problem with regard to infectious diseases; the drug leaves in milk and meat residues in treated animals, resulting in them being toxic, thus causing allergic reactions in hypersensitive organisms (Levy, 1987). More importantly, when lower levels of the drug are used in feed and foodstuffs consumed over a long period of time, the drug may be a conduit of the spread of drug-resistant micro-organisms (Cinquina *et al.*, 2003). It is reported that the use of tetracyclines in clinical practice is responsible for the selection of drug-resistant organisms (Chopra and Roberts, 2001). The first tetracycline-resistant bacterium *(shigella dysenteriae)* was isolated in 1953 (Watanabe, 1963; Falkow, 1975).

A wide range of tetracycline-resistant bacterial strains have been identified (Michalova *et al.*, 2004). Due to human food safety, tolerance concentration of many drugs in animal products have

been set by the European Union. The European regulation 2377/90 has set the maximum residues limits for tetracyclines in milk, meat and other food products (Commission Regulation, 1990).

2.1.2 Discovery and development of Tetracyclines

Both chlor-tetracycline and oxy-tetracycline were both discovered in the 1940s (Chopra and Roberts, 2001). These antibiotics were produced by *Streptomycin aureofaciens* and *S. rimosus,* respectively (Michalova *et al.* 2004). As the years went by, more tetracyclines were discovered (Methacycline, Doxycycline, Minocycline, Rolitetracycline, Lymecycline and Glycylclines) (Goldstein *et al.*, 1994). All the mentioned compounds are referred to as "typical tetracyclines" and belong to the first class of tetracycline antibiotics. The antibiotic interacts with the ribosomes of the bacteria, thus blocking protein synthesis and results in bacteriostatic activity (Sum *et al.*, 1998). Atypical tetracyclines such as Chelocardin, Anhydrotetracycline, Anyhydrochortetracycline and Thiatetracycline, all fall under the second class of tetracyclines and inhibit the bactericidal activity of attacking the cytoplasmic membrane (Oliva *et al.*, 1992; Chopra, 1994). Due to the cytotoxicity of these compounds and their low level inhibition, they are of no interest for therapy and are not authorised for use within the European Union and the Czech Republic (Emea, 1999).

Some tetracycline compounds produced in the early years (such as Chlomacyclines) are no longer marketed while others (such as Rolitetracycline, Lymecycline and Chlortetracycline) are no longer available in all countries of the world due to new tetracycline generations (Finch, 1997; Kucers and Bennett, 1987).

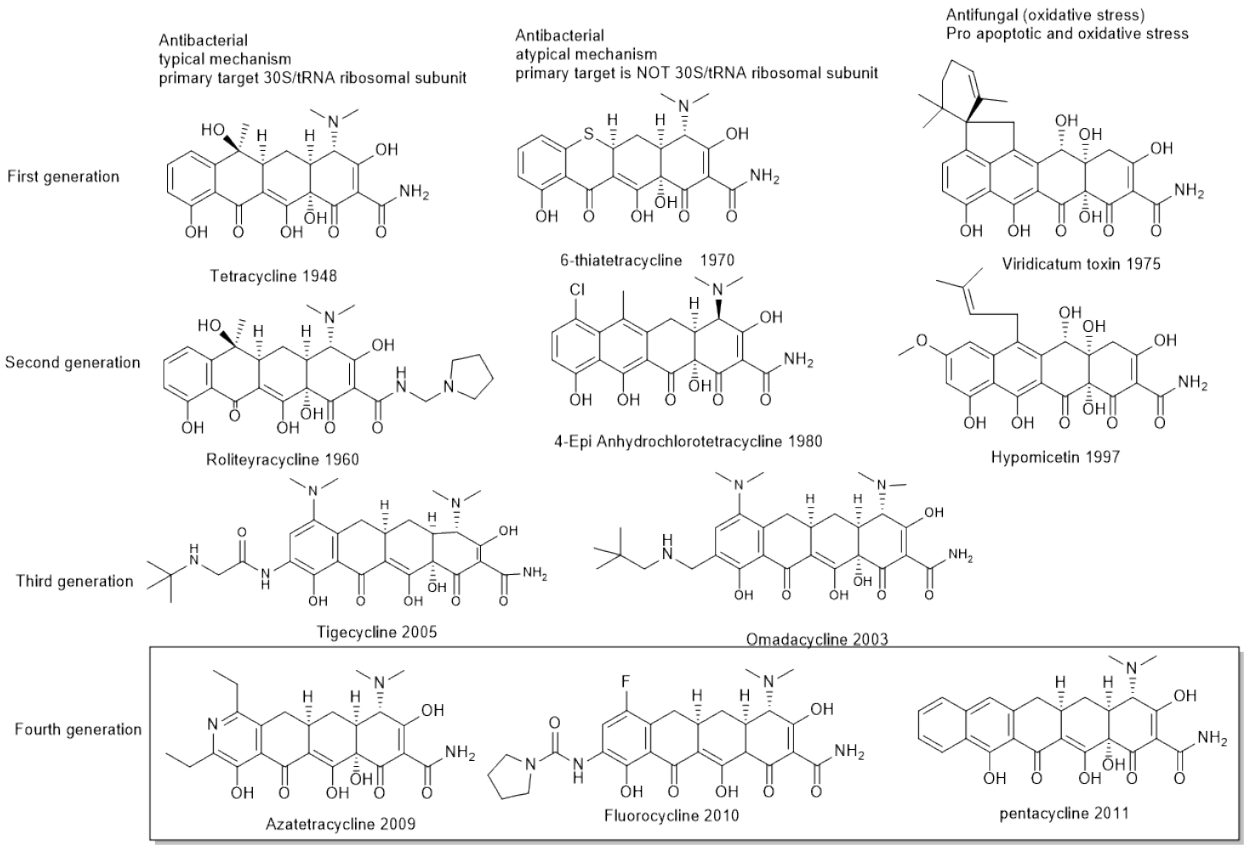

Figure 2.1: Chemical structures of different types of Tetracycline

Dr Benjamin Duggar, an employee of Lederle Laboratories, American Cynamid, was the first researcher to isolate the first compound belonging to the Tetracycline family, Chlortetracycline in late 1948 (Duggar, 1948). Chlortetracycline was first isolated from *Streptomyces aureofaciens* and because of the gold colouring of the bacteria, they were referred to as *auromycin* (Fabian *et al.*, 2014). Alexander Finlay (from Pfizer) discovered Oxytetracycline in the early 1950s from a soil bacterium *Streptomyces rimosus*, which has a second metabolite from Terramycin (Finlay *et al.*, 1950).

The chemical structure of antibiotics remained unknown until 1953, even though they were already on the market (Fabian *et al.*, 2014). Nobel Prize laureate Robert. B Woodward and the Pfizer team, for all their combined efforts and hard work, synthesized resulted formation chemical structures of oxytetracycline and chlortetracycline (Stephens *et al.*, 1952; 1954 and Hochstein *et al.*, 1953). This family of antibiotics was named "Tetracycline" due to their structures and are the naphthacene

core, comprising four aromatic rings (Stephens *et al.,* 1952). The difference between the two antibiotics oxytetracycline and tetracycline is that chlortetracycline lacks a hydroxyl group at C5 position on the ring B while on ring D at C7 position; it has a chlorine atom substituent present (Hochstein *et al.*, 1953).

However, due to chemical improvements, Pfizer-Woodward described the C7-deschloro derivative of chlortetracycline as being dominated against a large variety of bacterial pathogens, converted into tetracycline (Conover *et al.,* 1953) and is one of the simplest associate of the Tetracycline group of antibiotics. With time, in the broth of *S. aureafaciens* (Backus *et al*., 1954) and *S. rimosus* (Perlman *et al*., 1960), tetracycline was detected. Even with the revelation that tetracycline was an ancestor of chlortetracycline (McCormick *et al.,* 1960), soon after the discovery of the first generation of the group of tetracyclines, Pfizer and Lederle initiated the creation of a second generation of tetracycline compounds in which they increased antimicrobial potency, improved pharmacokinetic properties and decreased toxicity (Blackwood *et al.,* 1961).

Pfizer was led by a series of chemical modifications of ring C, which made the discovery of the semi-synthesis of methacycline (Boothe *et al.,* 1959; Blackwood *et al.,* 1961). The antibiotic is still used to date. Lederle discovered the precursor demeclocycline by analysing biogenesis mutant of chlortetracycline in *S. aureofaciens*. With time, it was derived to sanycycline, a form of tetracycline that has less effect against microbial activity and contains weak chemical features (McCormick *et al.,* 1960). Sanycycline was later transformed to form minocycline, which is one of the strongest and effective forms of tetracycline. During that time, and considering the fact that it was the last type of tetracycline to be introduced on the market in the 20th century (Martell and Boothe, 1967), the development of the third generation of antibiotics was of interest to researchers due to the emergence of antibiotic resistance.

During the late 1980s, Lederle Wyeth reviewed the Tetracycline programme. Tetracycline properties and mode of actions have been re-evaluated again according to recent knowledge obtained about the antibiotic (Tally *et al.,* 1995) and has led to the focus on the modification of C7 and C9 position of ring D of the sancycline core. The synthesis of a series of C9-aminotetracyclines

(bearing glycyl moiety), made a breakthrough (Sum *et al.,* 1994) and the t-butyl amino group, is the most potent antimicrobial. Tetraphase pharmaceuticals utilised Meyers chemistry (Sum *et al.,* 2008) to acquire the Fluorocycline, Eravacycline, which contains C7-fluoroand C9-pyrrolidirioace modification on ring D (Grossman *et al.,* 2012).

2.1.3 Mode of action

These antibiotics are broad spectrum and are both bactericidal and bacteriostatic (Zakeri and Wright, 2007). Tsankov *et al*. (2003) state that tetracyclines are infused through the cell wall of the bacteria, by passive diffusion and also through the cytoplasmic membrane, through an energy dependent process (Tsankov *et al.,* 2003). The bacteriostatic mode of action of the compounds are well categorised, however, bactericidal tetracyclines (e.g. Chelocardin) are less spoken of and understood (Schnappinger and Hillen, 1996).

Three major classes of bactericidal antibiotics (B-lactams, Quinolones and Aminoglycosides) that have cellular killing capabilities should be given some credit towards the production of toxic hydroxyl radical formation by bactericidal tetracyclines (Zakeri and Wright, 2007). The reversible inhibition of the protein synthesis is associated with antibacterial activity of typical tetracyclines (Laskin, 1967; Kersten and Frey, 1972). The antimicrobial functions of tetracyclines have been recognised towards the binding of the 30S ribosomal subunit close to the A site and subsequent inhibition of protein synthesis by preventing aminoacylated-tRNA docking (Maxwell, 1967; Brodesen *et al.,* 2000). Some ribosome proteins are involved (s3, s14 & s19), (Franklin, 1966; Buck and Cooperman, 1990) while some bases in the 16s rRNA such as e.g. G_{6931}, U_{1052}, C_{1054}, C_{1330} and C_{1138} are involved in the binding of tetracycline to ribosomes (Chopra *et al.,* 1992).

2.1.4 Tetracycline uptake

In order for tetracyclines to gain access to ribosomes, they must first transverse biological membranes gram negative and gram positive organisms (Zakeri and Wright, 2007). Tetracyclines have the ability to chelate divalent cations (commonly magnesium) and their cellular functions are pivotal towards ionic interactions (White and Cantor, 1971). Charges on tetracyclines play a key role in drug uptake due to their ionic states (Zakeri and Wright, 2007).

Tetracyclines cross the outer membrane by going through porin channels while in complex Mg^{2+} situations in order to penetrate the outer membrane of gram negative bacteria. Afterwards, the

tetracycline-Mg^{2+} complex dissociates, thus allowing the process to diffuse through the cytoplasmic membrane into the cytosol, where Mg^{2+} ion will be chelated in order to bind the ribosome (Schnappinge and Hilen, 1996).

2.2 Generations of Tetracycline

2.2.1 First generation of Tetracycline products (Chlortetracycline, Oxytetracycline and Tetracycline)

The first generation of tetracyclines described and established are chlortetracycline (1948), oxytetracycline (1951) and tetracycline (1953). They were all discovered in the late 1940s and early 1950s during the golden age period of antibiotic discovery due to the screening programmes of natural products (Zakeri and Wright, 2007). These compounds were made attractive to the pharmaceutical industry because of their ability to act against a plethora of microbes from diverse genetic, ecological and physiological backgrounds, falling under broad spectrum antibiotics (Bradford and Jones, 2012).

This generation of antibiotics does not have extreme side-effects and have a strong oral bioavailability and pharmacokinetic parameters, including how easy and inexpensive they are to produce (Aqwuh and MacGowan, 2006). All these features have made tetracycline, a highly favoured drug of choice for the fight against bacterial infections. A few years later, the second generation of tetracyclines were produced from soil-dwelling organisms and isolated as fermentation products produced by the organisms (Duggar, 1948).

2.2.2 Second generation of Tetracycline products (doxycycline and minocycline)

The second generation of antibiotics (doxycycline produced in 1967 and minocycline produced in 1971) display a higher antimicrobial function compared to tetracycline against a range of gram-positive and particularly, gram-negative bacteria (Brandford and Jones, 2012). Minocycline antibiotics have been found to have a 20-fold higher affinity to ribosomes compared to those of tetracycline (they are 5- fold less than those of tigecycline, and are more efficient than tetracycline in the inhibition on *in vitro* 2-7 fold) (Bereron *et al.,* 1998; Olson *et al.,* 2006). It has been suggested that minocycline binds analogously to the ribosome as tetracycline due to the fact that both minocycline and tetracycline have resemblance in their chemical structure while minocycline has the ability to compete with tetracycline for ribosome binding (Olson *et al.,* 2006).

The presence of the C7-dimethylamido group on ring D has improved the binding properties of minocycline and it may assist stacking interactions with C_{1054}. Minocyclines, which fall under the second generation of tetracyclines, are more lipophilic and display better absorption and pharmacokinetic parameters than their parent compounds (Aqwuh and MacGowan, 2006).

2.2.3 Third generation Tetracycline products (Glycylcyclines and Tigecycline)

The third generation of tetracycline products include glycylcyclines and tigecycline, produced in 1999. Compared to the first and second generations of tetracyclines, sancycline (DMG-DMDOT) and minocycline (DMG-MINO), they have similar minimal inhibitory concentrations against susceptible and resistant bacterial strains. These derivatives show improved inhibitory activities against a large range of gram-negative and gram-positive bacteria (Testa *et al.,* 1993; Barden *et al.,* 1994 and Sum *et al.,* 1994).

Glycylcycline products, such as tigecycline, have enhanced their ribosome binding properties as well as exhibiting 10- 30-fold lower than half the inhibitory concentrations during *in vitro* translation compared to tetracycline (Bergeon *et al.,* 1996; Olson *et al.,* 2006; Grossman *et al.,* 2012; Tenner *et al.,* 2013). It has been reported that tigecycline has a higher binding affinity for ribosomes compared to tetracycline. It also has 10- 100-fold, higher affinity than tetracycline (Olson *et al.,* 2006; Grossman *et al.,* 2012; Jenner *et al.,* 2013).

The similarity in chemical foot printing and hydroxyl-radical cleavage forms produced in the presence of each drug (Moazed and Ndler, 1987; Beur *et al.,* 2014), as well as the similar binding site of tigecycline with tetracycline, is also maintained by the opposition of tigecycline and tetracycline for ribosome binding (Olson *et al.,* 2006; Grossman *et al.,* 2012). The main difference between tetracycline and tigecycline is the 9-t-butylglycylamido and 7-dimethlamido attached to ring D. Furthermore, the modest overlap detected between tetracycline and the A-tRNA, moiety of tetracycline 9-t-batylglycylamido have gradually improved the steric overlap of tigecycline and the anticodon loop of the Aminoacyl-tRNA (Draper et *al.,* 2013).

2.2.4 Fourth generation Tetracycline products (Azatetracycline, Fluarocycline and Pentacycline)

Thousands of medicinal chemists around the world have been synthesised recently; there have been more than 300 tetracyclines tested produced, in particular in the USA and Japan (Draper et

al., 2013). Pentacycline antibacterials were made available by the Harvard University and a structural modification of doxycycline with five rings (azatetracycline and alkylaminotetracycline antibacterial were made available from other labs) (Grossman *et al.,* 2012).

The therapeutic usefulness of tetracyclines was introduced by Golub and McNamara 35 in 1983 (Brandford and Jones, 2012). Only tetracyclines can inhibit the activity of collagenase protease, produced by host tissues, which have been repeated in the implication of periodontal destruction (Olson *et al.,* 2006). The discovery of new properties of these drugs could provide a novel approach towards the treatment of diseases, including some medical disorders such as non-infectious corneal ulcers. Excessive collagen destruction by tetracyclines is involved, and seems to inhibit activity through a mechanism unrelated to the drug's antibacterial efficacy (Beur *et al.,* 2014). All chemical modified tetracyclines have been improved with the ability to remove the dimethylamino group from the C4 location on the A ring (Jenner *et al.,* 2013). In order to easily classify the new compounds, it is very important to understand the chemical properties that make it possible for tetracycline structure-based drugs to act as a chameleonic entity (Brandford and Jones, 2012).

Tetracyclines could be considered an optimus example of multi target drugs and the first well-documented in literature. Moreover, doxorubicin and all other anthracyclines are structurally correlated to tetracyclines and it is appropriate to classify both of them in the same scheme because of their chemical similarities, chemical physics properties and mechanisms of action as anticancer drugs (Beur *et al.,* 2014).

2.3 Resistance to Tetracyclines

2.3.1 Introduction

During the mid-1950s, a large number of pathogenic and commensal bacteria were vulnerable to tetracyclines (Levy, 1984) as shown by the outcome of 433 different members of the *Enterobacteriaceae* collected between 1917 and 1954, where about 2% were resistant to tetracyclines (Hughes and Datta, 1983). Before the widespread use of tetracycline, there were studies on naturally occurring environmental bacteria, which were representative of the existing population (Dancer and Platt, 1997). With results of genetic acquisition of *tet* genes, resistance to tetracyclines has risen in many pathogenic and commensal bacteria (Chopra and Roberts, 2001).

2.3.2 Resistance mechanisms of Tetracyclines

There are four main mechanisms that enable bacteria to acquire resistance to tetracycline. Innate mechanisms exist in some bacteria since some of the defined groups are based primarily on sequence homology (Guilaume *et al.,* 2004). Frequently occurring tetracycline resistances are determined in gram negative bacteria, however, group 1 drug H^+ antiporters containing 12 transmembrane helices are, by far, the largest group and comprise well organised Tetracycline efflux pumps such as TetA (Nguyen *et al.,* 2014).

The four main mechanisms that allow bacteria to acquire resistance are as follows: (i) through active efflux and they are able to reduce intracellular concentrations of the compound (Ball *et al.,* 1980); (ii) the disruption of tetracycline ribosomal interaction by ribosomal protection proteins (Burdett, 1991); (iii) through monohydroxylation with the occurrence of enzymatic inactivation of the drug (Yang *et al.,* 2004); and (iv) through 16s RNA mutation for the bacteria to change the target site (Ross *et al.,* 1998).

Furthermore, the most common types of resistance developed among environmental and pathogenic bacteria are ribosomal and tetracycline efflux proteins (Chopra and Roberts, 2001). *Tet* and *oxt* were among the 40 identified tetracycline resistance genes in July 2007 (Zakeri and Wright, 2007). About thirty-three different tetracycline resistance genes (*tet*) and about three oxytetracycline (*oxt*) genes have been categorised (Roberts, 2003). *Tet* genes were named the new *tet* gene since they showed less than 80% amino acids and were the only genes identified so far known (Levy *et al.,* 1999). Oxyteyracycline resistance genes were the first to be described in oxytetracycline producing organisms, which is shown by the nomenclature, however, there is no crucial difference between *tet* and *oxt* genes (Ohnaki *et al.,* 1985; Doyle *et al.,* 1991).

2.4 Tetracycline

2.4.1 History of Tetracycline

Benjamin Duggar was the first to discover the maiden member of the family of tetracyclines in 1945, and referred to it as Aureomycin (chlortetracycline), which is a product of natural fermentation of *Streptomyces aureofaciens*, which is naturally present in the soil. After two years, a second isolated tetracycline, Terramycin (referred to as oxytetracycline) was synthesised by the *Streptomyces rimosus* (Sociedade Brasileira de Pediatria, 2012).

In 1953, tetracycline molecules were obtained (they have the simplest structure of this antibiotic family maintaining its functions). They were obtained through a biological process followed by a chemical one, which consisted in obtaining a precursor molecule by fermentation, followed by a chemical reaction for introducing functional groups in the precursor molecule (Edward, 2005). It was observed that the basic structures of two antibiotics (Aureomycin and Terramycin) were the same, and the generic name (Tetracycline) was suggested. After this discovery, many studies were conducted to identify new strains of tetracyclines (Pereira-Maia *et al.,* 2010; Sociedade Brasileira de Pediatria, 2012).

The three antibiotics (tetracycline, oxytetracycline and chlortetracycline) were the basis for obtaining new derivatives and to design less toxic drugs with better therapeutic use. Several by-products were synthesised such as demeclocycline, rolitetracycline and the methacycline, considered as first generation Tetracyclines. An inconvenience of these antibiotics was the short time they could persist in the body. The second generation of tetracycline (doxycycline and minocycline) were used to overcome this inconvenience (Sociedade Brasileira de Pediatria, 2012). From 1950 to 1970, several members of the tetracycline family had been obtained, as natural or semisynthetic products. During the same period, tetracyclines remained among the most commonly used antibiotics in the United States (Pereira-Maia *et al.,* 2010).

2.4.2 Properties of Tetracycline

Tetracycline constitutes a large group of broad spectrum antibiotics obtained by fermentation of a specific bacteria *Streptomyces aureofaciens* and *Streptomyces rimosus* (tetracycline, chlortetracycline and oxytetracycline), semisynthetic processes (demeclocycline, rolitetracycline and methacycline) or synthetics (doxycycline and minocycline), with low molecular weight, good oral absorption and efficient hepatic excretion (Sociedade Brasileira de Pediatria, 2012). It acts as an inhibitor of protein synthesis by preventing the binding of aminoacyl-tRNA to the A site of the bacterial ribosome (Hasan *et al.,* 1985). All tetracyclines have the same spectrum and mechanism of action, adverse effects and similar tolerances by resilient organisms. However, they present some differences regarding pharmacokinetics (Sociedade Brasileira de Pediatria, 2012).

According to Pereira-Maia *et al*. (2010), the absolute configuration of the natural carbon atom C-4 is an essential requirement for the pharmacological action of these compounds. The presence of the amide grouping at C-2 was also considered as a structural feature required for the biological action of tetracyclines. Another important observation related to increased enzyme inhibitory power, was the absence of methyl groups and hydroxyl at position C-6 (Pereira-Maia *et al.*, 2010).

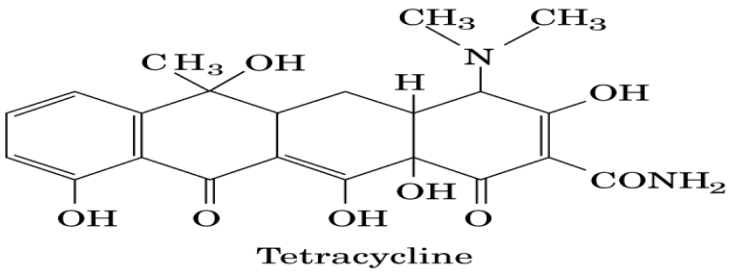

Figure 2.2: Structure of Tetracycline (C22H24N2O8)

2.4.3 Mode of action

The modes of action of tetracycline have been shown to inhibit the Mg^{2+} mediated ribosomal protein synthesis (30S subunit). In addition, the antibacterial effect is based on chelate formation, inhibition of bacterial oxidation and inhibition of nucleic acid synthesis. They are also bacteriostatic (Michalova *et al.,* 2004). It is well established that tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacylt RNA with bacterial ribosome (Chopra and Hawkey, 1992). Thus, to interact with their targets, these molecules need to traverse one or more membrane systems depending on whether the susceptible organism is gram positive or negative. Hence, a discussion of the mode of action of tetracyclines requires consideration of uptake and ribosomal binding mechanisms (Schappinger, 1996).

Also pertinent are explanations of the joint antibacterial - antiprotozoal activity of tetracyclines and the microbial selectivity of the class as a whole. Most of these issues have been considered at length (Franklin, 1966), thus the focus on new information. Tetracyclines traverse the outer membrane of gram-negative enteric bacteria through the OmpF and OmpC porin channels, as

positively charged cations (probably Magnesium)-Tetracycline coordination complexes (Chopra and Hawkey, 1992). The cationic metal ion-antibiotic complex is attracted by the Donnan potential across the outer membrane, leading to accumulation in the periplasm, where the metal ion-tetracycline complex probably dissociates to liberate uncharged tetracycline, a weakly lipophilic molecule able to diffuse through the lipid bilayer regions of the inner (cytoplasmic) membrane (Edline, 1991).

2.4.4 Chelation property of Tetracycline

Tetracyclines have high affinity to form chelates with polyvalent metallic cations (eg. Ca^{2+}, Mg^{2+}, Fe^{2+} and P^{3+}). Chelation of minerals by tetracycline also depends on the dose and route of administration. Divalent minerals are mostly chelated in the blood than in faeces and bones (which takes more time). Tetracyclines may chelate with divalent and trivalent metal ions, such as Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+}, and Al^{3+} at positions C-11 and C-12. The complexation constants for OTC with a few divalent metals may be found in Magnam *et al.* (1984); Lunestad and Goksøyr (1990); and Nova´k-Pe´kli *et al.* (1996). Chelation generally reduces bioavailability and the antibacterial effects of Tetracyclines. Complexation is, therefore, an important factor for assessing their environmental fate and effects, as divalent metals are often present in high concentrations *(*Magnam *et al.*,1984).

2.4.5 Absorption, distribution, metabolism and excretion of Tetracycline

These antibiotics are partially absorbed from the stomach and upper gastrointestinal tract. Food impairs absorption of all tetracyclines, except doxycycline and minocycline. Absorption of doxycycline and minocycline is improved with food (Zakeri and Wright, 2007). Since tetracyclines form insoluble chelates with calcium, magnesium and other metal ions, their simultaneous administration with milk (calcium), magnesium hydroxide, aluminium hydroxide or iron will interfere with absorption. Some tetracyclines are not completely absorbed and any drug remaining in the intestines may inhibit sensitive intestinal microorganisms and alter normal intestinal flora (Chopra and Hawkey, 1992).

Tetracyclines are distributed throughout body tissues and fluids in concentrations that reflect the lipid solubility of each individual agent. Minocycline and doxycycline are the most lipids soluble, while oxytetracycline is the least soluble. Tetracyclines penetrate the un-inflamed meninges and cross the placental barriers (Edward, 2005). Tetracyclines are metabolised in the liver and concentrated in the bile. Bile concentrations of the drugs could be up to five times those of the plasma. Doxycycline, minocycline and chlortetracycline are excreted primarily in the faeces (Yang *et al.,* 2004). The other tetracyclines are eliminated primarily in the urine by glomerular filtration. Obviously, these tetracyclines have greater urinary antibacterial activity than doxycycline that is excreted by non-renal mechanisms (Yang *et al.,* 2004).

2.4.6 Clinical uses

Tetracycline is the drug of choice in infections with mycoplasma pneumonia, chlamydia and rickettsia. It is used in combination regimens to treat gastric and duodenal ulcer conditions caused by *Helicobacter pylori*. Tetracyclines rapidly stop the shedding of vibrios in cholera, however, the bacterium's resistance has been proven during epidemics. Tetracycline, usually in combination with an aminoglycosides, is indicated for plague, tularaemia and brucellosis (Zakeri and Wright, 2007). It is indicated in the treatment of some spirochetes such as Lyme disease and Leptospirosis. Tetracyclines are, sometimes, employed in the treatment of protozoal infections such as those caused by *Entamoeba histolytica* or *Plasmodium falciparum*. Minocycline can eradicate the meningococcal carrier state. Demeclocycline inhibits the action of anti-diuretic hormone (ADH) in the renal tubule and has been used in the treatment of inappropriate secretion of ADH (Brandford and Jones, 2012).

2.4.7 Adverse effects of Tetracycline

Some of the adverse effects of tetracycline are as follows: gastric discomfort, epigastric distress commonly results from irritation of the gastric mucosa. This is often responsible for non-compliance in patients treated with such drugs. Effects on calcified tissue: deposition in the bone and primary dentition occurs during calcification in growing children (Bijan and Gerald, 2008). This causes discoloration and hypoplasia of the teeth and a temporary stunting of growth; there may be fatal hepatotoxicity: This side effect has been known to occur in pregnant female animals

that receive high doses of tetracyclines, especially if they were experiencing pyelonephritis (Edward, 2005).

Phototoxicity: Phototoxicity, such as severe sun-burn, occurs when a patient receiving tetracycline is exposed to the sun or ultraviolet rays. This toxicity is encountered most frequently with tetracycline, doxycycline and demeclocycline. Vestibular problems: dizziness, nausea and vomiting occur particularly with minocycline, which concentrates in the endolymph of the ear. Doxycycline may also cause vestibular effects (Brandford and Jones, 2012).

Pseudotumor cerebri: intracranial hypertension characterised by headache and blurred vision may also cause vestibular effects (Bijan and Gerald, 2008). Superinfections: overgrowth of candida in the vagina or resistant staphylococci in the intestine may also occur. Pseudomembranous colitis due to overgrowth of *Clostridium difficile* has also been reported.

Contraindications: Renally impaired patients should not be treated with any of the tetracyclines, except doxycycline. Accumulation of tetracycline may aggravate pre-existing azotaemia by interfering with protein synthesis, thus promoting amino acid degradation. Tetracyclines should not be employed in pregnant or breast feeding women or in children less than 8 years of age (Edward, 2005).

2.5 Essential role of minerals

All mammals require minerals for certain metabolism to occur in their body system. Among these minerals, the essential ones include P, Ca, Mg and Na. These minerals also serve as an intracellular buffer in the body fluids (Underwood, 1981). Limited supplies of phosphorus in the body could result in the rate and efficiency of growth tissues being delayed or even depressed. In other cases, multiple mineral deficiencies could result in aggravating the complexity of clinical symptoms in a combined selenium and vitamin E deficiency (Blood and Henderson, 1983).

2.5.1 Calcium (Ca)

2.5.1.1 Functions of Calcium (Ca)

Calcium is one of the most important minerals in the animal's body and 99% of it is found in the skeleton, including the teeth (Underwood and Suttle, 2001). Calcium functions mostly in the formation of the teeth and bones. When tetracycline is deposited on the bones, it tends to chelate calcium (Hale and Olson, 2005). Furthermore, Hale and Olson (2005) also reported that leakage of calcium in the animal's body contributes an important role in bone structure and deficiency in young animals as well as to the incomplete formation of the skeletal structure. Calcium also functions in blood clotting. Calcium's stomach absorption and bone tissue metabolism are important for maintaining an appropriate status of calcium and phosphorus (Michalek *et al.,* 2008). Calcium contributes to lowering cholesterol, helps in preventing muscle cramps and in the synthesising of Deoxyribose Nucleic Acid and Ribonucleic Acid (Underwood, 1981). With regard to animal organs, calcium provides a strong framework for supporting and protecting important organs (Underwood and Suttla, 2001).

2.5.1.2 Calcium deficiency

Deficiencies of calcium mostly affects young animals and results in abnormal growth and deformed bones. In older animals, it causes osteoporosis (Yami 2005). High amounts of calcium reduce the absorption and use of zinc. This deficiency might be primary or secondary in livestock but all of them result in osteodystrophy (Blood and Radostits, 1989). Deficiency of calcium is mostly expected if animals are given mainly grain rations and get small or no roughages (Blood and Radostits, 1989). According to Blood and Radostits (1989), high amounts of magnesium decrease calcium absorption, resulting in replacement of calcium in the bones and increased calcium excretion. Based on limited information about goats, it is likely that dairy breeds might be likely to be affected by manifestations of hypocalcaemia.

2.5.2 Magnesium (Mg)

2.5.2.1 Functions of Magnesium (Mg)

Magnesium is a crucial cation and is involved in enzymatic reactions. Magnesium is very important with regard to energy-requiring metabolism processes, nervous tissue conduction,

membrane integrity, protein synthesis, intermediary metabolism and hormone secretion (Laires *et al.*, 2004).

2.5.2.2 Magnesium deficiency

Magnesium is absorbed in the rumen in small amounts in ruminants and contributes in the maintenance of blood calcium concentrations and hypomagnesaemia could induce hypocalcaemia. Goats and some animals have little ability to manage blood magnesium concentrations if absorption is depressed. There are clinical syndromes that have been described with regard to this deficiency, however, all of them have hypomagnesaemia in common (Pugh, 2002). In goats, magnesium deficiency has led to a decrease in urine and milk production (Reis *et al.*, 2000).

2.5.3 Phosphorus (P)

2.5.3.1 Functions of Phosphorus

Phosphorus functions in bone structure, lipids and nucleic acid, as well as the development and maintenance of skeletal tissues. The important role of this mineral in the animal's body is in the maintenance of osmotic pressure, acid base balance and buffer capacity (Mamoon, 2008). Phosphorus also plays a role in energy regulation and contributes in protein synthesis, transport of fatty acids and the exchange of amino acids. Hale and Olson (2005) state that Phosphorus is involved in the chemical reaction of energy metabolism. The authors further maintain that Phosphorus deficiency results in reduced animal performance, decreased production, low milk production and reduced weight gain.

2.5.3.2 Phosphorus deficiency

In animals, phosphorus deficiency causes severe aphosphorosis, thus resulting in the development of stiffened movement posture, inflamed joints and unwillingness of animals to walk (Mokolopi and Beighle, 2006). The other effect is a decrease in blood plasma phosphate concentration as well as the response mechanism where withdrawal of calcium and phosphorus from bones cause animals to lose appetite and a decrease in body weight.

2.6 Importance of sheep and goats

Small ruminants are very important in Africa and could be used for food security, economic uses and even cultural activities. They also provide a major source of protein in the form of meat and milk (Haenlein, 2004). Goats play a unique role in providing support for some of the needy people in Africa and could play a powerful role in getting them out of poverty and towards prosperity (Peacock, 2005).

During harsh conditions, such as crop failure or family illnesses, small ruminants could be sold in order to buy food or medicine. This is an essential role in ensuring the security of family members (Peacock, 2005). Goats are kept in a wide range of agro-ecological conditions, often in mixed flock with sheep (Peacock, 2005). With increasing frequency of droughts in Southern Africa, many pastoralists are moving away from keeping cattle to keeping goats and sheep (Peacock, 2005). Some of the reasons are as follows: goats and sheep are relatively cheaper to acquire and reproduce faster; they are resistant to harsh environments; the animals are mainly maintained on natural pastures and represent the main source of minerals; and plants and animals depend eventually upon the soil for their mineral nutrients. Much of the pasture herbage available throughout the different regions of the world cannot totally satisfy all the mineral requirements of grazing ruminants, largely due to seasonal variations in availability and quality.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study area

This experimental research was conducted at the Molelwane farm of the School of Agriculture, Faculty of Natural and Agricultural Sciences, North-West University, Mafikeng Campus. The city of Mahikeng is found in the North West Province of the Republic of South Africa and is located at 25degrees 52 minutes south (25° 52´ S) and 25 degrees 38 minutes east (25° 38´ E).

3.2 Experimental design

20 female Boer goats (aged about 2 years or above) were used to conduct the study. Animals were randomly selected and placed into two groups of 10 animals each (the treatment and control groups). Both groups were housed in roofed kraals with enough water and feed. Lucerne was offered to the animals for three days. The animals were weighed before the start of the experiment and the weight used to calculate the recommended tetracycline dose for each goat. Before the injection of tetracycline, bone, blood and faecal samples were collected from all the groups of animals (at 0 hour). At 0 hour, after collecting the samples at North-West University Mafikeng Campus, tetracycline (10mg/kg) was injected intravenously in each of the experimental group goats, using the jugular vein.

3.3 Blood collection and analysis

3.3.1 Blood collection

The animals were allowed to rest to reduce the possible effect of excitement on blood concentrations (McDowell *et al*., 1982). Thereafter, blood was collected from the jugular vein using vacutainer sleeves and vacutainer needles. About 10 ml of blood was transferred into red stopper tubes at 0 hours before treating the animals. Subsequent samples were collected thereafter at 3, 6, 9, 24, 48, and 72 hours respectively post-treatment with tetracycline. Blood samples were stored for 24 hours at 4°C to allow adequate separation of the serum from the clot. Blood collected was centrifuged at 10 000 rpm and serum collected through a pipette and separated from the clot. Serum was stored at -20 °C until analysis.

3.3.2 Blood / serum analysis

Serum was transferred into a clean test tube using a clean pipette. Some 0.7 ml of serum in duplicate was added to 6.65 ml of stock trichloracetic acid in a clean test tube covered and mixed individually on an electric stirrer. The tubes were left to stand at room temperature for 5 minutes to precipitate the protein in the serum. Samples were then centrifuged at 2 600 rpm for 10 minutes, 5 ml of supernatant fluid from each sample was taken out and transferred into clean tubes using a clean pipette without disturbing the sediment. About 1.5 ml of ammonium molydate, 1.5 ml of hydroquinone and 1.5 ml of sodium sulphite were mixed with sample solutions. Each sample solution was mixed throughout and left at room temperature for 40 minutes, and poured into cuvettes and analysed. The absorbance was read at 646 nm in the Aquamate UV-Visible Spectrophotometer.

3.4 Faecal collection and analysis

3.4.1 Faecal collection

Faecal samples from the rectum of the animals were collected. Liquid paraffin was used to lubricate the hands and samples collected were immediately put in the sun (on aluminium plates) for drying.

3.4.2 Faecal analysis

Faecal analysis was carried out according to the method proposed by Mpho *et al.* (2017). After drying the faecal samples, they were ground through a 2 mm sieve. Every 1 g duplicate sample was weighed in acid-cleaned dry crucibles and the weight recorded. The weighed crucibles with fresh samples and the weight of empty crucibles were given as fresh weight of the sample in order to differentiate them. The crucibles were then heated in an oven at 106 °C for 16 hours (Mokopoli and Beighle, 2006).

Crucibles with dried faeces were allowed to cool for four hours in a desiccator and weighed in order to determine the dry weight. The crucibles were then placed into the Muffle furnace for ashing at a temperature of 800 °C for 16 hours. The samples were then allowed to cool down as for the dry weight above. The ash weight of the faecal sample was determined by weighing the crucible.

Of these samples, 1 ml of concentrated nitric acid was added to the crucible and left to evaporate until dryness on a drying element (medium heated hot plate). The crucibles were then returned to the Muffle furnace for 2 hours at a temperature of 600 °C. They were later removed after 2 hours and left to cool. Some 10 ml of 5M HCl were added to each crucible and allowed to evaporate at low heat of about 60 °C until only three ml was left in the crucible. The solution was transferred into a 100 ml volumetric flask to ensure that all the contents in every crucible were completely transferred.

The contents in the flask were mixed and left to stand overnight to give undigested matter time to settle at the bottom. Some 30 ml of the mixture was removed from the flask, using a pipette, without disturbing the sediment below and transferred into 100 ml test tubes for ICP analysis. Absorbance was read at 646 nm for Phosphorus, 282 nm for Magnesium and Calcium at 422 nm respectively in the Aquamate Spectrophotometer.

3.5 Bone sampling and analysis

3.5.1 Bone sampling

5 cm of bones were taken from the right middle locations of the 9^{th}, 10^{th}, 11^{th} and 12^{th} ribs. This process was repeated on the left middle location of corresponding ribs (Little and Mainson, 1977). Previous studies by Beighle *et al.* (1993) informed the choice of the right and middle locations of ribs. The studies revealed that the dorsal, ventral and middle rib locations could be used for the purpose of comparing bone mineral content as long as the site of the rib could be used each time when taking a sample biopsy. The middle location was considered the best area of the rib sample since it presented constant results (Beighle *et al.*, 1993). Biopsies was performed the middle location as well as between the vertebral attachment and the osteochondrial junction.

Animals were given 2-3 ml of Lignocaine as local anaesthetic (injected in the middle location of the rib up to the periosteum) and about 10 minutes allowed for the anaesthetic to take effect. The area was shaved and then disinfected with Betadine. The area over the side was surgically cut using a scalpel blade and about 2 cm incision made in the skin, over the side to be sampled and cut down from the muscle to the bone.

Trephine, a hole saw, of about 6 mm was used to collect the biopsy bone tissue. Trephine was introduced in the bone tissue until it fitted properly with the bone. With proper movement of the

Trephine, the core sample was collected from the rib and the muscle closed using catgut. The skin incision was closed using vertafil which is a suturing material. Rib bone samples were cleaned using sand paper and the cortical bone kept in a dry container for future analysis.

3.5.2 Bone analysis

Cortical bone samples were weighed first in dry crucibles of known weights. The fresh weight of the bones was the difference between the weights of the dried crucibles and the weight of crucibles containing fresh bones. Crucibles containing fresh bones were dried in an oven at 106 °C for 16 hours. They were then allowed to cool down for 2 hours in a desiccator (Bock, 1979). The weight of the air-dried bone was the difference in the weight of the dried crucible and the crucible containing the dried bone. The crucible with air-dried bones was placed in a muffle furnace for ashing at 600 °C for 16 hours. The furnace was allowed to cool for 60 minutes. The door was closed for the first 30 minutes and left open during the next 30 minutes.

The crucible was then removed and placed in a desiccator, the lid closed with a stopcock and left for 30 minutes. The stopcock was left closed and samples allowed to cool for 4 hours before being weighed. The difference between the dried crucible and the crucible with bone ash was the ash weight. The sample of the bone ash in the crucible was dissolved by adding 5 ml of 5MHC1, mixed to dissolve the ash and carefully transferred into a 100 ml volumetric flask, using an acid cleaned funnel. Distilled water was used to rinse the crucible several times and the funnel rinsed several times with water. The same water was added to the volumetric flask and the flask filled up to 100 ml. This procedure was repeated for all the samples. Volumetric flasks were inverted 2 to 3 times to ensure adequate mixing and the mixture transferred into the ICP machine for analysis.

3.6 Preparation of laboratory equipment

All the necessary equipment (crucibles, pipettes, glass beakers, cylinders, volumetric flask and red stopper tubes) used in the laboratory for analysis and digestion of faecal samples were mixed with 36% HC1 and left overnight to kill micro-organisms that could be present. They were rinsed with distilled water about 2 to 3 times and, after rinsing, they were dried in a hot oven for about 16 hours at 60 °C. The crucibles were cooled in a desiccator for 16 hours. Lastly, the crucibles were weighed to determine the empty weight and recorded thereafter.

3.7 Preparation of standards

3.7.1 Preparation of Phosphorus standards

In order to prepare 5 mg% of phosphorus standard, 5 g of 1000 ppm commercial stock standards was mixed with 95 ml of distilled water and 10 ml of stock standard mixed with 90 ml of distilled water to prepare 10 mg% standard. To prepare 20 mg% standard, 20 ml of stock standard was mixed with 80 ml of distilled water.

3.7.2 Preparation of Calcium standards

To prepare 5 mg% of calcium standard, 5 ml was drawn from 1000 ppm calcium commercial stock standard solution and added to 95 ml of distilled water in a volumetric flask. To prepare 10 mg% standard, 10 ml were taken from a 1000 ppm calcium commercial stock solution and added to 90 ml of distilled water and 20 mg% were prepared by adding 20 ml of 1000 ppm calcium commercial stock solution to 80 ml of distilled water in a volumetric flask.

3.7.3 Preparation of Magnesium solution

To prepare 0.5 mg% of magnesium standard, 0.5 ml% was taken from a 1000 ppm magnesium commercial stock standard solution and added to 95 ml of distilled water in a volumetric flask. A 1 mg% standard was prepared as follows: 1 ml of 1000ppm magnesium commercial stock solution was added to 99 ml of distilled water. A 2 mg% standard was prepared by adding 2 ml of 1000 ppm commercial stock solution to 98 ml of distilled water. A 3 mg% standard was prepared by adding 3 ml of 1000 ppm magnesium commercial stock solution to 97 ml of distilled water in a volumetric flask.

3.7.4 Conditions of the inductively coupled plasma mass spectrometry (ICP-MS)

All reagents used in the study were analytical grade. Ultrapure water was obtained from a Millipore water system (Millipore), ultrapure Nitric acid (HNO_3, Merck) was used to digest the samples. Stock standard solutions of arsenic and mercury containing 10 μg/mL in 2% HNO_3 were procured from Sigma Aldrich, USA, and prepared according to the procedure (Uluozlu *et al*., 2007). Certified Reference Materials (CRM) were purchased from National Institute of Standard Technology (NIST-8436) and used for the standardisation and validation of the method.

3.8 Statistical analysis

Data were analysed using Analysis of Variance (ANOVA) throughout the general linear model of the Statistical Package for the Social Sciences (version 10.0). The results were expressed as means and standard error of the mean (SEM). Comparisons between means were done using the t-tests and the probability value taken at $p<0.05$ or less. Correlation analysis was performed to check whether there was any association among P, Ca and Mg of bone, blood and faecal samples.

3.9. Ethical considerations

Ethical clearance was obtained from the Institutional Office (Nwu-00427-17-59) of the North-West University.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter focuses on the presentation of results. The results show a progressive increase of the level of calcium in serum from 0 to 3 hours and a significant increase between 3 and 72 hours. A peak concentration of 47.2 mg/ml was observed at 42 hours before decreasing after 72 hours, while in the control group, Calcium level showed a steady decline as shown in Figure 4.1 below. Significant differences ($P<0.05$) between the control and treated groups between 3 and 72 hours with regard to concentrations were observed.

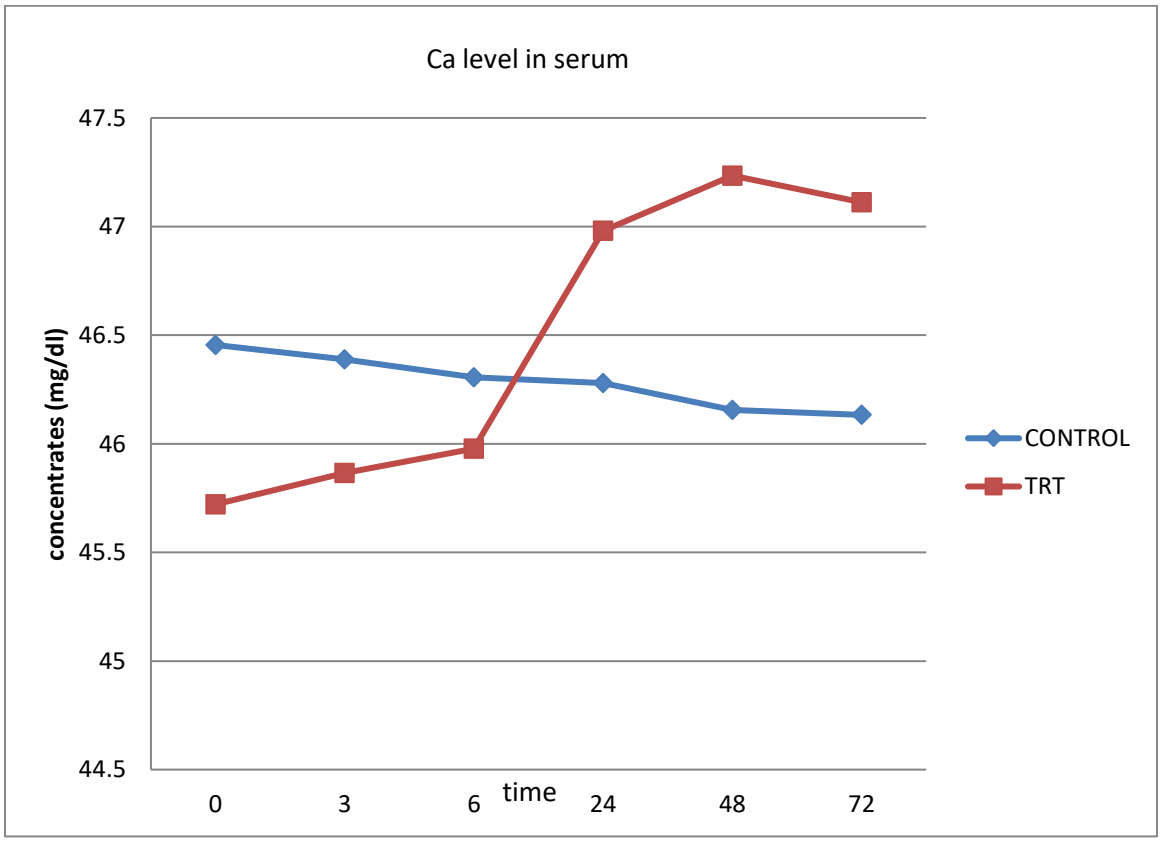

Figure 4.1: Mean concentration of serum calcium in treated/injected and controlled goats in mg/dl

Magnesium concentration declined in treated goats after intramuscular tetracycline injection between 0 and 24 hours and started to increase after 48 hours until 72 hours and had a significant difference over time as shown in Figure 4.2. The concentrations varied between 7.62 and 8.3 mg/l for treated and 6.164 and 6.34 mg/dl.

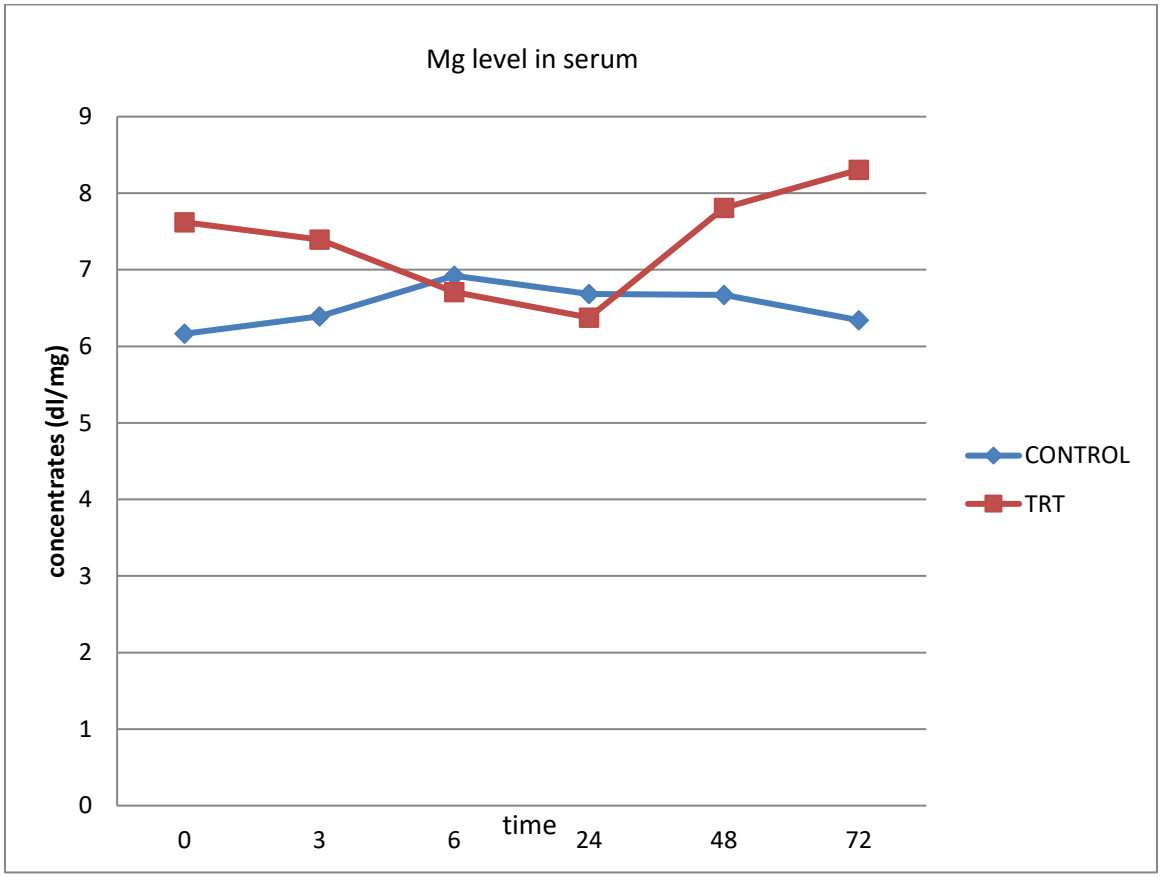

Figure 4.2: Mean serum magnesium concentration in goats (mg/dl)

Intramuscular tetracycline injection had an effect on serum phosphorus concentration in goats. A progressive increase of serum phosphorus concentration was observed in both groups (control and treated) over time (0-72 hours) but higher concentrations with statistically significant differences ($P<0.05$) were observed from 3 -72 hours in the treated group. The concentrations varied between 14.21 and 15.45 mg/dl among treated groups while in the control group, it varied between 14.11 and 14.7mg/dl (Figure 4.3).

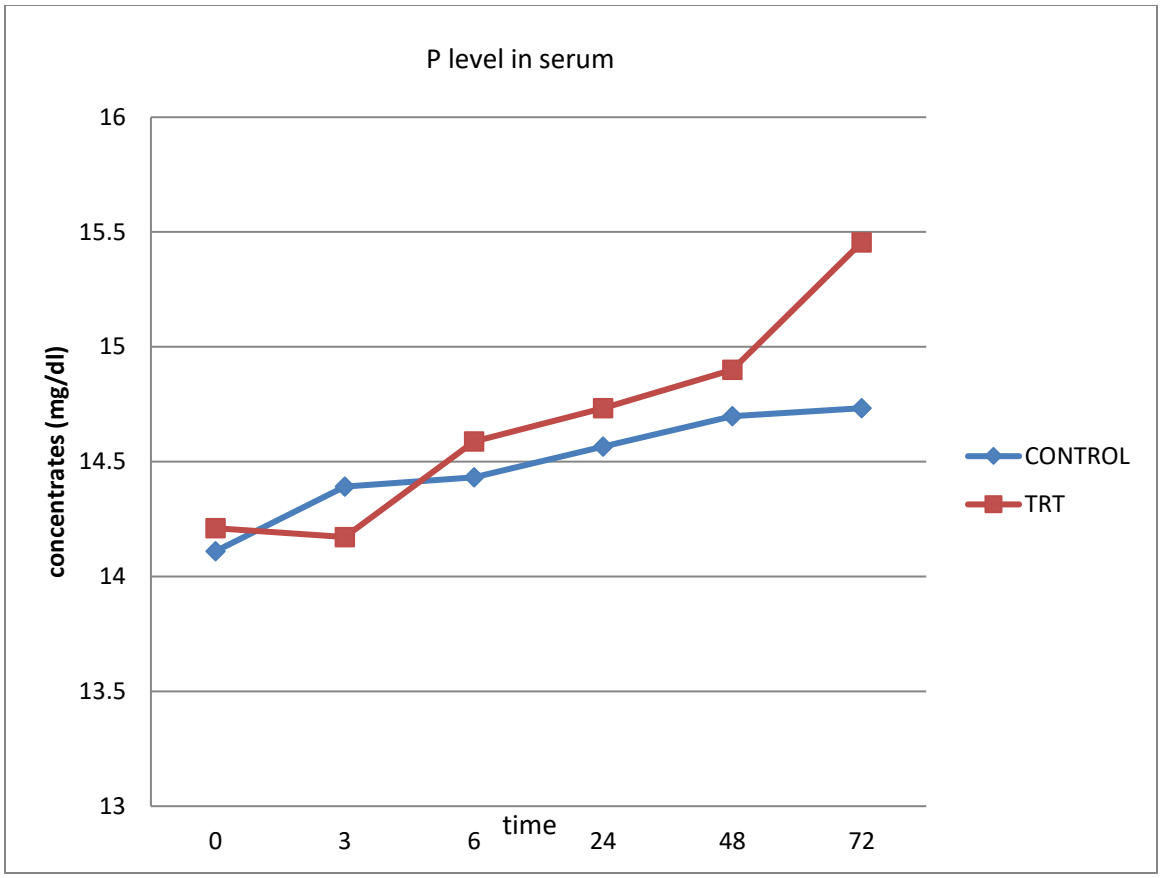

Figure 4.3: Results of the mean serum phosphorus concentration in goats in treated and control groups (mg/dl)

The results of the effect of tetracycline injection on faecal calcium concentrations (as shown in Figure 4.4) revealed that treated goats increased immediately after intramuscular tetracycline injection and reached a peak after 24 hours post-treatment and, declined at 48 hour and 72 hours with concentrations dropping from 240.28 to 170.54 mg/dl. No significant concentration variations were observed in the control group during the experiment (Figure 4.4).

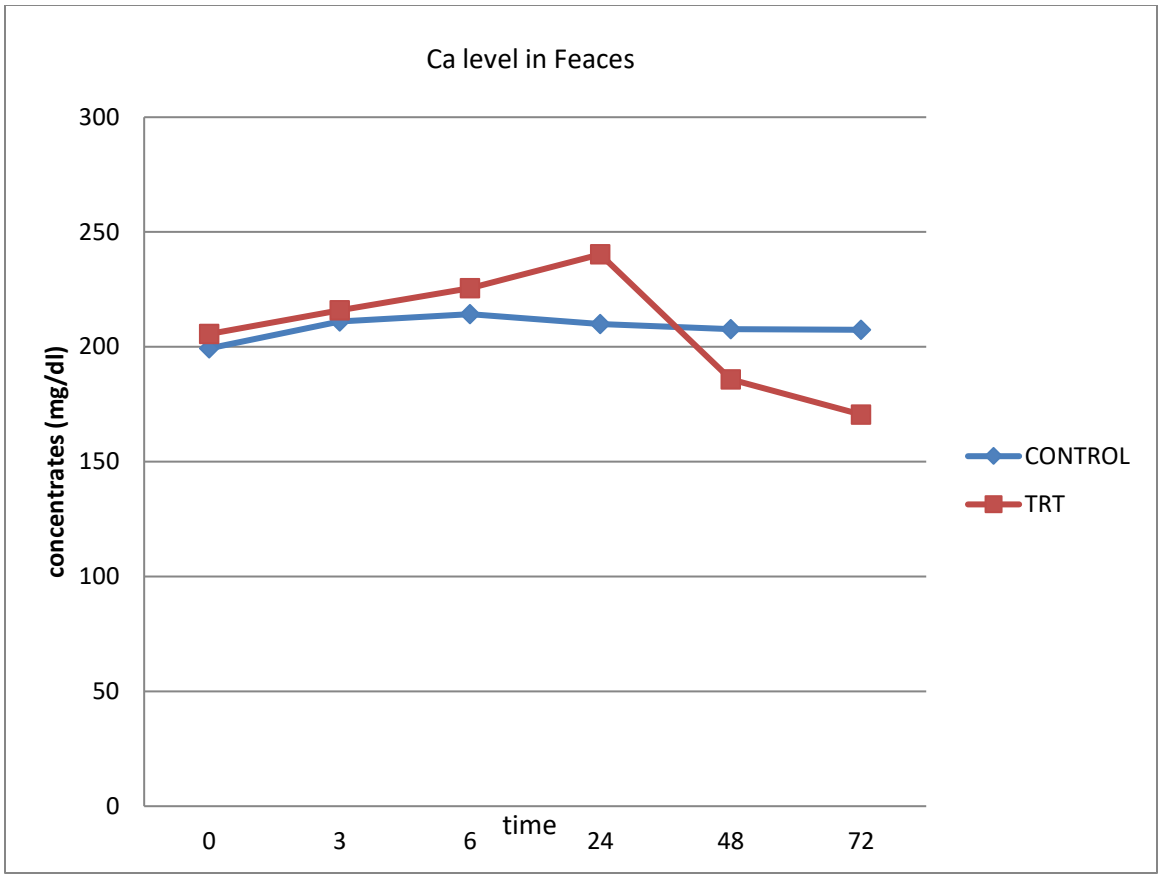

Figure 4.4: Results of the mean (mg/dl) of faecal Calcium in goats

Analysis of the effect of tetracycline injection on faecal concentration of Mg showed no significant differences between the treated and control groups. The concentration of faecal calcium among treated goats increased immediately after intramuscular tetracycline injection and reached a peak of 24.8mg/dl after 24 hours post-treatment and then declined progressively until 72 hours, thus reaching concentrations of 19.64 mg/dl. No significant concentration variations were observed in the control group (Figure 4.5).

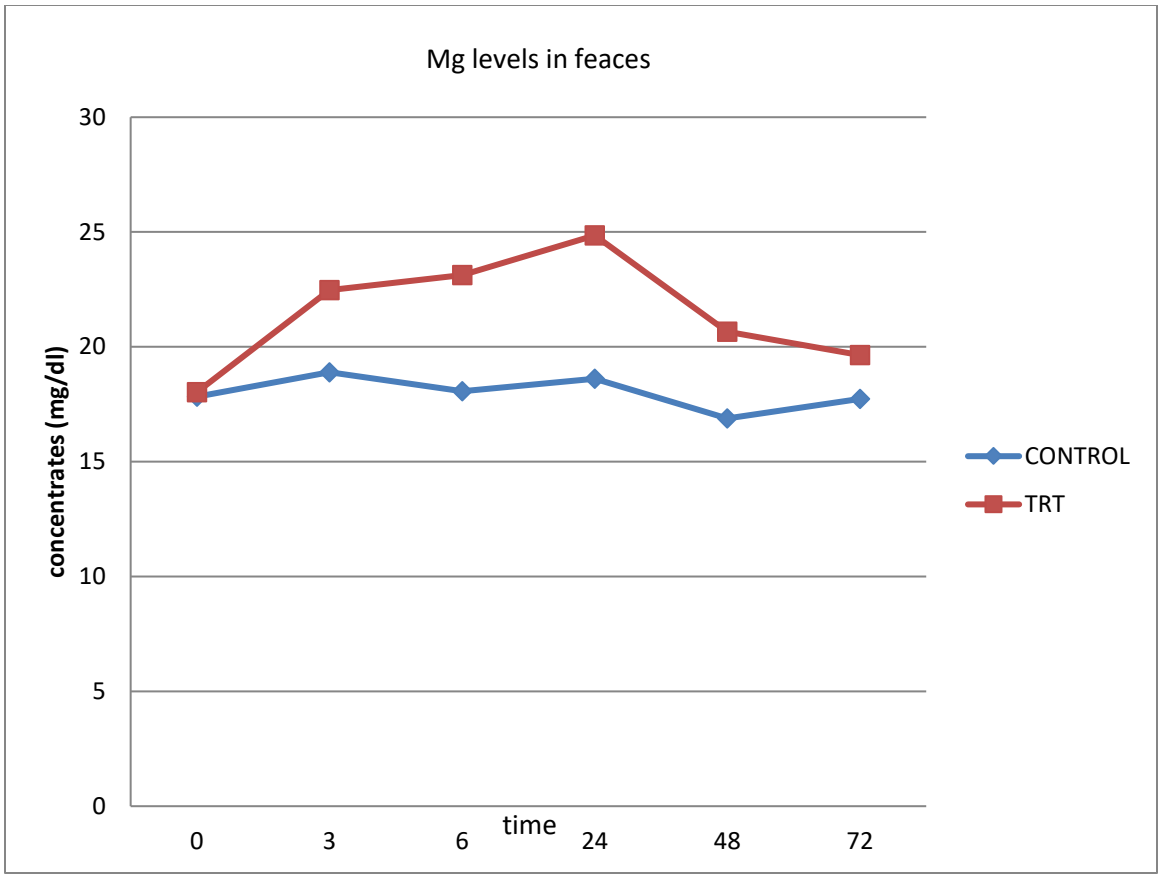

Figure 4.5: Summary of the means (mg/dl) of faecal magnesium concentration variations in goats treated with Tetracycline observed for 72 hours

The results revealed that faecal phosphorus concentration increased immediately after the intramuscular tetracycline injection until the end of the experiment. The concentrations varied from 20.85 at 0 hour to 31.9 at 72 hrs. There were significant differences ($P0<0.05$) of concentration within the treated group over time, while the control group did not show any significant variation of phosphorous concentrations over time (Figure 4.6).

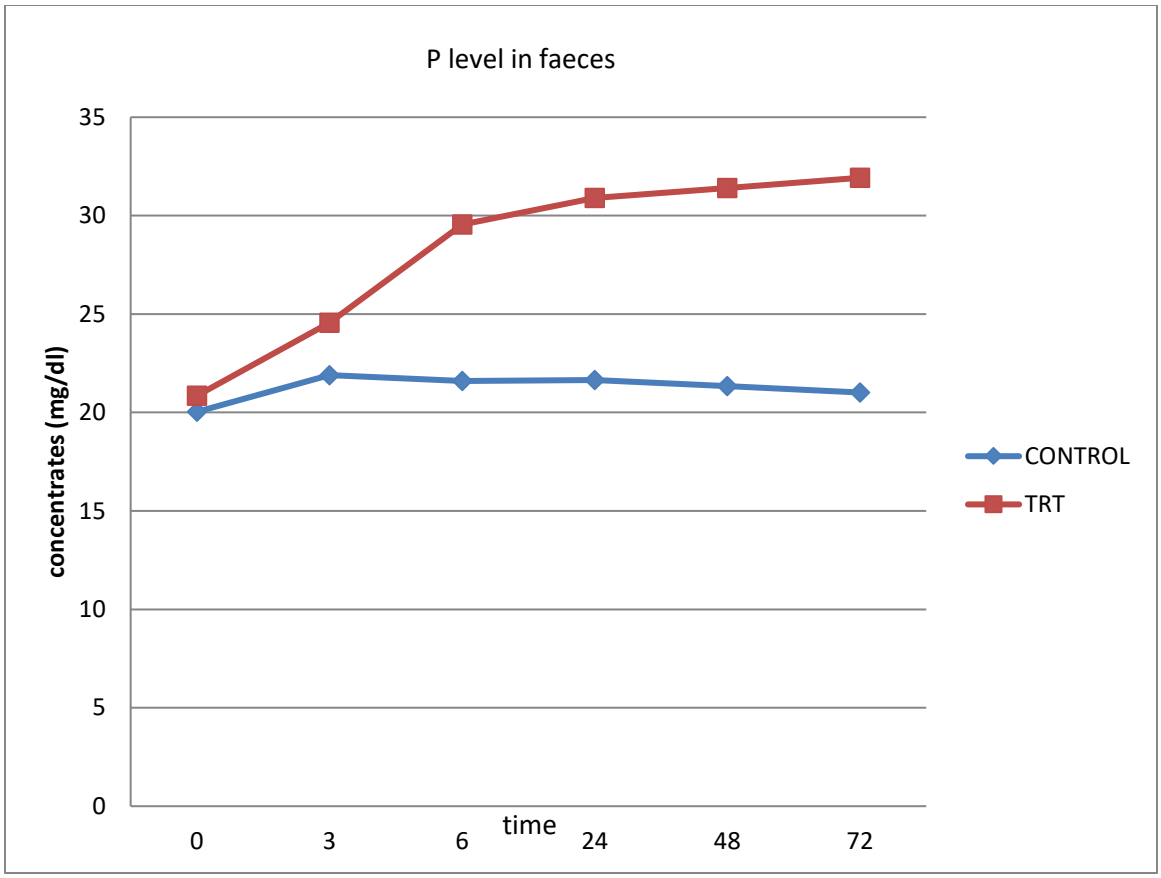

Figure 4.6: Summary of the means (mg/dl) of faecal phosphorous concentration variations in goats treated with tetracycline observed for 72 hours

In this study, mineral concentrations were also analysed in bones and the results obtained, showed that there were no significant effects ($P<0.05$) of tetracycline injection on bone calcium concentrations. No significant differences were observed between the control and treated groups (Figure 4.7). Concentrations varied between 1328.4 and 1345.15mg/dl and 1460.27 and 1580.2 mg/dl respectively in the treated and control groups.

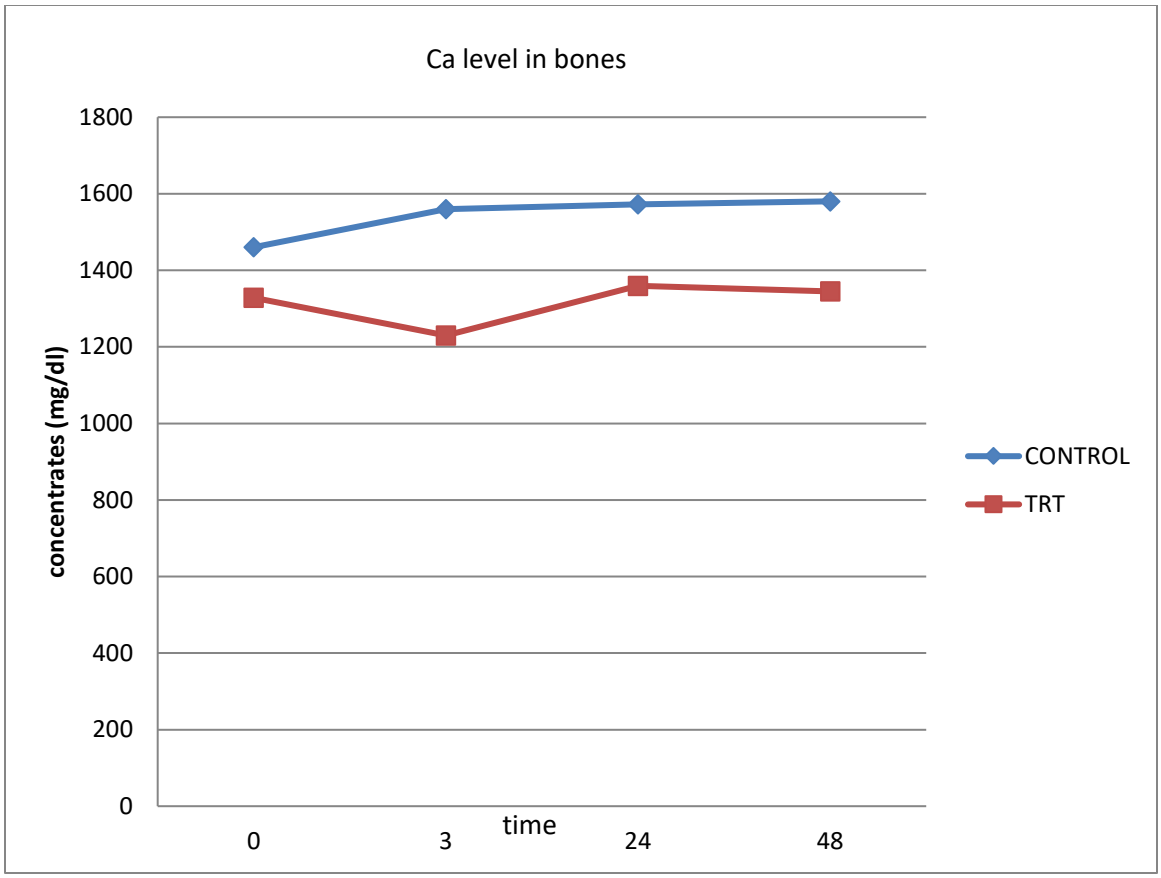

Figure 4.7: Summary of the means (mg/dl) of bone Calcium concentration variations in goats treated with tetracycline observed for 72 hours

Analysis of magnesium concentration in bone revealed a progressively significant decrease in the treated group, from hour 0 to hour 24, and then stabilised until the hour 72 compared to the control group (Figure 4.8). Concentrations were from 15.44 to 12.05 mg/dl and 16.2 to 15.29 mg/dl respectively in the treated and control groups. No significant differences were observed between the control and treated groups with regards to Mg concentrations in bones.

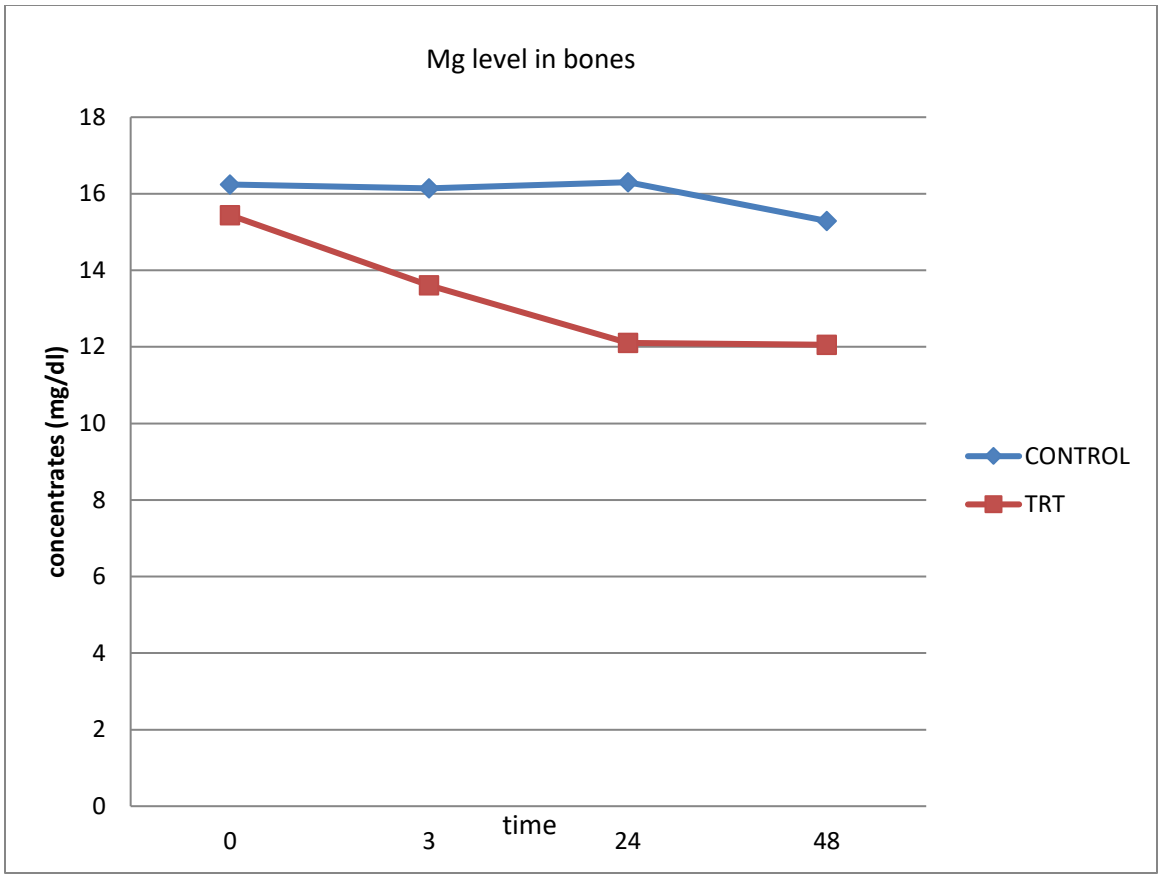

Figure 4.8: Summary of the means (mg/dl) of bone magnesium concentration variations in goats treated with tetracycline observed for 72 hours

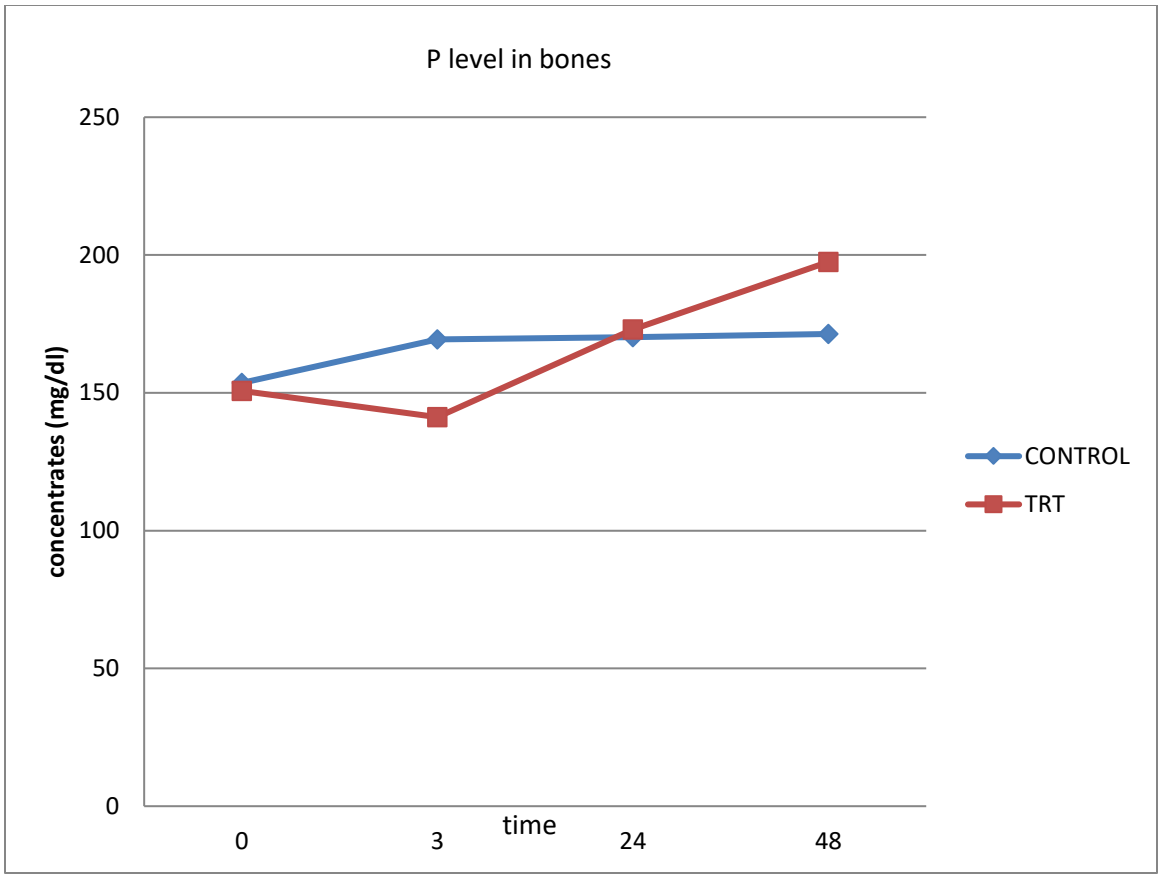

Figure 4.9: Summary of the means (mg/dl) of bone phosphorous concentration variations in goats treated with tetracycline observed for 72 hours

CHAPTER 5: DISCUSSION

5.1 Effect of Tetracycline injection of serum Calcium and Magnesium concentrations in the serum of goats

The results (see Figure 4.1) show that tetracycline injection (administered on goats) had an effect on serum calcium concentration. The increase in serum calcium concentration could be explained by the effect of the drug (tetracycline) on parathyroid hormones, thus causing an increase in serum calcium and a rise in calcium reabsorption by the kidney, thus leading to an increase of calcium in the serum. Similar findings were reported by Eduardo (1983). The results are not in line with those of Tras *et al.* (2006), who observed a significant decrease in the level of serum calcium after a few hours of administration of tetracycline injection to animals. However, the results of studies conducted on rats (Deore *et al.,* 2004), dogs (Tras *et al.,* 2006), and cattle (Hijikalai *et al.,* 2012). The decrease in the level of calcium serum of treated/injected animals could be explained by the fact that tetracycline, with its ability to chelate calcium, could form stable calcium complexes, which could lead to the reduction in its absorption from GIT and also in blood (Sasaki, 1998).

An increase in the concentration of serum calcium observed in this study could be explained by the fact that the injection triggered the mobilisation of calcium from other tissues. However, the concentration injected on the animals could not have been enough to form insoluble complexes with all mobilised calcium present in the serum thus, the increase of calcium concentration. Different researchers have reported the dose effect of tetracycline on the chelation of calcium and magnesium (Radwan *et al.,* 1998; Cappin, 2001; and Payne *et al.,* 2002).

In their study Kenneth *et al.* (2006) confirmed that all tetracyclines form insoluble complexes with calcium, Magnesium, iron and aluminium, markedly reduce their absorption in the system. The results obtained in this study are in agreement with previously conducted studies, where the increase of serum calcium was reported 24 hours into the experiment of administrating oxytetracycline in fish (Dobsikona *et al.,* 2013) and dogs (Trans *et al.,* 2006).

Previous studies have shown that the continuous use of tetracycline could cause different patterns of anaemic responses such as haemolytic and calcium, magnesium, phosphorus and iron deficiency

anaemia due to the chelating activity of the drug from the blood (Marcus *et al.,* 1985; Carrol, 2002).

5.2 Effect of Tetracycline injection on serum Magnesium (Mg) concentration

The trend of serum magnesium (Mg) concentration was similar to that of calcium in this study (Figure 4.2). After a slight decrease of Mg serum concentration in the first 3 hours, there was a significant increase until the end of the experiment. The reasons for this increase are similar to those mentioned for calcium concentration. The results obtained in this study are in line with those of Bhutto *et al.* (2017) who also noted similar results in goats, an increase in magnesium concentration was observed 20 hours after administrating high doses (20mg/k) of tetracycline. Bhutto *et al*. (2017) also maintain that tetracyclines have an effect on fatty tissues and adipose tissues are rich in magnesium. Thus, when fat tissue stores release triglycerides, it is possible to result in the release of more magnesium from adipose tissues into the blood, which could also explain the results obtained in this study.

However, long treatments due to improper removal of magnesium caused by the drug, could have an influence on the kidneys and could also possibly raise the level of magnesium in the blood. The high concentration of magnesium may end up causing hypermagnesemia, however, this is uncommon, as it mostly occurs if the animal has kidney failure in which instance unwanted magnesium cannot be excreted properly through the loop of Henle. Mammalian cells could be disturbed by tetracycline, which may cause the release of more magnesium in the blood since most of the body cells are the main sites for magnesium storage (Bhutto *et al.,* 2017).

5.3 Effect of Tetracycline injection on serum Phosphorus concentration in goats

The phosphorus in serum, was not significantly influenced in this study (Figure 4.3). It was observed that in the treated group, there was an increase of serum phosphorus from the beginning of the experiment until 24 hours to the end of the experiment. These results could be explained by the fact that tetracyclines have the ability to affect or damage the function of the kidney, thus resulting in the decline in the level of phosphorus and an increase in plasma concentration in the blood (Dobsikova *et al.,* 2013). However, this might need longer periods of treatment or overdose to see this occurring.

The current results are similar to those of Bhutt *et al.* (2017), who found that there are possibilities that the drug used to inject/treat goats could invigorate the intake of phosphorus concentration in the blood (Bhutto *et al.*, 2017). The results are also in line with previous studies on dogs, rats and goats (Sasaki, 1998; Deoare *et al.*, 2004; and Bhutto *et al.*, 2017). These authors observed a decrease in serum phosphorus after the injection of tetracycline. It was noted that the level of serum phosphorus P (<0.05) increased from 12 hours to 48 hours in dogs after post-treatment, while in goats and rats, an increase in serum phosphorus concentration was noted 3 hours after intramuscular injection of tetracycline. The route of injection influenced the difference between different experiments.

5.4 Effects of Tetracycline injection on faecal Calcium concentration in goats

The faecal calcium concentration injection of tetracycline in goats showed a slight increase at 24 hours after injection, and then afterwards, it decreased significantly till the end of the study (Figure 4.4). The increase observed in the first 24 hours could be linked to the increase in serum due to the mobilisation of calcium from different tissues and the failure of tetracycline to form adducts with all calcium molecules, thus leading to a threshold and the elimination through faeces. In addition, calcium being one of the important ions in the animal body, its main functions are to build bones, it brings about muscle contraction, it is important for nerve function and blood clotting. Due to the physiological functional importance of calcium in the body of animals, homeostasis could have corrected and stopped the loss, hence the decrease observed after 24 hours.

It is important to indicate that most biological elements, such as calcium, are not easily found in faeces, unless the animals have gastrointestinal disorders or the intestines have problems absorbing the minerals, then they will be flushed away and there will be high amounts of them in the faeces (Bhutto *et al.*, 2017). It should be noted that calcium is normally excreted through urine in the nephron in the kidneys and with about 98% stored in the bones and only 2% in the body. It is normal to find flushed traces of calcium in faeces (Nordin, 1976). Calcium loss, through faeces, could occur if there is a primary disturbance of the digestion of fats, leading to calcium being excreted in large quantities through faeces in the form of calcium soap (McCrudden and Fales, 1913). However, the loss of calcium in rickets, sometimes, leads to large amounts of phosphorus in faeces by loss of calcium phosphate.

5.5 Effects of Tetracycline on faecal Magnesium (Mg) concentration

Results obtained in this study (Figure 4.5) showed that there was no significant effect of tetracycline injection in goats with regard to the level of Mg in the faeces. Despite a slight increase in the first 24 hours after treatment, the concentrations decreased to normal by the end of the study. High concentration of magnesium in faeces could be due to the fact that the animal was unable to absorb divalent metal ions from the intestines or the animal could have kidney complications where magnesium was not re-absorbed properly (Berndt *et al.,* 2007).

Most of the magnesium found in the body is absorbed in the small intestine, through a passive paracellular mechanism, which is led by an electrochemical gradient and solvent drag (Baaij *et al*., 2012). Tetracycline is excreted mostly through urine, resulting in minimum removal of magnesium cations. More studies are required to investigate the effects of tetracycline on faecal samples of injected and control animals after 72hours of intramuscular injection.

5.6 Effects of Tetracycline on Phosphorus (P) concentration in faeces

This study revealed a significant increase in the level of faecal phosphorus after the injection of tetracycline. There was a significant difference ($P>0.05$) between the treated and control groups of goats over time. As was the case for calcium, the increase in the level of faecal phosphorus could be linked to the increase in the serum. Certainly, the mobilisation of phosphorus in serum led to low concentration of tetracycline in the system, thus leading to its removal through faeces. It is important to note that about 85% of animals' phosphate is stored in the bones and the small intestines, being the primary source of absorption, the process occurs through active tran-cellular and passive para-cellular processes (Hill *et al.*, 2013).

Berndt *et al.* (2007) maintain that the stomach/gut plays an important role in maintaining phosphate homeostasis by creating unidentified phosphatonin to respond to dietary phosphate loading (Berndt *et al.,* 2007). There is limited literature on the chelation effect of intramuscular injection of tetracycline on animals and its effects on phosphorus concentration in the faeces of injected animals. This study revealed very little about homeostasis of phosphorus and the effects of tetracycline injection.

With the increase of faecal phosphorus over time, it could be concluded that tetracyclines have an effect on phosphorus faecal concentration in goats. Sometimes, the increase in phosphorus

concentration in faeces could be due to the gut being affected through tran-cellular and para-cellular mechanisms and also the kidneys not excreting phosphorus properly.

5.7 Effects of Tetracycline on Calcium (Ca) concentration in bones

The results revealed no major changes of calcium concentrations in bones (Figure 4.7). This is an indication that tetracycline injection did not have chelation effect on bones with no significant difference ($P<0.05$) over time (between the treated/injected and control groups of goats injected with Tetracycline). The bones and teeth are the main reservoir of calcium for many physiological functions and the process is similar to magnesium and phosphorus (Brown, 2001). The gastrointestinal track and kidneys are the organs that control skeletal calcium through the regulatory pathway, and these results in mediation in bones by the osteoblast, the bone forming cell and the osteoclast, the bone resorbing cell (Joseph *et al.,* 2014). The only way calcium can reach the bones is by absorption through diet in the gastrointestinal or through supplements.

The bones and teeth are the major storage sites for calcium. Tetracycline intervenes with bone functions, when deposited in the bones, bone resorption and calcium increases. These findings are similar to those of Golub *et al.* (2013) and Gomes *et al.*1984), who found that this antibiotic has the ability to inhibit mammalian collagenolytic enzymes through a mechanism unrelated to the antibacterial efficacy of the drugs. There are possibilities that when tetracycline affects the parathyroid hormone, it increases the number and activity of osteoclasts, thus influencing bone reabsorption by the kidney. Similar findings were obtained by Eduardo (1983).

5.8 Effects of Tetracycline Magnesium concentrations in the bones of goats

The study revealed a progressive decrease in bone magnesium concentration during the period of the study (Figure 4.8), which could be linked to the increase in serum (largely due to the mobilisation from all tissues after injection of tetracycline). Magnesium is considered the second most common intracellular cation unlike other cations (such as calcium) that are regulated by the parathyroid hormone, however, for magnesium, there are no known hormones that have the capabilities or regulating it (Felsenfeld *et al.*, 2015). Thus, the results reveal that tetracycline has the ability to trigger the mobilisation of both calcium and magnesium and form adducts or chelation.

Magnesium has difficulties passing through narrow biological channels, which are readily traversed by calcium (Jahnen, 2002). The increase in magnesium concentration after 3 hours could be due to the fact that when tetracycline is deposited on the bones, it is known to cause depression in bone growth, which may lead to an increase in magnesium concentration. Magnesium is also an inhibitor of calcification in bones and soft tissues (Coburn and Levine, 1998).

5.9 Effects of Tetracycline on bone Phosphorus concentration in goats

The continuous use of tetracyclines and over dosing have been reported to cause acidosis, which results in an increase in phosphorus concentration in bones (Felsenfeld *et al.,* 2015). After 3 hours of tetracycline injection, the injected/treated goats showed an increase in phosphorus concentration in the bones; similar findings were also reported by Felsenfeld *et al,* (2015). There is currently no explanation for the increase in bones. According to Lederer *et al.* (2012) and Forster *et al.* (2013), phosphorus homeostasis could also be affected with a disturbance of the parathyroid hormone if its damaged may affect phosphorus levels.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The aim of this study was to examine the chelating effect of Tetracycline injection on serum, faeces and bone of goats. The results revealed that intramuscular injection of tetracycline had significant effects on Ca, Mg and P in the serum of boar goats over a given time interval. This effect resulted from divalent metal ion chelation by tetracycline. It is concluded the tetracycline injection triggered the mobilisation of the cations from different tissues in order to form adducts. However, the formation of adducts needed a certain concentration of tetracycline. Thus, in this study, only one dose of the injection was given to the animal. Hence, the increase in Ca, Mg and P in serum. The free cations were observed in faeces (the case of Calcium, P and Mg).

It is, therefore, concluded that there is a need for either increasing the concentration of the injection or repeating the treatment to effectively see the chelating effect of tetracycline since there was no significant effect of tetracycline on calcium concentration in bones and this can be a PhD topic by increasing the number of hours and dose. This could be seen in case of high concentrations or repeated treatment given to the animals. The researcher was unable to explain the reason behind the phosphorus in bones during the experiment. The experiment was performed 72 hours after a single injection. Thus, there is need to repeat the same experiment for a longer period of time. In addition, another research can be done by using different doses or repeated treatments should be done to effectively assess and come up with a conclusion on the chelating effect of Tetracycline. There is also a need to train veterinary practitioners and farmers on possible consequences of repeated or overdose treatment with tetracycline on goats or possibly, other animals.

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List of appendices

RESULT FOR CA IN SERUM

The mixed procedure

Model information

Data set WORK.SERUM

Model information	
Dependent variable	Ca
Covariance structure	Compound symmetry
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	8	1.45	0.2623
Time	5	40	5.62	0.0005
TRT*Time	5	40	3.27	0.0144

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		46.9077	2.0790	8	22.56	<.0001
TRT	**T**		43.3620	2.0790	8	20.86	<.0001
TRT*Time	**C**	**0**	54.8900	3.2060	32.8	17.12	<.0001
TRT*Time	**C**	**3**	46.3880	3.2060	32.8	14.47	<.0001

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT*Time	**C**	**6**	49.0600	3.2060	32.8	15.30	<.0001
TRT*Time	**C**	**24**	45.5740	3.2060	32.8	14.22	<.0001
TRT*Time	**C**	**48**	42.3740	3.2060	32.8	13.22	<.0001
TRT*Time	**C**	**72**	43.1600	3.2060	32.8	13.46	<.0001
TRT*Time	**T**	**0**	44.1640	3.2060	32.8	13.78	<.0001
TRT*Time	**T**	**3**	53.9800	3.2060	32.8	16.84	<.0001
TRT*Time	**T**	**6**	44.6420	3.2060	32.8	13.92	<.0001
TRT*Time	**T**	**24**	37.0500	3.2060	32.8	11.56	<.0001
TRT*Time	**T**	**48**	35.8500	3.2060	32.8	11.18	<.0001
TRT*Time	**T**	**72**	44.4860	3.2060	32.8	13.88	<.0001

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT	**C**		**T**		3.5457	2.9402	8	1.21	0.2623	Tukey	0.2623
TRT*Time	**C**	**0**	**C**	**3**	8.5020	3.7807	40	2.25	0.0301	Tukey-Kramer	0.5267
TRT*Time	**C**	**0**	**C**	**6**	5.8300	3.7807	40	1.54	0.1309	Tukey-Kramer	0.9191

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**0**	**C**	**24**	9.3160	3.7807	40	2.46	0.0181	Tukey-Kramer	0.3900
TRT*Time	**C**	**0**	**C**	**48**	12.5160	3.7807	40	3.31	0.0020	Tukey-Kramer	0.0731
TRT*Time	**C**	**0**	**C**	**72**	11.7300	3.7807	40	3.10	0.0035	Tukey-Kramer	0.1174
TRT*Time	**C**	**0**	**T**	**0**	10.7260	4.5339	32.8	2.37	0.0241	Tukey-Kramer	0.4507
TRT*Time	**C**	**0**	**T**	**3**	0.9100	4.5339	32.8	0.20	0.8422	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**6**	10.2480	4.5339	32.8	2.26	0.0306	Tukey-Kramer	0.5190
TRT*Time	**C**	**0**	**T**	**24**	17.8400	4.5339	32.8	3.93	0.0004	Tukey-Kramer	0.0148
TRT*Time	**C**	**0**	**T**	**48**	19.0400	4.5339	32.8	4.20	0.0002	Tukey-Kramer	0.0070
TRT*Time	**C**	**0**	**T**	**72**	10.4040	4.5339	32.8	2.29	0.0283	Tukey-Kramer	0.4964
TRT*Time	**C**	**3**	**C**	**6**	-2.6720	3.7807	40	-0.71	0.4838	Tukey-Kramer	0.9999
TRT*Time	**C**	**3**	**C**	**24**	0.8140	3.7807	40	0.22	0.8306	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**C**	**48**	4.0140	3.7807	40	1.06	0.2947	Tukey-Kramer	0.9948

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**3**	**C**	**72**	3.2280	3.7807	40	0.85	0.3983	Tukey-Kramer	0.9992
TRT*Time	**C**	**3**	**T**	**0**	2.2240	4.5339	32.8	0.49	0.6270	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**T**	**3**	-7.5920	4.5339	32.8	-1.67	0.1035	Tukey-Kramer	0.8690
TRT*Time	**C**	**3**	**T**	**6**	1.7460	4.5339	32.8	0.39	0.7027	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**T**	**24**	9.3380	4.5339	32.8	2.06	0.0475	Tukey-Kramer	0.6522
TRT*Time	**C**	**3**	**T**	**48**	10.5380	4.5339	32.8	2.32	0.0265	Tukey-Kramer	0.4772
TRT*Time	**C**	**3**	**T**	**72**	1.9020	4.5339	32.8	0.42	0.6776	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**C**	**24**	3.4860	3.7807	40	0.92	0.3620	Tukey-Kramer	0.9984
TRT*Time	**C**	**6**	**C**	**48**	6.6860	3.7807	40	1.77	0.0846	Tukey-Kramer	0.8248
TRT*Time	**C**	**6**	**C**	**72**	5.9000	3.7807	40	1.56	0.1265	Tukey-Kramer	0.9130
TRT*Time	**C**	**6**	**T**	**0**	4.8960	4.5339	32.8	1.08	0.2881	Tukey-Kramer	0.9940
TRT*Time	**C**	**6**	**T**	**3**	-4.9200	4.5339	32.8	-1.09	0.2858	Tukey-Kramer	0.9937

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**6**	**T**	**6**	4.4180	4.5339	32.8	0.97	0.3370	Tukey-Kramer	0.9975
TRT*Time	**C**	**6**	**T**	**24**	12.0100	4.5339	32.8	2.65	0.0123	Tukey-Kramer	0.2881
TRT*Time	**C**	**6**	**T**	**48**	13.2100	4.5339	32.8	2.91	0.0064	Tukey-Kramer	0.1748
TRT*Time	**C**	**6**	**T**	**72**	4.5740	4.5339	32.8	1.01	0.3204	Tukey-Kramer	0.9966
TRT*Time	**C**	**24**	**C**	**48**	3.2000	3.7807	40	0.85	0.4024	Tukey-Kramer	0.9993
TRT*Time	**C**	**24**	**C**	**72**	2.4140	3.7807	40	0.64	0.5268	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**0**	1.4100	4.5339	32.8	0.31	0.7578	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**3**	-8.4060	4.5339	32.8	-1.85	0.0728	Tukey-Kramer	0.7788
TRT*Time	**C**	**24**	**T**	**6**	0.9320	4.5339	32.8	0.21	0.8384	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**24**	8.5240	4.5339	32.8	1.88	0.0690	Tukey-Kramer	0.7639
TRT*Time	**C**	**24**	**T**	**48**	9.7240	4.5339	32.8	2.14	0.0395	Tukey-Kramer	0.5959
TRT*Time	**C**	**24**	**T**	**72**	1.0880	4.5339	32.8	0.24	0.8118	Tukey-Kramer	1.0000

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**48**	**C**	**72**	-0.7860	3.7807	40	-0.21	0.8364	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**0**	-1.7900	4.5339	32.8	-0.39	0.6955	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**3**	-11.6060	4.5339	32.8	-2.56	0.0153	Tukey-Kramer	0.3350
TRT*Time	**C**	**48**	**T**	**6**	-2.2680	4.5339	32.8	-0.50	0.6203	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**24**	5.3240	4.5339	32.8	1.17	0.2487	Tukey-Kramer	0.9881
TRT*Time	**C**	**48**	**T**	**48**	6.5240	4.5339	32.8	1.44	0.1596	Tukey-Kramer	0.9480
TRT*Time	**C**	**48**	**T**	**72**	-2.1120	4.5339	32.8	-0.47	0.6444	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**0**	-1.0040	4.5339	32.8	-0.22	0.8261	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**3**	-10.8200	4.5339	32.8	-2.39	0.0229	Tukey-Kramer	0.4376
TRT*Time	**C**	**72**	**T**	**6**	-1.4820	4.5339	32.8	-0.33	0.7458	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**24**	6.1100	4.5339	32.8	1.35	0.1870	Tukey-Kramer	0.9668
TRT*Time	**C**	**72**	**T**	**48**	7.3100	4.5339	32.8	1.61	0.1165	Tukey-Kramer	0.8944

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**72**	**T**	**72**	-1.3260	4.5339	32.8	-0.29	0.7718	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**3**	-9.8160	3.7807	40	-2.60	0.0131	Tukey-Kramer	0.3153
TRT*Time	**T**	**0**	**T**	**6**	-0.4780	3.7807	40	-0.13	0.9000	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**24**	7.1140	3.7807	40	1.88	0.0672	Tukey-Kramer	0.7630
TRT*Time	**T**	**0**	**T**	**48**	8.3140	3.7807	40	2.20	0.0337	Tukey-Kramer	0.5597
TRT*Time	**T**	**0**	**T**	**72**	-0.3220	3.7807	40	-0.09	0.9326	Tukey-Kramer	1.0000
TRT*Time	**T**	**3**	**T**	**6**	9.3380	3.7807	40	2.47	0.0179	Tukey-Kramer	0.3866
TRT*Time	**T**	**3**	**T**	**24**	16.9300	3.7807	40	4.48	<.0001	Tukey-Kramer	0.0031
TRT*Time	**T**	**3**	**T**	**48**	18.1300	3.7807	40	4.80	<.0001	Tukey-Kramer	0.0012
TRT*Time	**T**	**3**	**T**	**72**	9.4940	3.7807	40	2.51	0.0162	Tukey-Kramer	0.3624
TRT*Time	**T**	**6**	**T**	**24**	7.5920	3.7807	40	2.01	0.0514	Tukey-Kramer	0.6855
TRT*Time	**T**	**6**	**T**	**48**	8.7920	3.7807	40	2.33	0.0252	Tukey-Kramer	0.4764

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**T**	**6**	**T**	**72**	0.1560	3.7807	40	0.04	0.9673	Tukey-Kramer	1.0000
TRT*Time	**T**	**24**	**T**	**48**	1.2000	3.7807	40	0.32	0.7526	Tukey-Kramer	1.0000
TRT*Time	**T**	**24**	**T**	**72**	-7.4360	3.7807	40	-1.97	0.0562	Tukey-Kramer	0.7116
TRT*Time	**T**	**48**	**T**	**72**	-8.6360	3.7807	40	-2.28	0.0277	Tukey-Kramer	0.5033

RESULTS FOR MG IN SERUM

The Mixed Procedure

Model information	
Data set	WORK.SERUM
Dependent variable	Mg
Covariance structure	Compound Symmetry
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	8	1.96	0.1995
Time	5	40	5.60	0.0005
TRT*Time	5	40	2.93	0.0242

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		7.3627	0.5933	8	12.41	<.0001
TRT	**T**		8.5360	0.5933	8	14.39	<.0001
TRT*Time	**C**	**0**	8.1640	0.8799	30.1	9.28	<.0001
TRT*Time	**C**	**3**	7.3900	0.8799	30.1	8.40	<.0001
TRT*Time	**C**	**6**	6.9220	0.8799	30.1	7.87	<.0001
TRT*Time	**C**	**24**	6.6860	0.8799	30.1	7.60	<.0001
TRT*Time	**C**	**48**	6.6720	0.8799	30.1	7.58	<.0001
TRT*Time	**C**	**72**	8.3420	0.8799	30.1	9.48	<.0001
TRT*Time	**T**	**0**	7.6200	0.8799	30.1	8.66	<.0001
TRT*Time	**T**	**3**	9.3940	0.8799	30.1	10.68	<.0001
TRT*Time	**T**	**6**	6.7100	0.8799	30.1	7.63	<.0001
TRT*Time	**T**	**24**	6.3760	0.8799	30.1	7.25	<.0001

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT*Time	**T**	**48**	9.8100	0.8799	30.1	11.15	<.0001
TRT*Time	**T**	**72**	11.3060	0.8799	30.1	12.85	<.0001

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT	**C**		**T**		-1.1733	0.8390	8	-1.40	0.1995	Tukey	0.1995
TRT*Time	**C**	**0**	**C**	**3**	0.7740	1.0067	40	0.77	0.4465	Tukey-Kramer	0.9997
TRT*Time	**C**	**0**	**C**	**6**	1.2420	1.0067	40	1.23	0.2245	Tukey-Kramer	0.9826
TRT*Time	**C**	**0**	**C**	**24**	1.4780	1.0067	40	1.47	0.1499	Tukey-Kramer	0.9407
TRT*Time	**C**	**0**	**C**	**48**	1.4920	1.0067	40	1.48	0.1462	Tukey-Kramer	0.9370
TRT*Time	**C**	**0**	**C**	**72**	-0.1780	1.0067	40	-0.18	0.8605	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**0**	0.5440	1.2444	30.1	0.44	0.6651	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**3**	-1.2300	1.2444	30.1	-0.99	0.3308	Tukey-Kramer	0.9971

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**0**	**T**	**6**	1.4540	1.2444	30.1	1.17	0.2518	Tukey-Kramer	0.9886
TRT*Time	**C**	**0**	**T**	**24**	1.7880	1.2444	30.1	1.44	0.1611	Tukey-Kramer	0.9485
TRT*Time	**C**	**0**	**T**	**48**	-1.6460	1.2444	30.1	-1.32	0.1959	Tukey-Kramer	0.9709
TRT*Time	**C**	**0**	**T**	**72**	-3.1420	1.2444	30.1	-2.53	0.0171	Tukey-Kramer	0.3545
TRT*Time	**C**	**3**	**C**	**6**	0.4680	1.0067	40	0.46	0.6445	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**C**	**24**	0.7040	1.0067	40	0.70	0.4884	Tukey-Kramer	0.9999
TRT*Time	**C**	**3**	**C**	**48**	0.7180	1.0067	40	0.71	0.4798	Tukey-Kramer	0.9999
TRT*Time	**C**	**3**	**C**	**72**	-0.9520	1.0067	40	-0.95	0.3500	Tukey-Kramer	0.9981
TRT*Time	**C**	**3**	**T**	**0**	-0.2300	1.2444	30.1	-0.18	0.8546	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**T**	**3**	-2.0040	1.2444	30.1	-1.61	0.1177	Tukey-Kramer	0.8951
TRT*Time	**C**	**3**	**T**	**6**	0.6800	1.2444	30.1	0.55	0.5888	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**T**	**24**	1.0140	1.2444	30.1	0.81	0.4215	Tukey-Kramer	0.9995

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**3**	**T**	**48**	-2.4200	1.2444	30.1	-1.94	0.0612	Tukey-Kramer	0.7252
TRT*Time	**C**	**3**	**T**	**72**	-3.9160	1.2444	30.1	-3.15	0.0037	Tukey-Kramer	0.1064
TRT*Time	**C**	**6**	**C**	**24**	0.2360	1.0067	40	0.23	0.8158	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**C**	**48**	0.2500	1.0067	40	0.25	0.8051	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**C**	**72**	-1.4200	1.0067	40	-1.41	0.1661	Tukey-Kramer	0.9545
TRT*Time	**C**	**6**	**T**	**0**	-0.6980	1.2444	30.1	-0.56	0.5790	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**T**	**3**	-2.4720	1.2444	30.1	-1.99	0.0562	Tukey-Kramer	0.6992
TRT*Time	**C**	**6**	**T**	**6**	0.2120	1.2444	30.1	0.17	0.8659	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**T**	**24**	0.5460	1.2444	30.1	0.44	0.6640	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**T**	**48**	-2.8880	1.2444	30.1	-2.32	0.0273	Tukey-Kramer	0.4794
TRT*Time	**C**	**6**	**T**	**72**	-4.3840	1.2444	30.1	-3.52	0.0014	Tukey-Kramer	0.0435
TRT*Time	**C**	**24**	**C**	**48**	0.01400	1.0067	40	0.01	0.9890	Tukey-Kramer	1.0000

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**24**	**C**	**72**	-1.6560	1.0067	40	-1.65	0.1078	Tukey-Kramer	0.8814
TRT*Time	**C**	**24**	**T**	**0**	-0.9340	1.2444	30.1	-0.75	0.4587	Tukey-Kramer	0.9998
TRT*Time	**C**	**24**	**T**	**3**	-2.7080	1.2444	30.1	-2.18	0.0375	Tukey-Kramer	0.5749
TRT*Time	**C**	**24**	**T**	**6**	-0.02400	1.2444	30.1	-0.02	0.9847	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**24**	0.3100	1.2444	30.1	0.25	0.8050	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**48**	-3.1240	1.2444	30.1	-2.51	0.0177	Tukey-Kramer	0.3628
TRT*Time	**C**	**24**	**T**	**72**	-4.6200	1.2444	30.1	-3.71	0.0008	Tukey-Kramer	0.0268
TRT*Time	**C**	**48**	**C**	**72**	-1.6700	1.0067	40	-1.66	0.1050	Tukey-Kramer	0.8757
TRT*Time	**C**	**48**	**T**	**0**	-0.9480	1.2444	30.1	-0.76	0.4521	Tukey-Kramer	0.9997
TRT*Time	**C**	**48**	**T**	**3**	-2.7220	1.2444	30.1	-2.19	0.0366	Tukey-Kramer	0.5674
TRT*Time	**C**	**48**	**T**	**6**	-0.03800	1.2444	30.1	-0.03	0.9758	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**24**	0.2960	1.2444	30.1	0.24	0.8136	Tukey-Kramer	1.0000

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**48**	**T**	**48**	-3.1380	1.2444	30.1	-2.52	0.0172	Tukey-Kramer	0.3564
TRT*Time	**C**	**48**	**T**	**72**	-4.6340	1.2444	30.1	-3.72	0.0008	Tukey-Kramer	0.0260
TRT*Time	**C**	**72**	**T**	**0**	0.7220	1.2444	30.1	0.58	0.5661	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**3**	-1.0520	1.2444	30.1	-0.85	0.4046	Tukey-Kramer	0.9993
TRT*Time	**C**	**72**	**T**	**6**	1.6320	1.2444	30.1	1.31	0.1996	Tukey-Kramer	0.9727
TRT*Time	**C**	**72**	**T**	**24**	1.9660	1.2444	30.1	1.58	0.1246	Tukey-Kramer	0.9063
TRT*Time	**C**	**72**	**T**	**48**	-1.4680	1.2444	30.1	-1.18	0.2474	Tukey-Kramer	0.9877
TRT*Time	**C**	**72**	**T**	**72**	-2.9640	1.2444	30.1	-2.38	0.0237	Tukey-Kramer	0.4404
TRT*Time	**T**	**0**	**T**	**3**	-1.7740	1.0067	40	-1.76	0.0857	Tukey-Kramer	0.8279
TRT*Time	**T**	**0**	**T**	**6**	0.9100	1.0067	40	0.90	0.3714	Tukey-Kramer	0.9987
TRT*Time	**T**	**0**	**T**	**24**	1.2440	1.0067	40	1.24	0.2238	Tukey-Kramer	0.9824
TRT*Time	**T**	**0**	**T**	**48**	-2.1900	1.0067	40	-2.18	0.0356	Tukey-Kramer	0.5754

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**T**	**0**	**T**	**72**	-3.6860	1.0067	40	-3.66	0.0007	Tukey-Kramer	0.0306
TRT*Time	**T**	**3**	**T**	**6**	2.6840	1.0067	40	2.67	0.0110	Tukey-Kramer	0.2795
TRT*Time	**T**	**3**	**T**	**24**	3.0180	1.0067	40	3.00	0.0047	Tukey-Kramer	0.1469
TRT*Time	**T**	**3**	**T**	**48**	-0.4160	1.0067	40	-0.41	0.6816	Tukey-Kramer	1.0000
TRT*Time	**T**	**3**	**T**	**72**	-1.9120	1.0067	40	-1.90	0.0648	Tukey-Kramer	0.7526
TRT*Time	**T**	**6**	**T**	**24**	0.3340	1.0067	40	0.33	0.7418	Tukey-Kramer	1.0000
TRT*Time	**T**	**6**	**T**	**48**	-3.1000	1.0067	40	-3.08	0.0037	Tukey-Kramer	0.1234
TRT*Time	**T**	**6**	**T**	**72**	-4.5960	1.0067	40	-4.57	<.0001	Tukey-Kramer	0.0024
TRT*Time	**T**	**24**	**T**	**48**	-3.4340	1.0067	40	-3.41	0.0015	Tukey-Kramer	0.0574
TRT*Time	**T**	**24**	**T**	**72**	-4.9300	1.0067	40	-4.90	<.0001	Tukey-Kramer	0.0009
TRT*Time	**T**	**48**	**T**	**72**	-1.4960	1.0067	40	-1.49	0.1451	Tukey-Kramer	0.9359

RESULTS FOR P IN SERUM

The Mixed Procedure

Model information	
Data set	WORK.SERUM
Dependent variable	P
Covariance structure	Compound Symmetry
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	8	2.48	0.1542
Time	5	40	14.87	<.0001
TRT*Time	5	40	2.32	0.0606

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		15.2333	1.0411	8	14.63	<.0001
TRT	**T**		12.9163	1.0411	8	12.41	<.0001

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT*Time	**C**	**0**	18.1100	1.4390	25	12.59	<.0001
TRT*Time	**C**	**3**	7.3900	1.4390	25	5.14	<.0001
TRT*Time	**C**	**6**	17.7240	1.4390	25	12.32	<.0001
TRT*Time	**C**	**24**	17.9920	1.4390	25	12.50	<.0001
TRT*Time	**C**	**48**	14.4240	1.4390	25	10.02	<.0001
TRT*Time	**C**	**72**	15.7600	1.4390	25	10.95	<.0001
TRT*Time	**T**	**0**	14.4660	1.4390	25	10.05	<.0001
TRT*Time	**T**	**3**	9.3940	1.4390	25	6.53	<.0001
TRT*Time	**T**	**6**	14.1720	1.4390	25	9.85	<.0001
TRT*Time	**T**	**24**	13.7600	1.4390	25	9.56	<.0001
TRT*Time	**T**	**48**	13.1560	1.4390	25	9.14	<.0001
TRT*Time	**T**	**72**	12.5500	1.4390	25	8.72	<.0001

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT	**C**		**T**		2.3170	1.4724	8	1.57	0.1542	Tukey	0.1542

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**0**	**C**	**3**	10.7200	1.5389	40	6.97	<.0001	Tukey-Kramer	<.0001
TRT*Time	**C**	**0**	**C**	**6**	0.3860	1.5389	40	0.25	0.8032	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**C**	**24**	0.1180	1.5389	40	0.08	0.9393	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**C**	**48**	3.6860	1.5389	40	2.40	0.0214	Tukey-Kramer	0.4321
TRT*Time	**C**	**0**	**C**	**72**	2.3500	1.5389	40	1.53	0.1346	Tukey-Kramer	0.9238
TRT*Time	**C**	**0**	**T**	**0**	3.6440	2.0350	25	1.79	0.0854	Tukey-Kramer	0.8133
TRT*Time	**C**	**0**	**T**	**3**	8.7160	2.0350	25	4.28	0.0002	Tukey-Kramer	0.0055
TRT*Time	**C**	**0**	**T**	**6**	3.9380	2.0350	25	1.94	0.0643	Tukey-Kramer	0.7311
TRT*Time	**C**	**0**	**T**	**24**	4.3500	2.0350	25	2.14	0.0425	Tukey-Kramer	0.6007
TRT*Time	**C**	**0**	**T**	**48**	4.9540	2.0350	25	2.43	0.0224	Tukey-Kramer	0.4080
TRT*Time	**C**	**0**	**T**	**72**	5.5600	2.0350	25	2.73	0.0114	Tukey-Kramer	0.2482
TRT*Time	**C**	**3**	**C**	**6**	-10.3340	1.5389	40	-6.72	<.0001	Tukey-Kramer	<.0001

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**3**	**C**	**24**	-10.6020	1.5389	40	-6.89	<.0001	Tukey-Kramer	<.0001
TRT*Time	**C**	**3**	**C**	**48**	-7.0340	1.5389	40	-4.57	<.0001	Tukey-Kramer	0.0024
TRT*Time	**C**	**3**	**C**	**72**	-8.3700	1.5389	40	-5.44	<.0001	Tukey-Kramer	0.0002
TRT*Time	**C**	**3**	**T**	**0**	-7.0760	2.0350	25	-3.48	0.0019	Tukey-Kramer	0.0488
TRT*Time	**C**	**3**	**T**	**3**	-2.0040	2.0350	25	-0.98	0.3342	Tukey-Kramer	0.9972
TRT*Time	**C**	**3**	**T**	**6**	-6.7820	2.0350	25	-3.33	0.0027	Tukey-Kramer	0.0693
TRT*Time	**C**	**3**	**T**	**24**	-6.3700	2.0350	25	-3.13	0.0044	Tukey-Kramer	0.1104
TRT*Time	**C**	**3**	**T**	**48**	-5.7660	2.0350	25	-2.83	0.0090	Tukey-Kramer	0.2049
TRT*Time	**C**	**3**	**T**	**72**	-5.1600	2.0350	25	-2.54	0.0178	Tukey-Kramer	0.3485
TRT*Time	**C**	**6**	**C**	**24**	-0.2680	1.5389	40	-0.17	0.8626	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**C**	**48**	3.3000	1.5389	40	2.14	0.0381	Tukey-Kramer	0.5961
TRT*Time	**C**	**6**	**C**	**72**	1.9640	1.5389	40	1.28	0.2092	Tukey-Kramer	0.9776

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**6**	**T**	**0**	3.2580	2.0350	25	1.60	0.1219	Tukey-Kramer	0.8986
TRT*Time	**C**	**6**	**T**	**3**	8.3300	2.0350	25	4.09	0.0004	Tukey-Kramer	0.0095
TRT*Time	**C**	**6**	**T**	**6**	3.5520	2.0350	25	1.75	0.0932	Tukey-Kramer	0.8362
TRT*Time	**C**	**6**	**T**	**24**	3.9640	2.0350	25	1.95	0.0627	Tukey-Kramer	0.7233
TRT*Time	**C**	**6**	**T**	**48**	4.5680	2.0350	25	2.24	0.0339	Tukey-Kramer	0.5294
TRT*Time	**C**	**6**	**T**	**72**	5.1740	2.0350	25	2.54	0.0176	Tukey-Kramer	0.3447
TRT*Time	**C**	**24**	**C**	**48**	3.5680	1.5389	40	2.32	0.0256	Tukey-Kramer	0.4809
TRT*Time	**C**	**24**	**C**	**72**	2.2320	1.5389	40	1.45	0.1547	Tukey-Kramer	0.9452
TRT*Time	**C**	**24**	**T**	**0**	3.5260	2.0350	25	1.73	0.0955	Tukey-Kramer	0.8424
TRT*Time	**C**	**24**	**T**	**3**	8.5980	2.0350	25	4.23	0.0003	Tukey-Kramer	0.0065
TRT*Time	**C**	**24**	**T**	**6**	3.8200	2.0350	25	1.88	0.0722	Tukey-Kramer	0.7656
TRT*Time	**C**	**24**	**T**	**24**	4.2320	2.0350	25	2.08	0.0480	Tukey-Kramer	0.6391

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**24**	**T**	**48**	4.8360	2.0350	25	2.38	0.0254	Tukey-Kramer	0.4439
TRT*Time	**C**	**24**	**T**	**72**	5.4420	2.0350	25	2.67	0.0130	Tukey-Kramer	0.2755
TRT*Time	**C**	**48**	**C**	**72**	-1.3360	1.5389	40	-0.87	0.3905	Tukey-Kramer	0.9991
TRT*Time	**C**	**48**	**T**	**0**	-0.04200	2.0350	25	-0.02	0.9837	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**3**	5.0300	2.0350	25	2.47	0.0206	Tukey-Kramer	0.3855
TRT*Time	**C**	**48**	**T**	**6**	0.2520	2.0350	25	0.12	0.9024	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**24**	0.6640	2.0350	25	0.33	0.7469	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**48**	1.2680	2.0350	25	0.62	0.5389	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**72**	1.8740	2.0350	25	0.92	0.3659	Tukey-Kramer	0.9985
TRT*Time	**C**	**72**	**T**	**0**	1.2940	2.0350	25	0.64	0.5306	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**3**	6.3660	2.0350	25	3.13	0.0044	Tukey-Kramer	0.1109
TRT*Time	**C**	**72**	**T**	**6**	1.5880	2.0350	25	0.78	0.4425	Tukey-Kramer	0.9997

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**72**	**T**	**24**	2.0000	2.0350	25	0.98	0.3351	Tukey-Kramer	0.9973
TRT*Time	**C**	**72**	**T**	**48**	2.6040	2.0350	25	1.28	0.2124	Tukey-Kramer	0.9772
TRT*Time	**C**	**72**	**T**	**72**	3.2100	2.0350	25	1.58	0.1273	Tukey-Kramer	0.9072
TRT*Time	**T**	**0**	**T**	**3**	5.0720	1.5389	40	3.30	0.0021	Tukey-Kramer	0.0756
TRT*Time	**T**	**0**	**T**	**6**	0.2940	1.5389	40	0.19	0.8495	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**24**	0.7060	1.5389	40	0.46	0.6489	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**48**	1.3100	1.5389	40	0.85	0.3997	Tukey-Kramer	0.9992
TRT*Time	**T**	**0**	**T**	**72**	1.9160	1.5389	40	1.25	0.2204	Tukey-Kramer	0.9814
TRT*Time	**T**	**3**	**T**	**6**	-4.7780	1.5389	40	-3.10	0.0035	Tukey-Kramer	0.1168
TRT*Time	**T**	**3**	**T**	**24**	-4.3660	1.5389	40	-2.84	0.0071	Tukey-Kramer	0.2035
TRT*Time	**T**	**3**	**T**	**48**	-3.7620	1.5389	40	-2.44	0.0190	Tukey-Kramer	0.4017
TRT*Time	**T**	**3**	**T**	**72**	-3.1560	1.5389	40	-2.05	0.0469	Tukey-Kramer	0.6579

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**T**	**6**	**T**	**24**	0.4120	1.5389	40	0.27	0.7903	Tukey-Kramer	1.0000
TRT*Time	**T**	**6**	**T**	**48**	1.0160	1.5389	40	0.66	0.5129	Tukey-Kramer	0.9999
TRT*Time	**T**	**6**	**T**	**72**	1.6220	1.5389	40	1.05	0.2982	Tukey-Kramer	0.9951
TRT*Time	**T**	**24**	**T**	**48**	0.6040	1.5389	40	0.39	0.6968	Tukey-Kramer	1.0000
TRT*Time	**T**	**24**	**T**	**72**	1.2100	1.5389	40	0.79	0.4363	Tukey-Kramer	0.9996
TRT*Time	**T**	**48**	**T**	**72**	0.6060	1.5389	40	0.39	0.6958	Tukey-Kramer	1.0000

RESULT FOR CA IN FEACES

The Mixed Procedure

Model information

Data set	WORK.FEACES
Dependent variable	Ca
Covariance structure	Compound Symmetry

Model information	
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	8	15.13	0.0046
Time	5	40	16.80	<.0001
TRT*Time	5	40	10.79	<.0001

Least Squares Means							
Effect	**TRT**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
TRT	**C**		201.61	13.3989	8	15.05	<.0001
TRT	**T**		275.32	13.3989	8	20.55	<.0001
TRT*Time	**C**	**0**	199.35	24.2148	42.1	8.23	<.0001
TRT*Time	**C**	**3**	211.00	24.2148	42.1	8.71	<.0001
TRT*Time	**C**	**6**	244.24	24.2148	42.1	10.09	<.0001

Least Squares Means							
Effect	**TRT**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
TRT*Time	**C**	**24**	220.00	24.2148	42.1	9.09	<.0001
TRT*Time	**C**	**48**	167.71	24.2148	42.1	6.93	<.0001
TRT*Time	**C**	**72**	167.37	24.2148	42.1	6.91	<.0001
TRT*Time	**T**	**0**	460.36	24.2148	42.1	19.01	<.0001
TRT*Time	**T**	**3**	333.80	24.2148	42.1	13.79	<.0001
TRT*Time	**T**	**6**	284.98	24.2148	42.1	11.77	<.0001
TRT*Time	**T**	**24**	240.28	24.2148	42.1	9.92	<.0001
TRT*Time	**T**	**48**	165.03	24.2148	42.1	6.82	<.0001
TRT*Time	**T**	**72**	167.47	24.2148	42.1	6.92	<.0001

Differences of Least Squares Means										
Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT	**C**		**T**		-73.7067	18.9489	8	-3.89	0.0046	Tukey	0.0046
TRT*Time	**C**	**0**	**C**	**3**	-11.6500	31.2472	40	-0.37	0.7112	Tukey-Kramer	1.0000

Differences of Least Squares Means											
Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT*Time	**C**	**0**	**C**	**6**	-44.8960	31.2472	40	-1.44	0.1586	Tukey-Kramer	0.9485
TRT*Time	**C**	**0**	**C**	**24**	-20.6520	31.2472	40	-0.66	0.5125	Tukey-Kramer	0.9999
TRT*Time	**C**	**0**	**C**	**48**	31.6380	31.2472	40	1.01	0.3174	Tukey-Kramer	0.9965
TRT*Time	**C**	**0**	**C**	**72**	31.9780	31.2472	40	1.02	0.3123	Tukey-Kramer	0.9962
TRT*Time	**C**	**0**	**T**	**0**	-261.01	34.2450	42.1	-7.62	<.0001	Tukey-Kramer	<.0001
TRT*Time	**C**	**0**	**T**	**3**	-134.46	34.2450	42.1	-3.93	0.0003	Tukey-Kramer	0.0151
TRT*Time	**C**	**0**	**T**	**6**	-85.6300	34.2450	42.1	-2.50	0.0164	Tukey-Kramer	0.3686
TRT*Time	**C**	**0**	**T**	**24**	-40.9280	34.2450	42.1	-1.20	0.2387	Tukey-Kramer	0.9864
TRT*Time	**C**	**0**	**T**	**48**	34.3220	34.2450	42.1	1.00	0.3219	Tukey-Kramer	0.9968
TRT*Time	**C**	**0**	**T**	**72**	31.8820	34.2450	42.1	0.93	0.3572	Tukey-Kramer	0.9983

Differences of Least Squares Means											
Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT*Time	**C**	**3**	**C**	**6**	-33.2460	31.2472	40	-1.06	0.2937	Tukey-Kramer	0.9947
TRT*Time	**C**	**3**	**C**	**24**	-9.0020	31.2472	40	-0.29	0.7748	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**C**	**48**	43.2880	31.2472	40	1.39	0.1736	Tukey-Kramer	0.9598
TRT*Time	**C**	**3**	**C**	**72**	43.6280	31.2472	40	1.40	0.1703	Tukey-Kramer	0.9576
TRT*Time	**C**	**3**	**T**	**0**	-249.36	34.2450	42.1	-7.28	<.0001	Tukey-Kramer	<.0001
TRT*Time	**C**	**3**	**T**	**3**	-122.81	34.2450	42.1	-3.59	0.0009	Tukey-Kramer	0.0371
TRT*Time	**C**	**3**	**T**	**6**	-73.9800	34.2450	42.1	-2.16	0.0365	Tukey-Kramer	0.5855
TRT*Time	**C**	**3**	**T**	**24**	-29.2780	34.2450	42.1	-0.85	0.3974	Tukey-Kramer	0.9992
TRT*Time	**C**	**3**	**T**	**48**	45.9720	34.2450	42.1	1.34	0.1866	Tukey-Kramer	0.9677
TRT*Time	**C**	**3**	**T**	**72**	43.5320	34.2450	42.1	1.27	0.2106	Tukey-Kramer	0.9783
TRT*Time	**C**	**6**	**C**	**24**	24.2440	31.2472	40	0.78	0.4424	Tukey-Kramer	0.9997

Differences of Least Squares Means											
Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT*Time	**C**	**6**	**C**	**48**	76.5340	31.2472	40	2.45	0.0188	Tukey-Kramer	0.3989
TRT*Time	**C**	**6**	**C**	**72**	76.8740	31.2472	40	2.46	0.0183	Tukey-Kramer	0.3924
TRT*Time	**C**	**6**	**T**	**0**	-216.12	34.2450	42.1	-6.31	<.0001	Tukey-Kramer	<.0001
TRT*Time	**C**	**6**	**T**	**3**	-89.5600	34.2450	42.1	-2.62	0.0123	Tukey-Kramer	0.3053
TRT*Time	**C**	**6**	**T**	**6**	-40.7340	34.2450	42.1	-1.19	0.2409	Tukey-Kramer	0.9869
TRT*Time	**C**	**6**	**T**	**24**	3.9680	34.2450	42.1	0.12	0.9083	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**T**	**48**	79.2180	34.2450	42.1	2.31	0.0257	Tukey-Kramer	0.4844
TRT*Time	**C**	**6**	**T**	**72**	76.7780	34.2450	42.1	2.24	0.0303	Tukey-Kramer	0.5311
TRT*Time	**C**	**24**	**C**	**48**	52.2900	31.2472	40	1.67	0.1020	Tukey-Kramer	0.8695
TRT*Time	**C**	**24**	**C**	**72**	52.6300	31.2472	40	1.68	0.0999	Tukey-Kramer	0.8647
TRT*Time	**C**	**24**	**T**	**0**	-240.36	34.2450	42.1	-7.02	<.0001	Tukey-Kramer	<.0001

Differences of Least Squares Means											
Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT*Time	**C**	**24**	**T**	**3**	-113.80	34.2450	42.1	-3.32	0.0018	Tukey-Kramer	0.0709
TRT*Time	**C**	**24**	**T**	**6**	-64.9780	34.2450	42.1	-1.90	0.0646	Tukey-Kramer	0.7537
TRT*Time	**C**	**24**	**T**	**24**	-20.2760	34.2450	42.1	-0.59	0.5570	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**48**	54.9740	34.2450	42.1	1.61	0.1159	Tukey-Kramer	0.8970
TRT*Time	**C**	**24**	**T**	**72**	52.5340	34.2450	42.1	1.53	0.1325	Tukey-Kramer	0.9217
TRT*Time	**C**	**48**	**C**	**72**	0.3400	31.2472	40	0.01	0.9914	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**0**	-292.65	34.2450	42.1	-8.55	<.0001	Tukey-Kramer	<.0001
TRT*Time	**C**	**48**	**T**	**3**	-166.09	34.2450	42.1	-4.85	<.0001	Tukey-Kramer	0.0010
TRT*Time	**C**	**48**	**T**	**6**	-117.27	34.2450	42.1	-3.42	0.0014	Tukey-Kramer	0.0556
TRT*Time	**C**	**48**	**T**	**24**	-72.5660	34.2450	42.1	-2.12	0.0400	Tukey-Kramer	0.6130
TRT*Time	**C**	**48**	**T**	**48**	2.6840	34.2450	42.1	0.08	0.9379	Tukey-Kramer	1.0000

Differences of Least Squares Means											
Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT*Time	**C**	**48**	**T**	**72**	0.2440	34.2450	42.1	0.01	0.9943	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**0**	-292.99	34.2450	42.1	-8.56	<.0001	Tukey-Kramer	<.0001
TRT*Time	**C**	**72**	**T**	**3**	-166.43	34.2450	42.1	-4.86	<.0001	Tukey-Kramer	0.0010
TRT*Time	**C**	**72**	**T**	**6**	-117.61	34.2450	42.1	-3.43	0.0013	Tukey-Kramer	0.0543
TRT*Time	**C**	**72**	**T**	**24**	-72.9060	34.2450	42.1	-2.13	0.0392	Tukey-Kramer	0.6064
TRT*Time	**C**	**72**	**T**	**48**	2.3440	34.2450	42.1	0.07	0.9458	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**72**	-0.0960	34.2450	42.1	-0.00	0.9978	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**3**	126.56	31.2472	40	4.05	0.0002	Tukey-Kramer	0.0107
TRT*Time	**T**	**0**	**T**	**6**	175.38	31.2472	40	5.61	<.0001	Tukey-Kramer	<.0001
TRT*Time	**T**	**0**	**T**	**24**	220.08	31.2472	40	7.04	<.0001	Tukey-Kramer	<.0001
TRT*Time	**T**	**0**	**T**	**48**	295.33	31.2472	40	9.45	<.0001	Tukey-Kramer	<.0001

Differences of Least Squares Means											
Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT*Time	**T**	**0**	**T**	**72**	292.89	31.2472	40	9.37	<.0001	Tukey-Kramer	<.0001
TRT*Time	**T**	**3**	**T**	**6**	48.8260	31.2472	40	1.56	0.1260	Tukey-Kramer	0.9123
TRT*Time	**T**	**3**	**T**	**24**	93.5280	31.2472	40	2.99	0.0047	Tukey-Kramer	0.1484
TRT*Time	**T**	**3**	**T**	**48**	168.78	31.2472	40	5.40	<.0001	Tukey-Kramer	0.0002
TRT*Time	**T**	**3**	**T**	**72**	166.34	31.2472	40	5.32	<.0001	Tukey-Kramer	0.0002
TRT*Time	**T**	**6**	**T**	**24**	44.7020	31.2472	40	1.43	0.1603	Tukey-Kramer	0.9500
TRT*Time	**T**	**6**	**T**	**48**	119.95	31.2472	40	3.84	0.0004	Tukey-Kramer	0.0192
TRT*Time	**T**	**6**	**T**	**72**	117.51	31.2472	40	3.76	0.0005	Tukey-Kramer	0.0236
TRT*Time	**T**	**24**	**T**	**48**	75.2500	31.2472	40	2.41	0.0207	Tukey-Kramer	0.4240
TRT*Time	**T**	**24**	**T**	**72**	72.8100	31.2472	40	2.33	0.0249	Tukey-Kramer	0.4734
TRT*Time	**T**	**48**	**T**	**72**	-2.4400	31.2472	40	-0.08	0.9381	Tukey-Kramer	1.0000

RESULTS FOR MG IN FEACES

The Mixed Procedure

Model information	
Data set	WORK.FEACES
Dependent variable	Mg
Covariance structure	Compound Symmetry
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
TRT	1	8	2.34	0.1646
Time	5	40	1.98	0.1029
TRT*Time	5	40	0.38	0.8589

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		19.1737	2.4669	8	7.77	<.0001
TRT	**T**		24.5107	2.4669	8	9.94	<.0001
TRT*Time	**C**	**0**	17.8360	3.5054	27	5.09	<.0001
TRT*Time	**C**	**3**	18.8960	3.5054	27	5.39	<.0001
TRT*Time	**C**	**6**	22.0720	3.5054	27	6.30	<.0001
TRT*Time	**C**	**24**	21.6180	3.5054	27	6.17	<.0001
TRT*Time	**C**	**48**	16.8840	3.5054	27	4.82	<.0001
TRT*Time	**C**	**72**	17.7360	3.5054	27	5.06	<.0001
TRT*Time	**T**	**0**	26.0100	3.5054	27	7.42	<.0001
TRT*Time	**T**	**3**	25.4700	3.5054	27	7.27	<.0001
TRT*Time	**T**	**6**	26.4300	3.5054	27	7.54	<.0001
TRT*Time	**T**	**24**	28.8520	3.5054	27	8.23	<.0001
TRT*Time	**T**	**48**	20.6620	3.5054	27	5.89	<.0001
TRT*Time	**T**	**72**	19.6400	3.5054	27	5.60	<.0001

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT	**C**		**T**		-5.3370	3.4887	8	-1.53	0.1646	Tukey	0.1646
TRT*Time	**C**	**0**	**C**	**3**	-1.0600	3.8582	40	-0.27	0.7849	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**C**	**6**	-4.2360	3.8582	40	-1.10	0.2788	Tukey-Kramer	0.9931
TRT*Time	**C**	**0**	**C**	**24**	-3.7820	3.8582	40	-0.98	0.3329	Tukey-Kramer	0.9973
TRT*Time	**C**	**0**	**C**	**48**	0.9520	3.8582	40	0.25	0.8064	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**C**	**72**	0.1000	3.8582	40	0.03	0.9795	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**0**	-8.1740	4.9574	27	-1.65	0.1108	Tukey-Kramer	0.8799
TRT*Time	**C**	**0**	**T**	**3**	-7.6340	4.9574	27	-1.54	0.1352	Tukey-Kramer	0.9198
TRT*Time	**C**	**0**	**T**	**6**	-8.5940	4.9574	27	-1.73	0.0944	Tukey-Kramer	0.8420
TRT*Time	**C**	**0**	**T**	**24**	-11.0160	4.9574	27	-2.22	0.0348	Tukey-Kramer	0.5443
TRT*Time	**C**	**0**	**T**	**48**	-2.8260	4.9574	27	-0.57	0.5734	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**72**	-1.8040	4.9574	27	-0.36	0.7188	Tukey-Kramer	1.0000

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**3**	**C**	**6**	-3.1760	3.8582	40	-0.82	0.4153	Tukey-Kramer	0.9994
TRT*Time	**C**	**3**	**C**	**24**	-2.7220	3.8582	40	-0.71	0.4846	Tukey-Kramer	0.9999
TRT*Time	**C**	**3**	**C**	**48**	2.0120	3.8582	40	0.52	0.6049	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**C**	**72**	1.1600	3.8582	40	0.30	0.7652	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**T**	**0**	-7.1140	4.9574	27	-1.44	0.1628	Tukey-Kramer	0.9490
TRT*Time	**C**	**3**	**T**	**3**	-6.5740	4.9574	27	-1.33	0.1959	Tukey-Kramer	0.9704
TRT*Time	**C**	**3**	**T**	**6**	-7.5340	4.9574	27	-1.52	0.1402	Tukey-Kramer	0.9261
TRT*Time	**C**	**3**	**T**	**24**	-9.9560	4.9574	27	-2.01	0.0547	Tukey-Kramer	0.6854
TRT*Time	**C**	**3**	**T**	**48**	-1.7660	4.9574	27	-0.36	0.7244	Tukey-Kramer	1.0000
TRT*Time	**C**	**3**	**T**	**72**	-0.7440	4.9574	27	-0.15	0.8818	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**C**	**24**	0.4540	3.8582	40	0.12	0.9069	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**C**	**48**	5.1880	3.8582	40	1.34	0.1863	Tukey-Kramer	0.9673

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**6**	**C**	**72**	4.3360	3.8582	40	1.12	0.2678	Tukey-Kramer	0.9916
TRT*Time	**C**	**6**	**T**	**0**	-3.9380	4.9574	27	-0.79	0.4339	Tukey-Kramer	0.9996
TRT*Time	**C**	**6**	**T**	**3**	-3.3980	4.9574	27	-0.69	0.4989	Tukey-Kramer	0.9999
TRT*Time	**C**	**6**	**T**	**6**	-4.3580	4.9574	27	-0.88	0.3871	Tukey-Kramer	0.9990
TRT*Time	**C**	**6**	**T**	**24**	-6.7800	4.9574	27	-1.37	0.1827	Tukey-Kramer	0.9632
TRT*Time	**C**	**6**	**T**	**48**	1.4100	4.9574	27	0.28	0.7783	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**T**	**72**	2.4320	4.9574	27	0.49	0.6277	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**C**	**48**	4.7340	3.8582	40	1.23	0.2270	Tukey-Kramer	0.9833
TRT*Time	**C**	**24**	**C**	**72**	3.8820	3.8582	40	1.01	0.3204	Tukey-Kramer	0.9967
TRT*Time	**C**	**24**	**T**	**0**	-4.3920	4.9574	27	-0.89	0.3835	Tukey-Kramer	0.9989
TRT*Time	**C**	**24**	**T**	**3**	-3.8520	4.9574	27	-0.78	0.4439	Tukey-Kramer	0.9997
TRT*Time	**C**	**24**	**T**	**6**	-4.8120	4.9574	27	-0.97	0.3403	Tukey-Kramer	0.9976

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**24**	**T**	**24**	-7.2340	4.9574	27	-1.46	0.1560	Tukey-Kramer	0.9430
TRT*Time	**C**	**24**	**T**	**48**	0.9560	4.9574	27	0.19	0.8485	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**72**	1.9780	4.9574	27	0.40	0.6930	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**C**	**72**	-0.8520	3.8582	40	-0.22	0.8264	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**0**	-9.1260	4.9574	27	-1.84	0.0766	Tukey-Kramer	0.7862
TRT*Time	**C**	**48**	**T**	**3**	-8.5860	4.9574	27	-1.73	0.0947	Tukey-Kramer	0.8428
TRT*Time	**C**	**48**	**T**	**6**	-9.5460	4.9574	27	-1.93	0.0647	Tukey-Kramer	0.7369
TRT*Time	**C**	**48**	**T**	**24**	-11.9680	4.9574	27	-2.41	0.0228	Tukey-Kramer	0.4203
TRT*Time	**C**	**48**	**T**	**48**	-3.7780	4.9574	27	-0.76	0.4526	Tukey-Kramer	0.9997
TRT*Time	**C**	**48**	**T**	**72**	-2.7560	4.9574	27	-0.56	0.5828	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**0**	-8.2740	4.9574	27	-1.67	0.1067	Tukey-Kramer	0.8714
TRT*Time	**C**	**72**	**T**	**3**	-7.7340	4.9574	27	-1.56	0.1304	Tukey-Kramer	0.9131

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**72**	**T**	**6**	-8.6940	4.9574	27	-1.75	0.0908	Tukey-Kramer	0.8321
TRT*Time	**C**	**72**	**T**	**24**	-11.1160	4.9574	27	-2.24	0.0334	Tukey-Kramer	0.5309
TRT*Time	**C**	**72**	**T**	**48**	-2.9260	4.9574	27	-0.59	0.5599	Tukey-Kramer	1.0000
TRT*Time	**C**	**72**	**T**	**72**	-1.9040	4.9574	27	-0.38	0.7039	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**3**	0.5400	3.8582	40	0.14	0.8894	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**6**	-0.4200	3.8582	40	-0.11	0.9139	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**24**	-2.8420	3.8582	40	-0.74	0.4657	Tukey-Kramer	0.9998
TRT*Time	**T**	**0**	**T**	**48**	5.3480	3.8582	40	1.39	0.1734	Tukey-Kramer	0.9596
TRT*Time	**T**	**0**	**T**	**72**	6.3700	3.8582	40	1.65	0.1066	Tukey-Kramer	0.8790
TRT*Time	**T**	**3**	**T**	**6**	-0.9600	3.8582	40	-0.25	0.8048	Tukey-Kramer	1.0000
TRT*Time	**T**	**3**	**T**	**24**	-3.3820	3.8582	40	-0.88	0.3860	Tukey-Kramer	0.9990
TRT*Time	**T**	**3**	**T**	**48**	4.8080	3.8582	40	1.25	0.2200	Tukey-Kramer	0.9813

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**T**	**3**	**T**	**72**	5.8300	3.8582	40	1.51	0.1386	Tukey-Kramer	0.9287
TRT*Time	**T**	**6**	**T**	**24**	-2.4220	3.8582	40	-0.63	0.5337	Tukey-Kramer	1.0000
TRT*Time	**T**	**6**	**T**	**48**	5.7680	3.8582	40	1.49	0.1428	Tukey-Kramer	0.9334
TRT*Time	**T**	**6**	**T**	**72**	6.7900	3.8582	40	1.76	0.0861	Tukey-Kramer	0.8291
TRT*Time	**T**	**24**	**T**	**48**	8.1900	3.8582	40	2.12	0.0400	Tukey-Kramer	0.6105
TRT*Time	**T**	**24**	**T**	**72**	9.2120	3.8582	40	2.39	0.0218	Tukey-Kramer	0.4369
TRT*Time	**T**	**48**	**T**	**72**	1.0220	3.8582	40	0.26	0.7925	Tukey-Kramer	1.0000

RESULTS FOR P IN FEACES

The Mixed Procedure

Model information	
Data set	WORK.FEACES
Dependent variable	P
Covariance structure	Compound Symmetry
Subject effect	ID(TRT)

Model information	
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	8	11.31	0.0099
Time	5	40	0.26	0.9322
TRT*Time	5	40	2.22	0.0710

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		22.8237	2.1455	8	10.64	<.0001
TRT	**T**		33.0290	2.1455	8	15.39	<.0001
TRT*Time	**C**	**0**	20.0340	3.4378	35.4	5.83	<.0001
TRT*Time	**C**	**3**	19.3080	3.4378	35.4	5.62	<.0001
TRT*Time	**C**	**6**	26.5920	3.4378	35.4	7.74	<.0001
TRT*Time	**C**	**24**	24.6440	3.4378	35.4	7.17	<.0001

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT*Time	**C**	**48**	23.3420	3.4378	35.4	6.79	<.0001
TRT*Time	**C**	**72**	23.0220	3.4378	35.4	6.70	<.0001
TRT*Time	**T**	**0**	39.8540	3.4378	35.4	11.59	<.0001
TRT*Time	**T**	**3**	34.5500	3.4378	35.4	10.05	<.0001
TRT*Time	**T**	**6**	29.5500	3.4378	35.4	8.60	<.0001
TRT*Time	**T**	**24**	30.9020	3.4378	35.4	8.99	<.0001
TRT*Time	**T**	**48**	31.3960	3.4378	35.4	9.13	<.0001
TRT*Time	**T**	**72**	31.9220	3.4378	35.4	9.29	<.0001

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT	**C**		**T**		-10.2053	3.0342	8	-3.36	0.0099	Tukey	0.0099
TRT*Time	**C**	**0**	**C**	**3**	0.7260	4.1614	40	0.17	0.8624	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**C**	**6**	-6.5580	4.1614	40	-1.58	0.1229	Tukey-Kramer	0.9077
TRT*Time	**C**	**0**	**C**	**24**	-4.6100	4.1614	40	-1.11	0.2746	Tukey-Kramer	0.9926

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**0**	**C**	**48**	-3.3080	4.1614	40	-0.79	0.4313	Tukey-Kramer	0.9996
TRT*Time	**C**	**0**	**C**	**72**	-2.9880	4.1614	40	-0.72	0.4769	Tukey-Kramer	0.9998
TRT*Time	**C**	**0**	**T**	**0**	-19.8200	4.8618	35.4	-4.08	0.0002	Tukey-Kramer	0.0100
TRT*Time	**C**	**0**	**T**	**3**	-14.5160	4.8618	35.4	-2.99	0.0051	Tukey-Kramer	0.1507
TRT*Time	**C**	**0**	**T**	**6**	-9.5160	4.8618	35.4	-1.96	0.0582	Tukey-Kramer	0.7175
TRT*Time	**C**	**0**	**T**	**24**	-10.8680	4.8618	35.4	-2.24	0.0318	Tukey-Kramer	0.5355
TRT*Time	**C**	**0**	**T**	**48**	-11.3620	4.8618	35.4	-2.34	0.0252	Tukey-Kramer	0.4690
TRT*Time	**C**	**0**	**T**	**72**	-11.8880	4.8618	35.4	-2.45	0.0196	Tukey-Kramer	0.4014
TRT*Time	**C**	**3**	**C**	**6**	-7.2840	4.1614	40	-1.75	0.0877	Tukey-Kramer	0.8338
TRT*Time	**C**	**3**	**C**	**24**	-5.3360	4.1614	40	-1.28	0.2071	Tukey-Kramer	0.9768

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**3**	**C**	**48**	-4.0340	4.1614	40	-0.97	0.3382	Tukey-Kramer	0.9976
TRT*Time	**C**	**3**	**C**	**72**	-3.7140	4.1614	40	-0.89	0.3775	Tukey-Kramer	0.9988
TRT*Time	**C**	**3**	**T**	**0**	-20.5460	4.8618	35.4	-4.23	0.0002	Tukey-Kramer	0.0065
TRT*Time	**C**	**3**	**T**	**3**	-15.2420	4.8618	35.4	-3.14	0.0034	Tukey-Kramer	0.1092
TRT*Time	**C**	**3**	**T**	**6**	-10.2420	4.8618	35.4	-2.11	0.0423	Tukey-Kramer	0.6212
TRT*Time	**C**	**3**	**T**	**24**	-11.5940	4.8618	35.4	-2.38	0.0226	Tukey-Kramer	0.4387
TRT*Time	**C**	**3**	**T**	**48**	-12.0880	4.8618	35.4	-2.49	0.0178	Tukey-Kramer	0.3769
TRT*Time	**C**	**3**	**T**	**72**	-12.6140	4.8618	35.4	-2.59	0.0137	Tukey-Kramer	0.3162
TRT*Time	**C**	**6**	**C**	**24**	1.9480	4.1614	40	0.47	0.6422	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**C**	**48**	3.2500	4.1614	40	0.78	0.4394	Tukey-Kramer	0.9997

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**6**	**C**	**72**	3.5700	4.1614	40	0.86	0.3961	Tukey-Kramer	0.9992
TRT*Time	**C**	**6**	**T**	**0**	-13.2620	4.8618	35.4	-2.73	0.0099	Tukey-Kramer	0.2501
TRT*Time	**C**	**6**	**T**	**3**	-7.9580	4.8618	35.4	-1.64	0.1105	Tukey-Kramer	0.8847
TRT*Time	**C**	**6**	**T**	**6**	-2.9580	4.8618	35.4	-0.61	0.5468	Tukey-Kramer	1.0000
TRT*Time	**C**	**6**	**T**	**24**	-4.3100	4.8618	35.4	-0.89	0.3813	Tukey-Kramer	0.9989
TRT*Time	**C**	**6**	**T**	**48**	-4.8040	4.8618	35.4	-0.99	0.3298	Tukey-Kramer	0.9972
TRT*Time	**C**	**6**	**T**	**72**	-5.3300	4.8618	35.4	-1.10	0.2804	Tukey-Kramer	0.9932
TRT*Time	**C**	**24**	**C**	**48**	1.3020	4.1614	40	0.31	0.7560	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**C**	**72**	1.6220	4.1614	40	0.39	0.6988	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**0**	-15.2100	4.8618	35.4	-3.13	0.0035	Tukey-Kramer	0.1108
TRT*Time	**C**	**24**	**T**	**3**	-9.9060	4.8618	35.4	-2.04	0.0491	Tukey-Kramer	0.6666

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**24**	**T**	**6**	-4.9060	4.8618	35.4	-1.01	0.3198	Tukey-Kramer	0.9966
TRT*Time	**C**	**24**	**T**	**24**	-6.2580	4.8618	35.4	-1.29	0.2064	Tukey-Kramer	0.9762
TRT*Time	**C**	**24**	**T**	**48**	-6.7520	4.8618	35.4	-1.39	0.1736	Tukey-Kramer	0.9591
TRT*Time	**C**	**24**	**T**	**72**	-7.2780	4.8618	35.4	-1.50	0.1433	Tukey-Kramer	0.9328
TRT*Time	**C**	**48**	**C**	**72**	0.3200	4.1614	40	0.08	0.9391	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**0**	-16.5120	4.8618	35.4	-3.40	0.0017	Tukey-Kramer	0.0595
TRT*Time	**C**	**48**	**T**	**3**	-11.2080	4.8618	35.4	-2.31	0.0271	Tukey-Kramer	0.4895
TRT*Time	**C**	**48**	**T**	**6**	-6.2080	4.8618	35.4	-1.28	0.2100	Tukey-Kramer	0.9775
TRT*Time	**C**	**48**	**T**	**24**	-7.5600	4.8618	35.4	-1.55	0.1289	Tukey-Kramer	0.9149
TRT*Time	**C**	**48**	**T**	**48**	-8.0540	4.8618	35.4	-1.66	0.1065	Tukey-Kramer	0.8766
TRT*Time	**C**	**48**	**T**	**72**	-8.5800	4.8618	35.4	-1.76	0.0862	Tukey-Kramer	0.8266

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**72**	**T**	**0**	-16.8320	4.8618	35.4	-3.46	0.0014	Tukey-Kramer	0.0507
TRT*Time	**C**	**72**	**T**	**3**	-11.5280	4.8618	35.4	-2.37	0.0233	Tukey-Kramer	0.4472
TRT*Time	**C**	**72**	**T**	**6**	-6.5280	4.8618	35.4	-1.34	0.1879	Tukey-Kramer	0.9677
TRT*Time	**C**	**72**	**T**	**24**	-7.8800	4.8618	35.4	-1.62	0.1139	Tukey-Kramer	0.8911
TRT*Time	**C**	**72**	**T**	**48**	-8.3740	4.8618	35.4	-1.72	0.0937	Tukey-Kramer	0.8473
TRT*Time	**C**	**72**	**T**	**72**	-8.9000	4.8618	35.4	-1.83	0.0756	Tukey-Kramer	0.7919
TRT*Time	**T**	**0**	**T**	**3**	5.3040	4.1614	40	1.27	0.2098	Tukey-Kramer	0.9778
TRT*Time	**T**	**0**	**T**	**6**	10.3040	4.1614	40	2.48	0.0176	Tukey-Kramer	0.3829
TRT*Time	**T**	**0**	**T**	**24**	8.9520	4.1614	40	2.15	0.0376	Tukey-Kramer	0.5916
TRT*Time	**T**	**0**	**T**	**48**	8.4580	4.1614	40	2.03	0.0488	Tukey-Kramer	0.6698
TRT*Time	**T**	**0**	**T**	**72**	7.9320	4.1614	40	1.91	0.0638	Tukey-Kramer	0.7486

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**T**	**3**	**T**	**6**	5.0000	4.1614	40	1.20	0.2366	Tukey-Kramer	0.9858
TRT*Time	**T**	**3**	**T**	**24**	3.6480	4.1614	40	0.88	0.3859	Tukey-Kramer	0.9990
TRT*Time	**T**	**3**	**T**	**48**	3.1540	4.1614	40	0.76	0.4529	Tukey-Kramer	0.9997
TRT*Time	**T**	**3**	**T**	**72**	2.6280	4.1614	40	0.63	0.5313	Tukey-Kramer	1.0000
TRT*Time	**T**	**6**	**T**	**24**	-1.3520	4.1614	40	-0.32	0.7470	Tukey-Kramer	1.0000
TRT*Time	**T**	**6**	**T**	**48**	-1.8460	4.1614	40	-0.44	0.6597	Tukey-Kramer	1.0000
TRT*Time	**T**	**6**	**T**	**72**	-2.3720	4.1614	40	-0.57	0.5719	Tukey-Kramer	1.0000
TRT*Time	**T**	**24**	**T**	**48**	-0.4940	4.1614	40	-0.12	0.9061	Tukey-Kramer	1.0000
TRT*Time	**T**	**24**	**T**	**72**	-1.0200	4.1614	40	-0.25	0.8076	Tukey-Kramer	1.0000
TRT*Time	**T**	**48**	**T**	**72**	-0.5260	4.1614	40	-0.13	0.9000	Tukey-Kramer	1.0000

RESULTS FOR CA IN BONE

The Mixed Procedure

Model information

Data set	WORK.BONE
Dependent variable	Ca
Covariance structure	Compound Symmetry
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	2	0.15	0.7365
Time	3	6	0.73	0.5697
TRT*Time	3	6	0.11	0.9485

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		1317.37	207.65	2	6.34	0.0240

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**T**		1203.95	207.65	2	5.80	0.0285
TRT*Time	**C**	**0**	1460.27	375.00	7.87	3.89	0.0047
TRT*Time	**C**	**3**	1559.87	375.00	7.87	4.16	0.0033
TRT*Time	**C**	**24**	1270.79	375.00	7.87	3.39	0.0098
TRT*Time	**C**	**48**	978.55	375.00	7.87	2.61	0.0316
TRT*Time	**T**	**0**	1328.41	375.00	7.87	3.54	0.0078
TRT*Time	**T**	**3**	1229.79	375.00	7.87	3.28	0.0115
TRT*Time	**T**	**24**	1359.45	375.00	7.87	3.63	0.0069
TRT*Time	**T**	**48**	898.15	375.00	7.87	2.40	0.0440

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT	**C**		**T**		113.42	293.66	2	0.39	0.7365	Tukey	0.7365
TRT*Time	**C**	**0**	**C**	**3**	-99.6050	509.92	6	-0.20	0.8516	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**C**	**24**	189.48	509.92	6	0.37	0.7230	Tukey-Kramer	0.9999

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**0**	**C**	**48**	481.72	509.92	6	0.94	0.3813	Tukey-Kramer	0.9681
TRT*Time	**C**	**0**	**T**	**0**	131.86	530.33	7.87	0.25	0.8100	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**3**	230.47	530.33	7.87	0.43	0.6755	Tukey-Kramer	0.9997
TRT*Time	**C**	**0**	**T**	**24**	100.82	530.33	7.87	0.19	0.8540	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**48**	562.12	530.33	7.87	1.06	0.3206	Tukey-Kramer	0.9450
TRT*Time	**C**	**3**	**C**	**24**	289.09	509.92	6	0.57	0.5913	Tukey-Kramer	0.9982
TRT*Time	**C**	**3**	**C**	**48**	581.32	509.92	6	1.14	0.2977	Tukey-Kramer	0.9242
TRT*Time	**C**	**3**	**T**	**0**	231.46	530.33	7.87	0.44	0.6742	Tukey-Kramer	0.9996
TRT*Time	**C**	**3**	**T**	**3**	330.08	530.33	7.87	0.62	0.5513	Tukey-Kramer	0.9968
TRT*Time	**C**	**3**	**T**	**24**	200.43	530.33	7.87	0.38	0.7155	Tukey-Kramer	0.9999
TRT*Time	**C**	**3**	**T**	**48**	661.72	530.33	7.87	1.25	0.2480	Tukey-Kramer	0.8900
TRT*Time	**C**	**24**	**C**	**48**	292.23	509.92	6	0.57	0.5874	Tukey-Kramer	0.9980

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**24**	**T**	**0**	-57.6200	530.33	7.87	-0.11	0.9162	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**3**	40.9950	530.33	7.87	0.08	0.9403	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**24**	-88.6600	530.33	7.87	-0.17	0.8714	Tukey-Kramer	1.0000
TRT*Time	**C**	**24**	**T**	**48**	372.64	530.33	7.87	0.70	0.5025	Tukey-Kramer	0.9934
TRT*Time	**C**	**48**	**T**	**0**	-349.86	530.33	7.87	-0.66	0.5283	Tukey-Kramer	0.9954
TRT*Time	**C**	**48**	**T**	**3**	-251.24	530.33	7.87	-0.47	0.6486	Tukey-Kramer	0.9994
TRT*Time	**C**	**48**	**T**	**24**	-380.89	530.33	7.87	-0.72	0.4934	Tukey-Kramer	0.9926
TRT*Time	**C**	**48**	**T**	**48**	80.4000	530.33	7.87	0.15	0.8833	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**3**	98.6150	509.92	6	0.19	0.8530	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**24**	-31.0400	509.92	6	-0.06	0.9534	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**48**	430.26	509.92	6	0.84	0.4312	Tukey-Kramer	0.9821

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**T**	**3**	**T**	**24**	-129.65	509.92	6	-0.25	0.8078	Tukey-Kramer	1.0000
TRT*Time	**T**	**3**	**T**	**48**	331.64	509.92	6	0.65	0.5395	Tukey-Kramer	0.9958
TRT*Time	**T**	**24**	**T**	**48**	461.30	509.92	6	0.90	0.4005	Tukey-Kramer	0.9743

RESULTS FOR MG IN BONE

The Mixed Procedure

Model information	
Data set	WORK.BONE
Dependent variable	Mg
Covariance structure	Compound Symmetry
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	2	1.58	0.3355
Time	3	6	2.41	0.1654
TRT*Time	3	6	0.71	0.5819

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		17.6762	1.8969	2	9.32	0.0113
TRT	**T**		14.3025	1.8969	2	7.54	0.0171
TRT*Time	**C**	**0**	16.2450	2.3755	4.44	6.84	0.0016
TRT*Time	**C**	**3**	19.1400	2.3755	4.44	8.06	0.0008
TRT*Time	**C**	**24**	20.0300	2.3755	4.44	8.43	0.0007
TRT*Time	**C**	**48**	15.2900	2.3755	4.44	6.44	0.0021
TRT*Time	**T**	**0**	15.4400	2.3755	4.44	6.50	0.0020
TRT*Time	**T**	**3**	13.6100	2.3755	4.44	5.73	0.0033
TRT*Time	**T**	**24**	16.1050	2.3755	4.44	6.78	0.0017
TRT*Time	**T**	**48**	12.0550	2.3755	4.44	5.07	0.0054

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT	**C**		**T**		3.3737	2.6826	2	1.26	0.3355	Tukey	0.3355
TRT*Time	**C**	**0**	**C**	**3**	-2.8950	2.3352	6	-1.24	0.2614	Tukey-Kramer	0.8928
TRT*Time	**C**	**0**	**C**	**24**	-3.7850	2.3352	6	-1.62	0.1562	Tukey-Kramer	0.7298
TRT*Time	**C**	**0**	**C**	**48**	0.9550	2.3352	6	0.41	0.6968	Tukey-Kramer	0.9998
TRT*Time	**C**	**0**	**T**	**0**	0.8050	3.3595	4.44	0.24	0.8213	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**3**	2.6350	3.3595	4.44	0.78	0.4726	Tukey-Kramer	0.9879
TRT*Time	**C**	**0**	**T**	**24**	0.1400	3.3595	4.44	0.04	0.9686	Tukey-Kramer	1.0000
TRT*Time	**C**	**0**	**T**	**48**	4.1900	3.3595	4.44	1.25	0.2741	Tukey-Kramer	0.8902
TRT*Time	**C**	**3**	**C**	**24**	-0.8900	2.3352	6	-0.38	0.7162	Tukey-Kramer	0.9999
TRT*Time	**C**	**3**	**C**	**48**	3.8500	2.3352	6	1.65	0.1503	Tukey-Kramer	0.7163
TRT*Time	**C**	**3**	**T**	**0**	3.7000	3.3595	4.44	1.10	0.3268	Tukey-Kramer	0.9348
TRT*Time	**C**	**3**	**T**	**3**	5.5300	3.3595	4.44	1.65	0.1680	Tukey-Kramer	0.7175

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**C**	**3**	**T**	**24**	3.0350	3.3595	4.44	0.90	0.4126	Tukey-Kramer	0.9745
TRT*Time	**C**	**3**	**T**	**48**	7.0850	3.3595	4.44	2.11	0.0957	Tukey-Kramer	0.4940
TRT*Time	**C**	**24**	**C**	**48**	4.7400	2.3352	6	2.03	0.0887	Tukey-Kramer	0.5303
TRT*Time	**C**	**24**	**T**	**0**	4.5900	3.3595	4.44	1.37	0.2370	Tukey-Kramer	0.8451
TRT*Time	**C**	**24**	**T**	**3**	6.4200	3.3595	4.44	1.91	0.1215	Tukey-Kramer	0.5870
TRT*Time	**C**	**24**	**T**	**24**	3.9250	3.3595	4.44	1.17	0.3015	Tukey-Kramer	0.9159
TRT*Time	**C**	**24**	**T**	**48**	7.9750	3.3595	4.44	2.37	0.0701	Tukey-Kramer	0.3838
TRT*Time	**C**	**48**	**T**	**0**	-0.1500	3.3595	4.44	-0.04	0.9663	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**3**	1.6800	3.3595	4.44	0.50	0.6408	Tukey-Kramer	0.9991
TRT*Time	**C**	**48**	**T**	**24**	-0.8150	3.3595	4.44	-0.24	0.8191	Tukey-Kramer	1.0000
TRT*Time	**C**	**48**	**T**	**48**	3.2350	3.3595	4.44	0.96	0.3850	Tukey-Kramer	0.9650
TRT*Time	**T**	**0**	**T**	**3**	1.8300	2.3352	6	0.78	0.4630	Tukey-Kramer	0.9879

Differences of Least Squares Means

Effect	TRT	Time	_TRT	_Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
TRT*Time	**T**	**0**	**T**	**24**	-0.6650	2.3352	6	-0.28	0.7854	Tukey-Kramer	1.0000
TRT*Time	**T**	**0**	**T**	**48**	3.3850	2.3352	6	1.45	0.1974	Tukey-Kramer	0.8097
TRT*Time	**T**	**3**	**T**	**24**	-2.4950	2.3352	6	-1.07	0.3264	Tukey-Kramer	0.9430
TRT*Time	**T**	**3**	**T**	**48**	1.5550	2.3352	6	0.67	0.5302	Tukey-Kramer	0.9952
TRT*Time	**T**	**24**	**T**	**48**	4.0500	2.3352	6	1.73	0.1336	Tukey-Kramer	0.6741

RESULTS FOR P IN BONE

The Mixed Procedure

Model information	
Data set	WORK.BONE
Dependent variable	P
Covariance structure	Compound Symmetry
Subject effect	ID(TRT)
Estimation method	REML
Residual Variance Method	Profile

Model information

Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRT	1	2	0.15	0.7343
Time	3	6	4.22	0.0633
TRT*Time	3	6	0.55	0.6674

Least Squares Means

Effect	TRT	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
TRT	**C**		154.38	25.0341	2	6.17	0.0253
TRT	**T**		140.58	25.0341	2	5.62	0.0303
TRT*Time	**C**	**0**	169.39	33.3498	5.25	5.08	0.0033
TRT*Time	**C**	**3**	190.49	33.3498	5.25	5.71	0.0020
TRT*Time	**C**	**24**	170.15	33.3498	5.25	5.10	0.0033
TRT*Time	**C**	**48**	87.5000	33.3498	5.25	2.62	0.0447
TRT*Time	**T**	**0**	150.66	33.3498	5.25	4.52	0.0056

Least Squares Means							
Effect	**TRT**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
TRT* Time	**T**	**3**	141.17	33.3498	5.25	4.23	0.0074
TRT* Time	**T**	**24**	172.99	33.3498	5.25	5.19	0.0030
TRT* Time	**T**	**48**	97.5100	33.3498	5.25	2.92	0.0310

Differences of Least Squares Means

Effect	**TRT**	**Time**	**_TRT**	**_Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
TRT	**C**		**T**		13.7987	35.4035	2	0.39	0.7343	Tukey	0.7343
TRT* Time	**C**	**0**	**C**	**3**	-21.0950	35.9818	6	-0.59	0.5791	Tukey-Kramer	0.9977
TRT* Time	**C**	**0**	**C**	**24**	-0.7550	35.9818	6	-0.02	0.9839	Tukey-Kramer	1.0000
TRT* Time	**C**	**0**	**C**	**48**	81.8900	35.9818	6	2.28	0.0632	Tukey-Kramer	0.4224
TRT* Time	**C**	**0**	**T**	**0**	18.7350	47.1638	5.25	0.40	0.7068	Tukey-Kramer	0.9998
TRT* Time	**C**	**0**	**T**	**3**	28.2200	47.1638	5.25	0.60	0.5745	Tukey-Kramer	0.9974
TRT* Time	**C**	**0**	**T**	**24**	-3.6000	47.1638	5.25	-0.08	0.9420	Tukey-Kramer	1.0000

Least Squares Means											
Effect	**TRT**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**				
TRT* Time	**C**	**0**	**T**	**48**	71.8800	47.1638	5.25	1.52	0.1853	Tukey-Kramer	0.7759
TRT* Time	**C**	**3**	**C**	**24**	20.3400	35.9818	6	0.57	0.5924	Tukey-Kramer	0.9982
TRT* Time	**C**	**3**	**C**	**48**	102.99	35.9818	6	2.86	0.0287	Tukey-Kramer	0.2321
TRT* Time	**C**	**3**	**T**	**0**	39.8300	47.1638	5.25	0.84	0.4352	Tukey-Kramer	0.9820
TRT* Time	**C**	**3**	**T**	**3**	49.3150	47.1638	5.25	1.05	0.3415	Tukey-Kramer	0.9484
TRT* Time	**C**	**3**	**T**	**24**	17.4950	47.1638	5.25	0.37	0.7252	Tukey-Kramer	0.9999
TRT* Time	**C**	**3**	**T**	**48**	92.9750	47.1638	5.25	1.97	0.1030	Tukey-Kramer	0.5579
TRT* Time	**C**	**24**	**C**	**48**	82.6450	35.9818	6	2.30	0.0614	Tukey-Kramer	0.4139
TRT* Time	**C**	**24**	**T**	**0**	19.4900	47.1638	5.25	0.41	0.6958	Tukey-Kramer	0.9997
TRT* Time	**C**	**24**	**T**	**3**	28.9750	47.1638	5.25	0.61	0.5646	Tukey-Kramer	0.9970
TRT* Time	**C**	**24**	**T**	**24**	-2.8450	47.1638	5.25	-0.06	0.9541	Tukey-Kramer	1.0000
TRT* Time	**C**	**24**	**T**	**48**	72.6350	47.1638	5.25	1.54	0.1814	Tukey-Kramer	0.7684

Least Squares Means											
Effect	**TRT**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**				
TRT* Time	**C**	**48**	**T**	**0**	-63.1550	47.1638	5.25	-1.34	0.2356	Tukey-Kramer	0.8560
TRT* Time	**C**	**48**	**T**	**3**	-53.6700	47.1638	5.25	-1.14	0.3044	Tukey-Kramer	0.9248
TRT* Time	**C**	**48**	**T**	**24**	-85.4900	47.1638	5.25	-1.81	0.1269	Tukey-Kramer	0.6353
TRT* Time	**C**	**48**	**T**	**48**	-10.0100	47.1638	5.25	-0.21	0.8399	Tukey-Kramer	1.0000
TRT* Time	**T**	**0**	**T**	**3**	9.4850	35.9818	6	0.26	0.8009	Tukey-Kramer	1.0000
TRT* Time	**T**	**0**	**T**	**24**	-22.3350	35.9818	6	-0.62	0.5576	Tukey-Kramer	0.9968
TRT* Time	**T**	**0**	**T**	**48**	53.1450	35.9818	6	1.48	0.1901	Tukey-Kramer	0.7974
TRT* Time	**T**	**3**	**T**	**24**	-31.8200	35.9818	6	-0.88	0.4105	Tukey-Kramer	0.9771
TRT* Time	**T**	**3**	**T**	**48**	43.6600	35.9818	6	1.21	0.2706	Tukey-Kramer	0.9017
TRT* Time	**T**	**24**	**T**	**48**	75.4800	35.9818	6	2.10	0.0807	Tukey-Kramer	0.4991