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Thesis or Dissertation Title: Effects of Eight Weeks of 6-OXOTM Supplementation on Serum Hormone Profiles and on Serum and Urinary Clinical Safety Markers in Resistance-Trained Males.

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ABSTRACT

Effects of Eight Weeks of 6-OXO^{TM} Supplementation on Serum Hormone Profiles and on Serum and Urinary Clinical Safety Markers in Resistance-Trained Males

Daniel A. Rohle

Committee Chairperson: Darryn S. Willoughby, Ph.D.

The purpose of this study was to determine the effects of 6OXO™, an aromatase inhibitor, in a dose dependent manner on serum hormone levels and clinical safety markers in resistance trained males. Sixteen healthy trained subjects, who participated in a resistance training protocol, had blood samples taken at weeks 0, 1, 3, 8, and 11. These samples were analyzed for total testosterone, free testosterone, DHT, estradiol, estriol, estrone, SHBG, LH, FSH, GH, and cortisol. There were no significant differences between groups ($p>0.01$). However, total testosterone concentration, free testosterone concentration, and DHT concentration increased over the course of the study ($p=0.009$). Measures of body composition did not change with supplementation ($p>0.05$). Safety markers were seen to not be adversely affected with ingestion of 6OXO™ ($p>0.01$). 6OXO™ supplementation appears to be safe and increases total testosterone, free testosterone, and DHT concentrations independent of the two different doses.

Effects of Eight Weeks of 6-OXO^{TM} Supplementation on Serum Hormone Profiles and on Serum and Urinary Clinical Safety Markers in Resistance-Trained Males

by

Daniel A Rohle, BS BA

A Thesis

Approved by the Department of Health, Human Preformance, and Recreation

Richard B. Kreider, Ph.D

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Master of Science in Education

Approved by the Thesis Committee

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Accepted by the Graduate School
December 2005

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ACKNOWLEDGMENTS

I would like to thank my parents, without them I would not be here today, nor would I have had the opportunities that have been presented to me. I would also like to thank them for putting up with me because I know how stubborn and angry I can be. I would like to thank Dr. Darryn Willoughby for helping me with the research project design, the research, and the analysis. Without him I would not have been able to accomplish as much as I did. I would like to thank all my friends, who I called up trying to explain the statistical aspects I figured out, and who just sat there and listened. Finally I would like to thank my computer. You only blew up once while I was working on this, and I am grateful that nothing was lost.

CHAPTER ONE

Introduction

Athletes have long been looking for a way to gain an edge in competition, which has lead many to turn to anabolic steroids. Anabolic steroids are defined as testosterone or testosterone derivatives that are used for their ability to create a state of nitrogen retention in lean mass by stimulation of protein synthesis and/or by a decrease in protein breakdown. It has been previously thought that anabolic steroids did not cause an increase in muscle size and strength, but now more recent studies like Bhasin et. al. (1997, 2000, 2001, 2001) and Sinh-Hikim et al. (2002, 2003) have shown that testosterone at supra-physiological levels increases muscle size and strength. Schroeder et al. showed that exogenous testosterone derivatives could increase muscle size but not muscle qualities (2003). These studies have shown the effect that high levels of testosterone and testosterone derivatives have in males.

Testosterone is the male sex hormone that is derived from cholesterol. Biosynthesis begins in the adrenal cortex where cholesterol is converted through a multi-step process to dehydroepiandrosterone (DHEA) and androstenedione. This compound is then converted to testosterone in the Leydig cells that are located in the testis. See Figure 1. Testosterone and other C-19 androgens are precursors for the family of estrogen compounds (estradiol, estriol, and estrone). Testosterone can also be converted to the compound dihydrotestosterone (DHT). See Figure 2. Testosterone and the other C-19 androgens are converted into estradiol by the action of the enzyme aromatase.

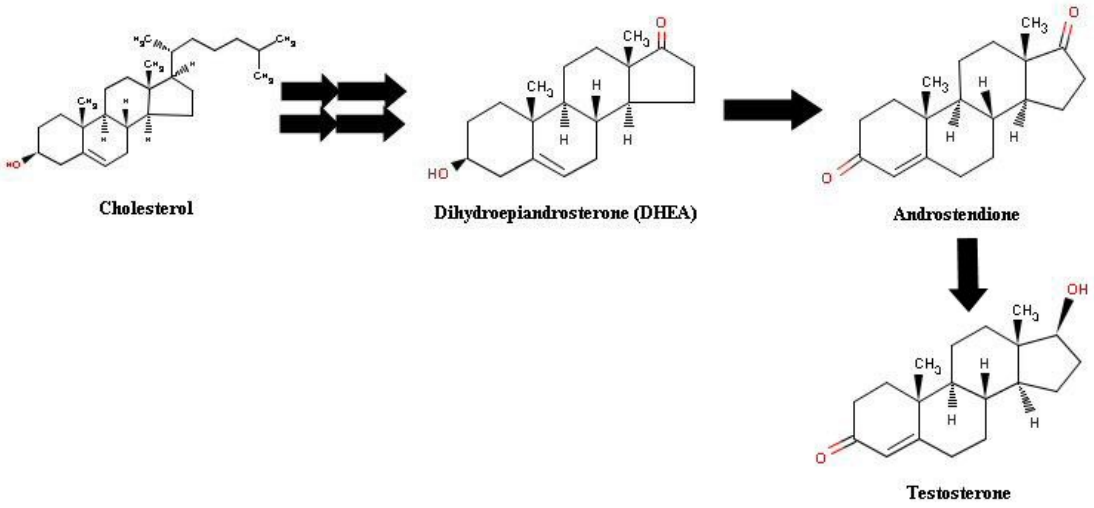

Figure 1. Basic synthesis of testosterone.

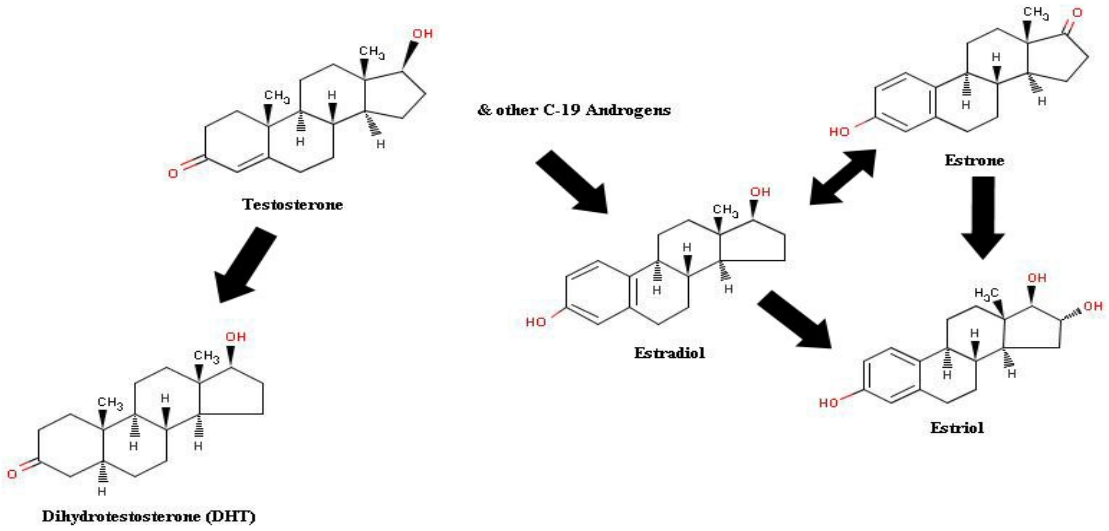

Figure 2. Basic synthesis of dihydrotestosterone and the estrogens: estradiol, estrone, and estriol.

Testosterone production and release is regulated by the pulsing action of gonadotropin releasing hormone (GnRH). Gonadotropin releasing hormone is produced in the hypothalamus that is controlled by higher brain centers to release GnRH in a pulsing mechanism. Less frequent pulses causes the pituitary to release FSH and more

frequent pulses stimulate LH release. These hormones control testosterone production and estradiol production. These sex hormones can feed back to the pituitary, hypothalamus, and higher brain centers in a negative fashion. Too much of these hormones causes their own production to shut down and too little of these hormones help stimulate their release. This is a fail safe switch so that the preset physiological levels never get too high or too low.

Testosterone is not completely freely circulating in the blood once it is produced. Testosterone is almost 100% bound in blood to proteins with 40% bound to albumin, 40% bound to a β-globulin called gonadal steroid-binding globulin or sex hormone binding globulin (SHBG), and 17% is bound to other proteins. The small fraction of testosterone that is not bound is considered the free testosterone and is the bioactive component of the hormone. When measuring testosterone levels it is usually separated into measurements of total testosterone (TT) and free testosterone (FT).

In order to use the endocrine system of the body to maximize free testosterone, supplements companies have tried to find compounds that either increase testosterone or that can mimic its effects. There have been herbal remedies like tribulus terrestris extract that are purported to support testosterone production. There have also been pro-hormones like DHEA, androstenedione, and other compounds that are precursors to testosterone or are precursors to designer testosterone derivatives like 1-androstenedione and 1-testosterone. The compounds are alleged to increase testosterone or to increase the concentration of compounds that can act like testosterone. The newest wave of supplements is compounds that have no androgenic or estrogenic activity. These

supplements are aromatase inhibitors like chrysin, formestane, and 6-OXO™ chemically known as androst-4-ene-3,6,17-trione. See Figure 3.

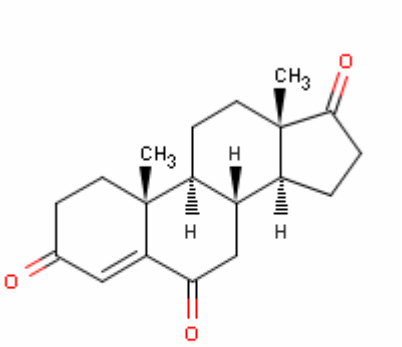

Figure 3. Chemical structure for androst-4-ene-3,6,17-trione.

Numazawa and colleagues (1987, 1996) have shown that 6-OXO™ irreversibly binds to the aromatase enzyme thereby causing a halt in estradiol production. Since this compound is naturally occurring it is available over the counter via the FDA's Dietary Supplement Act. Therefore use of this compound could lead to a decrease in estradiol synthesis, which could lead to an increase in testosterone concentration as less is converted to estradiol.

Purpose of the Study

1. To determine the efficacy of an 8-week oral supplementation period with either 300 mg/day or 600 mg/day of 6-OXO™ on serum hormone levels (total and free testosterone, dihydrotestosterone, estradiol, estrione, estrone, sex hormone binding globulin, leutinizing hormone, and follicle stimulating hormone) and on serum and urinary clinical markers.

2. To assess the safety profile of 6-OXO™ supplementation by way of evaluating the systemic hemodynamic effects (heart rate, blood pressure, rate pressure product) and serum and urinary clinical markers [a comprehensive CBC panel including hematocrit, hemoglobin, and leukocyte differentials, lipids (triglycerides and total cholesterol with sub-fractions), albumin, glucose, BUN,

creatinine, sodium, potassium, chloride, carbon dioxide, calcium, total protein, globulin, total bilirubin, ALP, ALT, and AST] in response to both supplementation protocols.

3. To evaluate the safety profile and hemodynamic effects after a 3-week washout period following both supplementation protocols.

Hypothesis

Ho_1 With supplementation of 6-OXO™ total testosterone, free testosterone, estradiol, estriol, estrone, and dihydrotestosterone concentrations will be similar between groups.

Ho_2 Supplementation with 6-OXO™ will not cause any significant changes in clinical safety markers at either concentration level of 6-OXO™.

Ho_3 Leutinizing hormone and follicle stimulating hormones will increase with 6-OXO™ supplementation and will not significantly differ between the two supplement concentration levels

Ho_4 Body composition will not differ between the different supplement concentrations of 6-OXO™.

Ho_5 Any hormonal changes that were seen in the dosage groups will not be different from hormonal changes that occur in a control group.

Delimitations

1. Sixteen apparently healthy, resistance-trained (regular resistance training for at least three years) males between the ages of 21-45 years will be allowed to participated in study.

2. At weeks 0, 1, 3, 8, and 10 venous blood samples, total body mass and body composition will be determined, and urine samples will be obtained in mid-stream.

3. The 16 participants will be equally divided, matched by age and body weight, and then assigned an 8-week supplementation protocol consisting of the oral ingestion of either 300 mg/day (100 mg AM, 200 mg PM, with meals) or 600 mg/day (300 mg AM, 300 mg PM, with meals) of 6-OXO™ (ErgoPharm, Champaign, IL).

4. The subjects will take supplements every day during the supplementation period of 8 weeks, and after the supplementation period a 3-week washout period will be required.

5. Subjects will participate in a four day a week unsupervised resistance training protocol that they will record along with detailed dietary records.

6. Weekly hemodynamic safety measurements (heart rate, blood pressure, and rate pressure product) will be obtained by the laboratory nurse.

7. All supervised testing and training will be conducted in the Exercise & Sport Nutrition Laboratory (ESNL) and Exercise and Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University, in Waco, TX.

8. A control group of 8 male resistance trained subjects that had participated in the Muscle Tech study using the same resistance training protocol and experimental testing procedures will have their blood samples taken from storage in the -80°C freezer and analyzed for specific hormonal levels.

Assumptions

1. All participants will follow all training program guidelines and only perform the exercises and repetitions instructed to them for the duration of the study.

2. All participants will take their prescribed dose of the 6-OXO^{TM} for the entire investigation period.

3. Participants will fast overnight for eight hours prior to coming in for testing.

4. Participants are all apparently healthy with no contraindications to any of the prescribed exercises and testing procedures.

5. Participants will make no changes to their current dietary habits.

Definitions

- Albumin – produced mainly in the liver, it is the most abundant protein in the blood. it helps keep the blood from leaking out of blood vessels. When albumin levels drop, fluid may collect in the ankles, lungs, or abdomen.

- Globulin – another major type of protein found in the blood. It is important for its immunologic responses, especially its gamma component (IgA, IgG, IgM, and IgE). Some globulins are formed by the liver while others are formed by the immune system.

- Testosterone – the male sex hormone. Formed in greatest quantities by the testes, and is known as one of primary anabolic hormones.

- Estrogen – the generic name for the female sex hormones. Consists of three compounds: estradiol, estriol, and estrone.

- Gonadotropin Releasing Hormone – a hormone released from the hypothalamus that activates the anterior pituitary to release follicle stimulating hormone and leutinizing hormone.

- Leutinizing Hormone – a hormone released from the anterior pituitary. It is released by a pulsing mechanism that controls the production of testosterone specifically in the Leydig cells.

- Follicle Stimulating Hormone – a hormone release from the anterior pituitary. It is released by a pulsing mechanism that controls the production of the estrogenic steroids.

- Aromatase – a P450 cytochrome enzyme that produces the estrogenic steroids from C-19 androgens.

- C-19 Androgens – male sex steroids that contain exactly 19 carbon atoms.

CHAPTER TWO

Review of Literature

Aromatase and Estrogen Biosynthesis

Aromatase, a unique cytochrome P450 enzyme, is the enzyme in the human body that creates C18 steroids (estrogens) from C19 steroids (androgens). The aromatase enzyme is encoded by the gene designated CYP19 and is separated into two main proteins. The first is cytochrome P450 arom, a hemeprotein that creates the C18 steroids that contain a phenolic A-ring according to Simpson et al. (1994). The second protein is NADPH-cyctochrome P450 reductase. This protein functions to transfer reducing equivalents to the P450 arom. The catalysis process consists of three successive oxygenations, then the eventual loss of the methyl group at position C19 as formic acid and water to form the phenolic A ring. This process uses three moles of NADPH and three moles of oxygen to convert one mole of substrate (androstenedione, for example) to one mole of product (estrone). See Figure 4.

The structure of aromatase is being closely studied in attempts to regulate the formation of estrogens. The reason for this is because estrogens are known to cause the reproduction and estrogen-dependent tumor proliferation in cancers like breast cancer (Simpson et al., 1997; Reed 1994). Many studies have focused on the structure and function aspects of aromatase in order to elucidate the important regions of the aromatase enzyme that are important for catalytic function. One method that has helped researchers understand aromatase is site directed mutagenesis (Auvray et al., 2002; Zhou et al., 1990). The mutant form of human aromatase that had a phenylalanine, at residue 406,

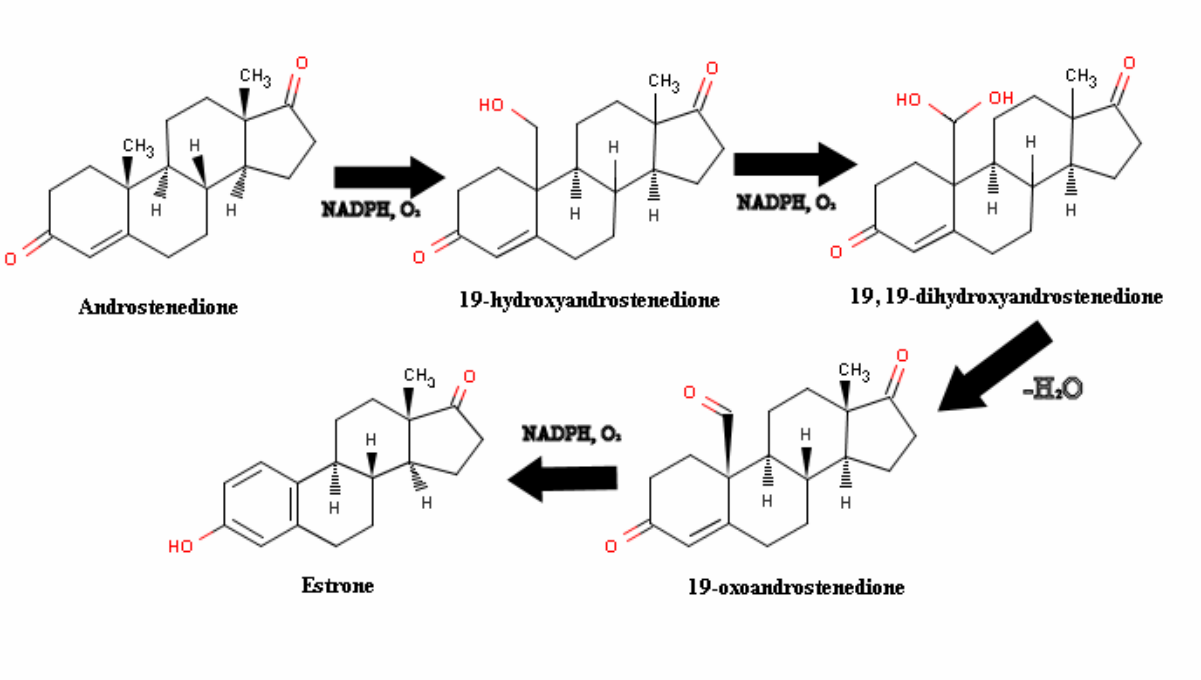

Figure 4. Synthesis of an estrogenic steroid (estrone) from a C-19 androgen (androstenedione).

mutated into an arginine residue caused the enzyme to become totally inactive, and a mutation at residue 308, which was a proline mutated into a phenylalanine, caused the enzyme to have a major reduction in Vmax (Zhou, 1990). These two mutations allow for researchers to understand specific areas of aromatase that are important for function allowing for specific targeting of these areas to create better aromatase inhibitors. As research has progressed human aromatase structure, specifically at the active site, is becoming more understood. See Figure 5 obtained from Auvray et al. (2002). A mutation at the 309 residue of aromatase, which is the transfer of an aspartic acid residue to either an alanine or asparagine residue, caused a loss of activity for androstenedione and testosterone but not for nor-testosterone (Auvray et al., 2002). This mutation provides an insight into how the 309 residue might interact with a compound,

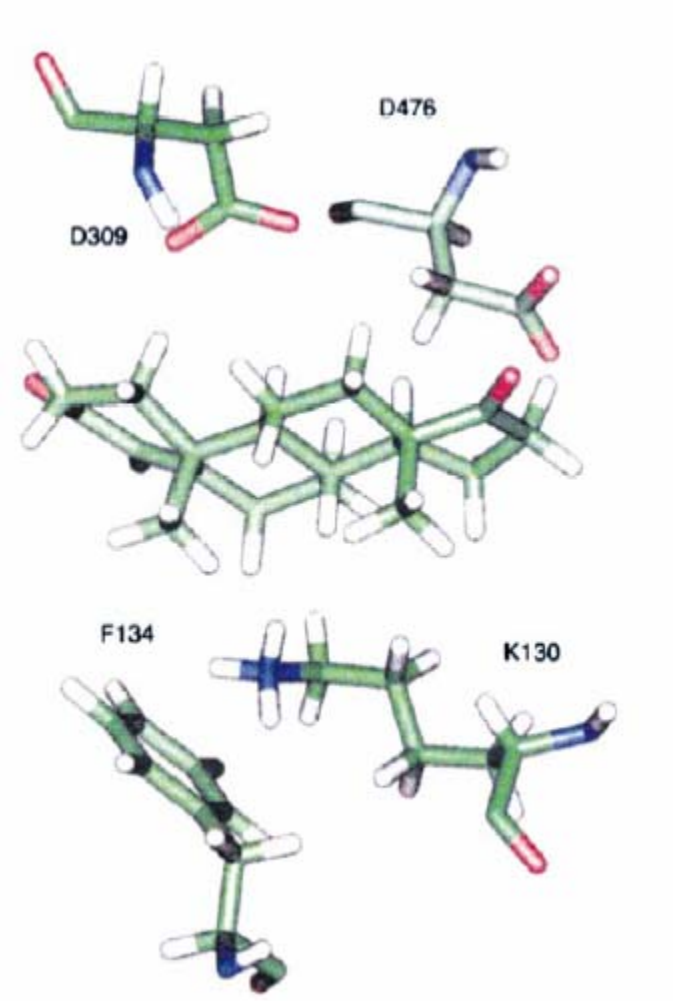

Figure 5. Positions of specific residues in the active site of human aromatase around androstenedione. (Auvray et. al., 2002)

and how a compound that is chemically similar to testosterone have a completely different phenotype. The mutated aromatase does not have activity for androstenedione and testosterone, but because of the difference in structure of nor-testosterone from the other two, it can still be aromatized. The chemical properties of testosterone derivatives can interact with aromatase in different ways. This would cause some compounds such as 6-OXO™ to appear to be steroidal in nature but because of its specific chemical structure it does not act like testosterone and will irreversibly bind aromatase. These

testosterone derivatives might have steric interactions to residues or form strong bonds to other residues like the aspartic acid residue at position 476 which when mutated causes aromatase to lose its activity (Auvray, 2002). The acidic residue might work to stabilize the steroid compound when it is in the active site of aromatase. In the pursuit of better aromatase inhibitors, knowledge of this residue gives researchers an area of focus. These mutational studies have worked to create a working knowledge of the active site in aromatase.

Besides using site directed mutagenesis researchers also have used different compounds to understand the mechanics of the active site of aromatase. The aromatase enzyme seems to have a hydrophobic binding pocket extending into the plane of the substrate. Nagaoka et al. (2003) found that elongation of an acetoxy group in a series of 4-acyloxy steroids or a methyl group in a series of 4B-alkyl steroids decreased the affinity for aromatase principally in relation to carbon number of the acyl or alkyl function. Their findings give a better understanding of the aromatase active site, and how addition of specific moieties can lead to different interactions with the aromatase enzyme. Nagaoka also reported that the binding pocket (active site) in the region of the beta position on the fourth carbon of 5-ene steroids can accommodate small lipophillic groups such as a methyl group (2003). This information can be useful in forming a more effective inhibitor of aromatase. Numazawa et al. (2003) found that a combination of 2β- and 2α - moieties are essential for the formation of a thermodynamically stable inhibitor-aromatase complex. They also found that the 2β-methyl moiety could possibly prevent the aromatase reaction due to steric hindrance. Both of these studies give possible compounds that could be used to inhibit estrogen production. The regulation of

aromatase can lead to regulation of testosterone levels. Excess levels of testosterone are usually transferred into estrogen compounds to maintain the body's homeostatic testosterone-estrogens ratio. With the inclusion of aromatase inhibitors estrogen levels might be kept at a minimum while testosterone levels might be increased.

Aromatase Inhibitors

Aromatase inhibitors are mainly used to block estrogen synthesis. Since aromatase has two areas that can be targeted, the steroid binding area and the iron-containing heme area, there have arisen two classifications of aromatase inhibitors, Type I or steroidal inhibitors and Type II or nonsteroidal inhibitors. Type I inhibitors are analogues to androgens. They work by irreversibly binding (suicide or mechanism-based inactivators) to aromatase at the substrate binding site of aromatase (Lombardi 2002). Because they are related to the C-19 androgens of the body they compete with the body's androgens. Formastane has been shown to be an effective competitive aromatase inhibitor by Wiseman et al. (1993). Examples of Type I inhibitors include testololactone, formestane, exemestane, atamestane, and plomestane. See Figure 6. The binding of these compounds to aromatase causes the enzyme to lose it catalytic properties and the only way for estrogen production to occur is for the biosynthesis of new aromatase enzymes. A subclass of Type I inhibitors that compete with the active site of aromatase are flavonoids. These plant derived compounds have been seen to inhibit the production of estrogens (Jeong, 1999; Kellis, 1984). Type II inhibitors of aromatase act by reversibly binding to the enzyme's cytochrome P450 moiety's heme group (Lombardi, 2002). These compounds are aminoglutethimide, rogletimide, fadrozole, anastrozole, and letrozole. See Figure 7.

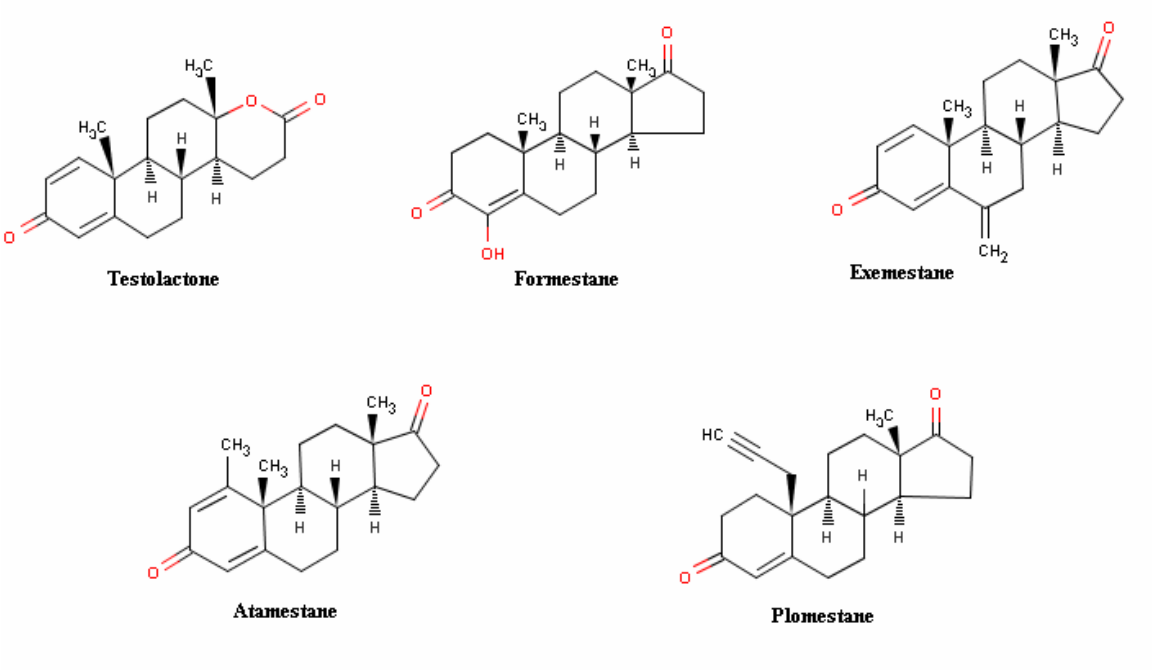

Figure 6. Chemical structures for some Type I aromatase inhibitors.

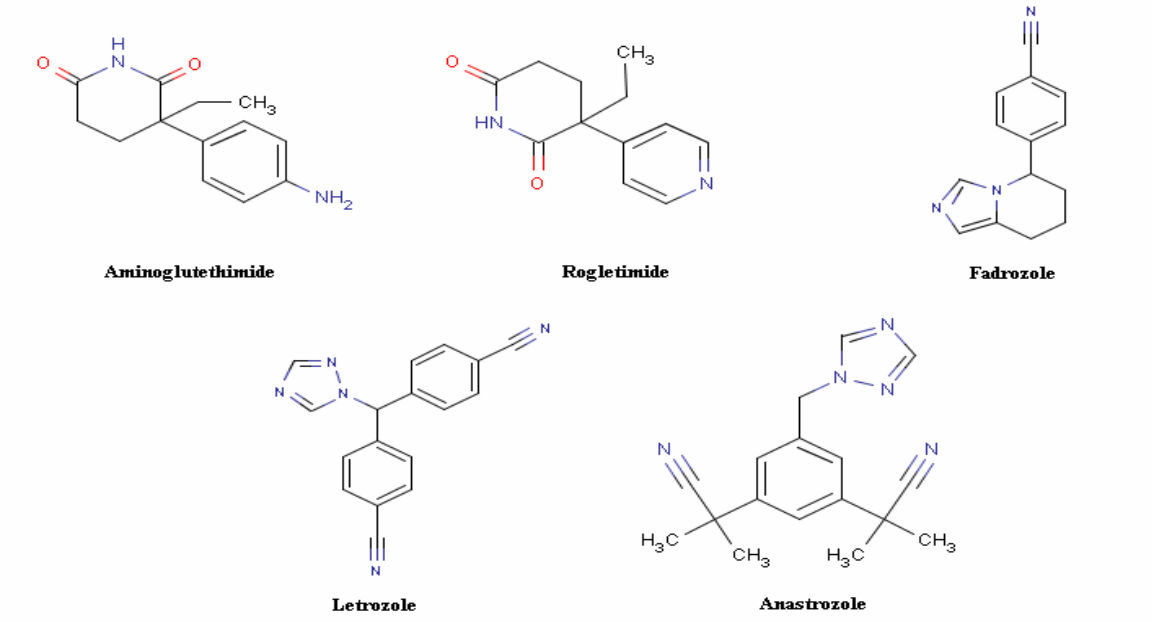

Figure 7. Chemical structure for some Type II aromatase inhibitors.

Because the Type II inhibitors bind reversibly to aromatase the enzyme can still produce estrogens without requiring new biosynthesis of aromatase. These compounds were created to help in the treatment of breast cancer in woman (Chen, 1998, 2003; Long,

1998; Choueiri, 2004). In breast cancer patients, aromatase is expressed at a higher level in breast tumors. Estrogens are thought to increase growth rates of estrogen dependent tumors by inducing peptide growth factors responsible for proliferation of cancer cells. In the beginning the first aromatase inhibitors were not very specific and would target more P450 cytochromes then just the aromatase enzyme's cytochrome. Aminoglutethimide was first used and caused profound suppression of adrenal steroid synthesis (Traina, 2004) and so using the above mentioned techniques scientists have tried to improve aromatase inhibitors. The newest aromatase inhibitors have better selectivity to aromatase. Exemestane is one of the newer Type I inhibitors. It works by irreversibly binding to aromatase and has more specificity than older aromatase inhibitors according to Lombardi (2002). As more and better inhibitors of aromatase are made to treat estrogen dependent cancers, new uses of aromatase inhibitors are being looked at in men.

Aromatase Expression in Men

Estrogens are produced by the ovary in the premenopausal woman; however, estrogen formation for men and postmenopausal women circulating estrogen levels can be attributed to peripheral tissue (Simpson, 2003). Tissues other than the gonads that synthesize estrogens are fat, muscle, brain, skin, and bone. Fat and muscle are considered to be the main the produces of estrogens because of their large mass. Adipose production of estrogen has been studied and estrogen was seen to be produced specifically by the stromal cells (Cleland, 1983; Simpson, 1983) but muscle's contribution to the circulating estrogenic steroid pool is not well understood (Larionov et al., 2003). Fat is considered a major source of estrogen production because it can

aromatize androgens (Cleland, 1983), it stores steroids (Deslypere, 1985), it is a major component of body mass, and obesity is related to estrogens levels in the blood. In men it has been seen that with obesity plasma levels of testosterone and C-19 steroid are low (Tchernof, 1995; Couillard, 2000). Using RT-PCR Larinov et al. (2003) showed that muscle does contribute to the circulating levels of estrogens. Fat cells contribute more but based on the proportion of lean body mass gives rise to the amount of estrogens produced. The formation of estrogen in muscle may have systemic physiological effects and contribute to estrogen-dependent pathological conditions in men. This had lead to the idea of using aromatase inhibitors as a possible treatment mechanism in human males. Estrogen produced in the testis may involve a negative feedback regulation of androgen biosynthesis which might be accomplished by reducing the activity of 17α hydroxylase/c17, 20-lyase (one of the enzymes responsible for androgen synthesis), or by a mitogenic role of estrogens in Sertoli and Leydig cell proliferation (Larinov 2003).

Estrogen also plays a role in diseases of the prostate. Estrogens appear to accumulate and their receptors are in a higher concentration in benign prostate hypertrophy patients (Brodie, 2001). Brodie (2001) found that in the testes of normal men aromatase immunostain was always associated with Leydig cells and not with the Sertoli cells. Aromatase activity was reported as 0.014-0.55 pmol estrogen per mg/h and the highest levels were reported in men aged 18-20 years of age. Based on these findings Brodie (2001) felt that any relationship between increased levels of estrogens with age and prostate problems comes from peripheral aromatization rather than testicular.

Aromatase Inhibitors in Males

Testosterone deficiencies in human males have lead to a number of disease states that can possibly be treated with aromatase inhibition. In young boys supplementation with aromatizable and nonaromatizable compounds significantly increased measures of whole body protein synthesis (Maruas, 1998, 2003). The results suggest that androgens have a direct effect on whole body protein pools. When given testosterone there was an increase in GH levels and when the boys were given non-aromatizable compounds like DHT the increase in GH production is not seen (Maruas, 2000). This suggests that GH levels are related to testosterone's aromatization to estrogens. When eugonadal boys were given the aromatase inhibitors, exemestane (Mauras, 2003) and anastrozole (Mauras, 2000), there was a comparable suppression of estradiol with a parallel increase in testosterone and free testosterone concentrations. The decrease in estrogen levels was accompanied by not only an increase in total and free testosterone, but also by an increase in gonadotropins. There were no changes in SHBGs and DHEAs concentrations. According to Mauras (2000, 2003) there were in changes in GH, IGFBP-3, glucose, insulin, and bone growth factors. These results show that in eugonadal boys aromatase inhibition was well tolerated and could possibly be used to treat hypogonadal disease states.

In men with low or borderline-low serum testosterone levels, aromatase inhibition was studied by Leder et al. (2004). In this study, anastrozole was used because it is a potent and selectively orally administered aromatase inhibitor. Estradiol is considered to be a crucial mediator of hormonal feedback in the pituitary and hypothalamus in men and with a decrease in estradiol levels it should cause an increase in circulating levels of

testosterone. Testosterone levels increased with aromatase inhibition as did DHT levels. Bioavailable levels of testosterone were shown to increase by more than 100% in some of the men in the groups that received anastrozole. This study showed that inhibition of aromatase caused an increase in testosterone and LH while modestly decreasing estrogen. Harden & MacLusky (2004) did a case report on a gentleman with low testosterone and found that with administration of an aromatase inhibitor caused an increase in testosterone, luteinizing hormone, and follicle-stimulating hormone. A decrease in SHBG was seen and estrogen remained low.

Some problems could arise when using a Type I aromatase inhibitor. Because Type I aromatase inhibitors are closely related to testosterone and the C-19 androgens cross reactivity with the specific quantitative methods that measure for testosterone may occur. In a study on testolactone by Cummings and colleagues (1998) the aromatase inhibitor caused an increase in androgen level. When the researchers delved deeper into the analysis of the androgens they were saw that some of the large increase was caused by the presence of testolactone. This study cautions researchers to pay close attention to cross reactivity that can occur when looking at androgen levels when using Type I aromatase inhibitors.

Aromatase Inhibition Effects on Gonadotropin Releasing Hormone and Leutinizing Hormone

Production of testosterone in the Leydig cells is directly controlled by the action of leutinizing hormone. Leutinizig hormone controls the production of testosterone through a pulsing effect. When the amplitude of LH pulses increases it causes more testosterone to be produced. The LH pulses are controlled by pulses of gonadotropin

releasing hormone that is secreted by the hypothalamus. An increased amplitude of GnRH pulses causes an increase in LH pulses which then stimulates testosterone production. With aromatase inhibition, estrogen formation is suppressed. This has given researchers the ability to look at the endocrine system of sex hormone stimulation by controlling the amount of circulating sex hormones. In a study done by Hayes et al. (2000), they looked hormone release when estradiol production is inhibited. Hayes found that there was a rise in mean LH levels with aromatase inhibition (2000). It was also seen that with GnRH blockade LH secretion remained unchanged from baseline. Hayes proposed that estradiol works to increase GnRH pulse frequency and decreases the pituitary's receptiveness to GnRH. Schnorr et al. (2001) saw that in order for testosterone to affect GnRH and LH release it needed to be aromatized. Similar results were seen by Wickman (2001) and Veldhuis (1987). Wickman was able to see the changes in LH concentrations with aromatase inhibition (2001) as was Veldhuis (1987). Therefore, when estradiol levels are decreased due to aromatase inhibition the feedback will cause testosterone to increase in the body.

Testosterone Physiological Effects in Muscle

In pubescent boys testosterone helps with maturation of muscle and bone, stimulating body and pubic hair growth, and deepening of the voice. Testosterone's role in muscle maturation and hypertrophy is unknown. However, if it actually directly affects muscle and the exact mechanism for the effects are not known. Numerous studies (Bhasin et al., 1996, 1997, 2000; Sinh-Hikim et al., 2002, 2003; Schroeder et al., 2003) have shown that when testosterone is supplemented muscle mass and strength increased. Bhasin et al. (2001) showed that testosterone supplementation increased muscle mass and

strength in a dose dependent manner. Testosterone, in humans, increases muscle mass by increasing myonuclear number (Sinh-Hikim et al., 2002, 2003), by muscle fiber hypertrophy (Sinh-Hikim et al., 2002), by an increase in protein synthesis, and by an increase in muscle satellite cell number (Sinh-Hikim et al., 2003). Testosterone supplementation and resistance training has been shown to increase lean muscle mass in HIV men who have low testosterone levels (Bhasin, 2000). In men with HIV muscle wasting is a big concern. Yet, even though it have been shown by Bhasin (2000) that exogenous testosterone and resistance training causing an increase in lean mass, the reverse is not true. For example, in men infected with the HIV virus, (Roubenoff et al., 2002), it was found that serum free testosterone did not effect the change in lean body mass and resting energy expenditure that is associated with muscle wasting. They found that even though these patients had decreased levels of free serum testosterone it was not a direct indicator of muscle catabolism (Roubenoff et al., 2002). This seems contrary to articles that discuss the action of testosterone in muscle. Based on the studies of Bhasin and Sink-Hikim, one would expect to observe a loss of LBM and strength associated with decreased concentrations of testosterone. Roy et al. (2002) concluded that decreases that are seen in strength that occur during the aging process are more based upon the age and fat-free mass and not serum levels of testosterone. As human males age hormone levels decrease along with strength and lean muscle mass (Roy, 2002). It is interesting how testosterone can increase muscle hypertrophy, but when testosterone decreases or is low there is not a direct correlation to muscle wasting. This shows that more research needs to be done on the specific action of testosterone in muscle.

Testosterone and Exercise

Testosterone release is affected by resistance exercise. Concentric and eccentric contractions have different affects on hormone release (Durand et al., 2003). In Durand's study free and total testosterone was seen to increase with their resistance training protocol, but neither concentric nor eccentric contractions alone were better at increasing the amount of testosterone in the body (2003). During a constant load, multi-exercise protocol total testosterone levels increased in response to the contractions and did not significantly increase in either, as was seen over a pre-exercise, post-exercise, and post 15 minute time period (Durand, 2003). In contrast, when testosterone levels were studied over night after heavy resistance training there was a 24% reduction in LH and 12 to 15% reduction in testosterone (Nindl, 2001). Leutinizing hormone causes the release of testosterone in the body. So a decrease in LH would cause a decrease in testosterone. Smilios (2003) recorded that there were no changes in testosterone concentrations in three different exercise protocols (2003). In another study looking at supplementation of a specific carbohydrate protein liquid on three consecutive days of resistance training, it was found that testosterone concentrations increased following exercise for both groups. The testosterone concentration then decreased to resting levels with the placebo group and fell below resting values during supplementation (Kraemer et al., 1998). There was a negative correlation between insulin and testosterone (Kraemer et al., 1998). In a study by Volek and Kraemer (1997) the effects of resistance training and nutrition on testosterone levels where studied. The found that post exercise testosterone levels increases (Volek et al., 1997) and that nutrition did not seem to affect testosterone release at all. Instead they seemed to find that testosterone levels at rest could be increased with

changes in nutrition (Volek et al., 1997). Testosterone release varies greatly with resistance exercise. More research needs to be done on the role that testosterone plays in muscle so that these inconsistencies can be explained.

Androst-4-ene-3,6,17-trione (6-OXO™).

Androst-4-ene-3,6,17-trione was first looked at by Schwarzel as a possible aromatase inhibitor (1973). Androst-4-ene-3,6,17-trione has been shown to be an irreversible aromatase inhibitor (Numazawa, 1987; Numazawa, 1996). Because Androst-4-ene-3,6,17-trione has been shown to be an aromatase inhibitor it can be inferred that the effects seen with other aromatase inhibitors will been seen with androst-4-ene-3,6,17-trione. The chemical structure of Androst-4-ene-3,6,17-trione is very similar to that of other aromatase inhibitors. See Figure 8 for examples. Androst-4-ene-3,6,17-trione has

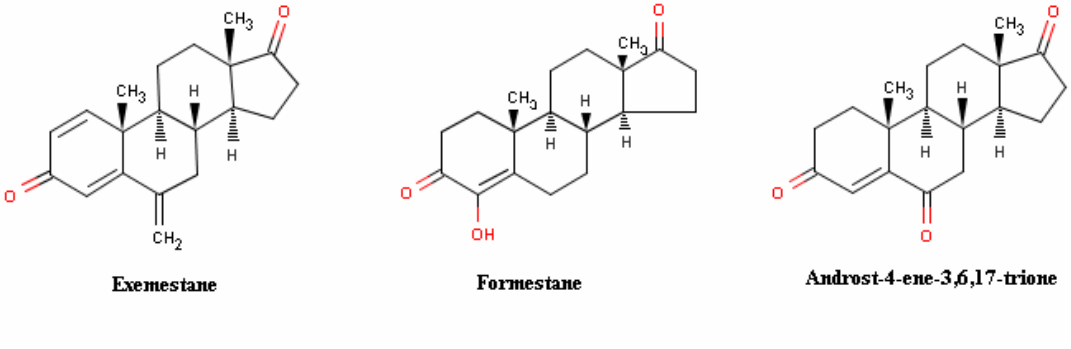

Figure 8. The chemical structures of three aromatase inhibitors.

a very similar structure to androstenedione which might cause it to completely inhibit other receptors that have an affinity to C-19 androgens, specifically 5-α-reductase. Numazawa (1987) saw that in human placenta microsomes androst-4-ene-3,6,17-trione was converted to a 3-β reduced metabolite that was prevented by 5-α-reductase. This

suggests that androst-4-ene-3,6,17-trione might compete with testosterone at certain enzymes and receptors.

In order to asses the validity of such claims a study was conducted assessing blood hormone responses over a 3-week period of 600 mg a day of androst-4-ene-3,6,17-trione (6-OXO™). The researchers found that total blood testosterone concentrations changed from baseline by 47%, while free blood testosterone increased by 126% from baseline. There were no significant changes noted for estradiol (Inclendon, 2003). In order to test the safety of 6-OXO™ supplementation serum clinical safety markers were assessed and shown to respond within normal limits (Inclendon, 2003). Yet because of the small sample size of 6 men, the power of the study is limited.

Conclusion

The endocrine system of the body is a complex arrangement. Aromatase is the enzyme that converts the C-19 androgens to the estrogenic steroids. Because many disease states are dependent on estrogen formation a family of compounds known as aromatase inhibitors has arisen. These inhibitors have been used in women to treat estrogen dependent cancers and disease states and in men to treat low testosterone levels and estrogen dependent disease states. Athletes have tried to increase their testosterone levels with anabolic steroids in order to give them a competitive edge. Exercise alone does not seem to conclusively increase testosterone so in an effort to increase their testosterone levels naturally some athletes have looked to taking aromatase inhibitors. One of the newest naturally derived aromatase inhibitor is androst-4-ene-3,6,17-trione (6-OXO™). This compound is a proven aromatase inhibitor, but not much research has

been done in healthy eugonadal male resistance trained populations that supplement with 6-OXO™.

CHAPTER THREE

Methodology

Subjects

Sixteen apparently healthy, resistance-trained, which was defined as regular resistance training for at least three years, males between the ages of 21-45 years were allowed to volunteer. All participants provided written informed consent and were cleared for participation by passing a mandatory medical screening by the laboratory nurse. Participants with contraindications to exercise as outlined by the American College of Sports Medicine (ACSM) and/or who have consumed any nutritional supplements (excluding multi-vitamins) 2 months prior to the study were not allowed to participate. All eligible subjects signed university-approved informed consent documents and approval was granted by the Institutional Review Board for Human Subjects. Additionally, all experimental procedures involved in the study conformed to the ethical consideration of the Helsinki Code. The subjects were explained the purpose of the training program, the protocol to be followed, and the experimental procedures to be used.

Baseline Testing

All supervised testing and training was conducted in the Exercise & Sport Nutrition Laboratory (ESNL) and Exercise and Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University, in Waco, TX. Venous blood samples were obtained from the antecubital

vein into a 10 ml collection tube using a standard vacutainer apparatus. Blood samples were allowed to stand at room temperature for 10 min and then centrifuged. The serum was removed and frozen at -20°C for later analysis. Urine samples were obtained in mid-stream into a 50 ml collection tube using a standard collection protocol. Urine samples were frozen at -20°C for later analysis. Blood and urine samples were obtained at week 0 and after weeks 1, 3, 8, and 11 (3-week washout period) after a 12-hour fast and standardized to the same time of day for each sample. Using a Dade Dimension clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE), Abbott Cell Dyne 3500 hematology analyzer (Abbott Laboratories, Chicago, IL), and Bayer Clinitek 200 Plus urine analyzer (Bayer Diagnostics, Tarrytown, NY), blood and urine samples were assayed for general clinical chemistry markers (i.e., glucose, total protein (Tot Pro), blood urea nitrogen (BUN), creatinine, BUN/creatinine ratio, uric acid, AST (Alanine aminotranferease), ALT (aspartate aminotransferase), CK (creatine kinase), LDH (lactate dehydrogenase), GGT (γ-Glutamyltransferase), albumin, globulin, calcium (Ca), total bilirubin (Tot Bil), alkaline phosphatase, triglycerides, cholesterol, HDL (high density lipoproteins), LDL (low density lipoproteins) while whole blood samples were assayed for standard cell blood counts with percentage differentials (i.e., hemoglobin, hematocrit, red blood cell counts (RBCC), MCV, MCH, MCHC, RDW, white blood cell counts (WBCC) (neutrophils, lymphocytes, monocytes, eosinophils, basophils). These assays helped evaluate the effects of the supplementation regimen on general markers of tissue degradation, immune function, and clinical safety.

In addition, using enzyme-linked immunoabsorbent assays (ELISA) and enzyme-immunoabsorbent assays (EIA), serum samples were also assayed for the various levels

of hormones (total and free testosterone, dihydrotestosterone, estradiol, estrione, estrone, sex hormone binding globulin, leutinizing hormone, follicle stimulating hormone, growth hormone, and cortisol) with a Wallac Victor-1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays were performed at either 405 or 450 nm wavelength against a known standard curve. Theses assays helped evaluate the effects of supplementation on the interaction between testosterone and estrogen hormones and by providing insight into aromatase enzyme activity.

During both the supplementation and washout periods the participants' resistance-training sessions and dietary intake was not supervised; however, it was required that all participants keep detailed training and dietary records and not change their routine dietary habits or level of physical activity. The principal investigator evaluated training records by examination to see if subjects were properly filling out work out cards, and dietary records were analyzed by the laboratory dietician using Food Processor Software. Additionally, mandatory weekly hemodynamic safety measurements (heart rate and blood pressure) were obtained by the laboratory nurse.

Total body mass and body composition were determined at week 0 and after weeks 1, 3, 8, and 11. Total body mass (kg) was determined on a digital weight scale. Percent body fat (PBF), fat mass (FM), fat-free mass (FFM), lean mass (LM), total body water (TBW) (total, intracellular (ICF), and extracellular (ECF)), bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) were determined using DEXA (Hologic).

Independent and Dependent Variables

Table 1 shows the general research design protocols that were administered in this study. The independent variables were the nutritional supplement 6-OXO™ , the dosage of the supplement given for each group, and the number of testing/evaluation times during the course of the study (weeks 0, 1, 3, 8, 11). Dependent variables included anthropometric variables (total body weight, percent body fat, fat mass, fat-free mass), hemodynamic variables (heart rate and blood pressure), serum hormone variables (total and free testosterone, dihydrotestosterone, estradiol, estrione, estrone, sex hormone binding globulin, leutinizing hormone, and follicle stimulating hormone), and serum and urinary clinical marker variables (glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, AST, ALT, CK, LDH, GGT, albumin, globulin, sodium, chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, HDL, LDL, hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, RDW, neutrophils, lymphocytes, monocytes, eosinophils, basophils).

Follow-Up Testing (Weeks 1, 3, 8, 11)

All participants reported back to the ESNL after 1, 3, 8, and 11 weeks of their training and supplementation regimen. During these testing sessions, the participants provided the ESNL staff with completed dietary record forms and training logs. In addition, the same clinical and body composition assessments were conducted using the same procedures as outlined previously in the baseline testing protocol. A flowchart of all the testing for the entire testing period can be seen in Table 1 below.

Upon completing the 8-week supplementation protocol, all participants underwent a three-week washout period in which the supplement was not ingested. At the end of the

washout period, all testing and assessment procedures were performed in the identical fashion as weeks 0, 1, 3, and 8.

Table 1

Flowchart for Timetable of Testing Assessments

Familiarization and Entry	Week 0	Week 1	Week 3	Week 8	Week 11 (wash-out)
Phone interview Familiarization session Informed Consent Form Demographic Form Health History Form Activity Form Dietary Form General Exam to Determine Qualifications to Participate in Study Determination of Height and Body Weight Randomization Into Groups Instructions for Supplementation Protocol	Baseline Blood Collection Baseline Urine Collection Baseline Hemodynamic Measures Baseline Anthro Measures Baseline Body Composition Baseline Diet Analysis Baseline Activity Evaluation	Blood Collection Urine Collection Hemodynamic Measures Anthro Measures Body Composition Diet Analysis Activity Evaluation	Blood Collection Urine Collection Hemodynamic Measures Anthro Measures Body Composition Diet Analysis Activity Evaluation	Blood Collection Urine Collection Hemodynamic Measures Anthro Measures Body Composition Diet Analysis Activity Evaluation	Blood Collection Urine Collection Hemodynamic Measures Anthro Measures Body Comp Diet Analysis Activity Evaluation

Training Protocol

Participants were required to participate in a 4-day per week resistance-training program split into two upper and two lower body workouts per week for a total of 8 weeks. This 8-week training protocol was periodized in 4-week increments consisting of selected exercises for the following muscle groups: chest, back, shoulders, biceps, triceps, abs, quadriceps, hamstrings, gluteals, calves, and lower back. Each exercise consists of

three sets of either 10 repetitions (weeks 1-4) or 8 repetitions (weeks 4-8) performed with as much weight as the participant can perform per set (typically 60-85% 1RM). Rest periods between exercises lasted longer than 3 minutes and the rest periods between sets lasted no longer than 2 minutes. Documentation of all training was completed in training logs as well as in conversations with an ESNL staff member on a weekly basis.

Supplementation Protocol

The supplement protocol consisted of the oral ingestion of either 300 mg/day (100 mg AM, 200 mg PM, with meals) or 600 mg/day (300 mg AM, 300 mg PM, with meals) of 6-OXO™ (ErgoPharm, Champaign, IL). For days where no exercise occurs, the supplements were ingested in the same timely fashion. After the supplementation period, a 3-week washout period was required.

Reported Side Effects from Supplements

With each weekly visit to the laboratory to meet with the research nurse for the assessment of hemodynamic safety markers, participants reported by questionnaire whether they tolerated the supplement, supplementation protocol, as well as report any medical problems/symptoms they may have encountered throughout the protocol of the study.

Control Group Demographics

Eight resistance trained males that fulfilled all requirements needed to participate in this research study were randomly selected from a placebo group in the Muscle Tech study. The Muscle Tech study was another study done to see if certain supplements increase physical performance and have positive changes in hormone levels. The Muscle

Tech was a double blind placebo controlled study. These subjects had participated in the same resistance training program during their respective supplement period. All experimental procedures including blood draws collection procedures and hormone analysis were identical to the above procedures. The blood samples collected from these subjects at weeks 0, 4, and 8 had been stored at in a -80°C freezer for a period of less than 1 year. Procedures used to store the Muscle Tech control samples were identical to those used in this study.

Methods and Materials

Dietary Inventories/Analysis

Participants recorded all food and fluid intake on dietary record forms for 4 days prior to baseline testing, week-4 testing, and week-8 testing. Dietary intake (total calories, total grams of carbohydrates, protein, and fat) was assessed using the Food Processor III Nutrition Software by a registered dietician.

Participants

Approximately 16 apparently healthy, resistance-trained, which is defined as regular resistance training for at least three years, males between the ages of 21-45 years were recruited to participate in this study. The participants were randomly assigned to one of two groups consisting of approximately eight men in each group.

Body Mass

The participant's body mass was obtained using a calibrated digital scale with a precision of $\pm$ 0.02 kg.

Blood and Urine Samples

Participants donated approximately 10 ml of fasting venous blood during each testing session. Blood samples were obtained using standard phlebotomy procedures using standard sterile venipuncture of an antecubital vein by laboratory technicians trained in phlebotomy. The phlebotomists and lab technicians wore personal protective clothing (latex gloves) when handling blood samples. Participants were seated in a phlebotomy chair and their arms were cleaned with a sterile alcohol wipe and sterile gauze. A standard rubber tourniquet was then placed on the brachium. The antecubital vein was palpitated and a 23 gauge sterile needle attached to a plastic vacutainer holder was inserted into the vein using standard procedures. Two serum separation vacutainer tube (red top) and one EDTA vacutainer tube (purple top) were inserted into the vacutainer holder for blood collection in succession using multiple sample phlebotomy techniques. Once the samples were obtained, the vacutainer holder and needle was removed. The needle was discarded as hazardous waste in a plastics sharps container. The site of the blood draw was cleaned with sterile gauze and a sterile Band-Aid was placed on the site. The blood collection tubes were labeled and placed in a test tube rack. Laboratory technicians (who have received blood borne pathogen training) centrifuged the serum samples, transferred the serum into labeled serum storage containers, and then prepared the samples for storage into a refrigerator or freezer for subsequent analysis. The serum was removed and frozen at -20°C for later analysis. Urine samples were obtained in mid-stream into a 50 ml collection tube using a standard collection protocol. Urine samples were frozen at -20°C for later analysis. Blood and urine samples were obtained at week 0 and after weeks 1, 3, 8, and 11 (3-week washout period) after a 12-

hour fast and standardized to the same time of day for each sample. Using a Dade Dimension clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE), Abbott Cell Dyne 3500 hematology analyzer (Abbott Laboratories, Chicago, IL), and Bayer Clinitek 200 Plus urine analyzer (Bayer Diagnostics, Tarrytown, NY), blood and urine samples were assayed for general clinical chemistry markers (i.e., glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, AST, ALT, CK, LDH, GGT, albumin, globulin, sodium, chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, HDL, LDL) while whole blood samples were assayed for standard cell blood counts with percentage differentials (i.e., hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, RDW, white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils). These assays helped evaluate the effects of the supplementation regimen on general markers of tissue degradation, immune function, and clinical safety.

In addition, using enzyme-linked immunoabsorbent assays (ELISA) and enzyme-immunoabsorbent assays (EIA), serum samples were also assayed for the various levels of hormones (total and free testosterone, dihydrotestosterone, estradiol, estrione, estrone, sex hormone binding globulin, leutinizing hormone, and follicle stimulating hormone) with a Wallac Victor-1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays were performed at either 405 or 450 nm wavelength against a known standard curve. Theses assays were intended to help evaluate the effects of supplementation on the interaction between testosterone and estrogen hormones and by providing insight into aromatase enzyme activity.

Total Body Water & Body Composition

Body composition was determined using a calibrated Hologic 4500W Dual-Energy X-ray Absorptiometry (DEXA) by licensed personnel with limited x-ray technology training under the supervision of Richard B. Kreider, PhD, MX. The DEXA body composition test involved having the participant lie down on their back in a standardized position in a pair of shorts and a t-shirt or a gown. A low dose of radiation scanned their entire body for approximately 7 minutes. The DEXA then segmented regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments for determination of fat, soft tissue (muscle) and bone mass. Radiation exposure from DEXA for the whole body scan was approximately 1.5mR per scan. This was similar to the amount of natural background radiation a person receives in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose was less than 5mR for the entire study.

Heart Rate and Blood Pressure

Resting heart rate was determined by palpitation of the radial artery using standard procedures. Blood pressure was assessed in the supine position after resting for 7 minutes using a mercurial sphygmomanometer using standard procedures.

Equipment

Digital Scale

Total body weight was determined using a digital scale accurate to $\pm$ 0.02 kg. The scale was calibrated by placing certified 25-kg weights and balancing the scale. Other than general instructions, special skills were not required to measure bodyweight.

Dual Energy X-Ray Absorptiometer (DEXA)

Body composition measurements were determined by qualified personnel (in compliance with state regulations) using a Hologic 4500W dual energy x-ray Absorptiometer (*Waltman, MA*). This system segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments (i.e. bone mass, fat mass, and fat free/soft tissue mass). Quality control (QC) calibration procedures were performed on a spine phantom (*Hologic X-CALIBER Model DPA/QDR-1 anthropometric spine phantom*) daily. In addition, weekly calibration procedures were performed on a density step calibration phantom.

Mercurial Sphygmomanometer

Blood pressure was assessed by auscultation of the brachial artery using a mercurial sphygmomanometer using standard clinical procedures.

Clinical Blood Analyzers

Dade Dimension clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE), Abbott Cell Dyne 3500 hematology analyzer (Abbott Laboratories, Chicago, IL), and Bayer Clinitek 200 Plus urine analyzer (Bayer Diagnostics, Tarrytown, NY), blood and urine samples were assayed for general clinical chemistry markers (i.e., glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, AST, ALT, CK, LDH, GGT, albumin, globulin, sodium, chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, HDL, LDL) while whole blood samples were assayed for standard cell blood counts with percentage differentials (i.e., hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, RDW, white blood

cell counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils. The above mentioned equipment were used to analyze the blood and urine obtained.

Hormone Analysis

Enzyme-linked immunoabsorbent assays (ELISA) and enzyme-immunoabsorbent assays (EIA), serum samples will also be assayed for the various levels of hormones (total and free testosterone, dihydrotestosterone, estradiol, estrione, estrone, sex hormone binding globulin, leutinizing hormone, follicle stimulating hormone, growth hormone, and cortisol) with a Wallac Victor-1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at either 405 or 450 nm wavelength against a known standard curve. The assays were done with kits obtained from DSLabs (Webster, TX) and Alpco Diagnostics (Windham, NH).

Data Analysis

Statistical Analysis

Statistical analyses were performed by utilizing separate 2 x 5 [Group (300 mg, 600 mg] x Test (wks 0, 1, 3, 8, 11) factorial multivariate analyses of variance (MANOVA). Box M tests were performed to test for differences in covariance matrices. Bartlett's Test of Sphericity were performed to test that the variance/covariance matrix of the dependent variables is circular in form which will allow for accurate interpretation of univariate ANOVAs. Levene's Test of Equality of Error were performed to test for equality of variance for each dependent variable. Further analysis of the main effects for Group and Test were performed by separate one-way ANOVAs and using Sidak pairwise comparisons. Significant between-group differences were then determined by involving

the Tukey HSD Post Hoc Test when the variances are equal and a Games-Howell Post Hoc Test was used when the variances are not equal. The changes from post- to pre-training for each criterion variable was then analyzed with a one-way ANOVA, Sidak pairwise comparison, and between-group differences were determined with a Tukey HSD Post Hoc Test when the variances are equal, and a Games-Howell Post Hoc Test will be used when the variances are not equal. All statistical procedures were performed using SPSS 13.0 software and a probability level of < 0.05 will be adopted throughout. The same statistically measures were applied to the control group and a 3x5 [Group (300 mg, 600 mg, Control] x Time Points (1, 2) factorial multivariate analyses of variance (MANOVA).

CHAPTER FOUR

Results

The subjects were all male with mean age of 26.6 ± 4.9 years, height of 180.2 ± 6.3 cm, and body weight of 87.3 ± 13.2 kg. All subjects were healthy, trained (regular resistance training for at least three years) individuals. All 16 subjects were able to complete the required dosing regimen, training protocols, and testing procedures with little to no side effects. The subjects all complied with the work out regimen and supplement protocol. There were also no significant differences in dietary intake over the course of the study ($p>0.05$).

Whole Blood Clinical Safety Markers

Whole blood clinical safety markers were measured from blood samples obtained 5 times over the course of the 11-week study. Table 2 shows the measured mean values, including the standard deviations (±SD), for these markers. The results indicate that there were no significant differences in whole blood clinical safety markers over the course of the study ($p>0.01$). However, the results did show that the 600 mg group had higher baselines values for hematocrit ($p=0.030$), absolute monocytes ($p=0.027$), and absolute basophils ($p=0.005$), than the 300 mg group that persisted throughout the study. This led us to accept the null hypothesis that supplementation does not adversely change safety markers from baseline.

Serum Clinical Safety Markers

Serum clinical safety markers were measured from blood samples obtained 5 times over the course of the 11-week study. Table 3 contains the mean ±SD values for those measures. Results showed that no significant differences were located for any of the serum clinical safety markers over the course of the study ($p > 0.01$). However, the 600 mg group was shown to have significantly higher baseline values for total cholesterol ($p=0.001$), low density lipoprotein ($p=0.01$), blood urea nitrogen ($p=.016$), GGT, calcium, total blood protein, and albumin ($p=0.000$) than the 300 mg group that persisted throughout the study. This led us to accept the null hypothesis that supplementation does not adversely change safety markers from baseline.

Hemodynamic Clinical Safety Markers

At each testing session subjects had their heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) determined in order to assess the hemodynamic safety of supplementation with 6OXO™. Table 4 contains the mean values of the hemodynamic measures ± SD. There were no significant differences in hemodynamic measures over the course of the study ($p>0.01$). However, the results did show that the 300 mg group had statistically significant higher baseline SBP than the 600 mg group ($p=0.041$) that persisted throughout the study. This led us to accept the null hypothesis that supplementation does not adversely change safety markers from baseline.

Urine Clinical Safety Markers

Each subject also submitted a urine sample analyzed, at each of the 5 testing sessions for 11 weeks, for changes in clinical safety markers that could be construed as

Table 2

Whole Blood Clinical Chemistry Markers

	Group 1 (300 mg)					Group 2 (600 mg)				
Week	0	1	3	8	11	0	1	3	8	11
WBCC (K/µL)	5.3±1.6	5.0±1.1	5.3±1.7	5.2±2.0	4.5±1.5	5.5±.96	4.6±.91	5.0±1.0	4.9±.67	5.1±.84
RBCC (M/µL)	5.0±.51	5.0±.50	4.6±.84	5.0±.37	4.9±.46	5.2±.37	5.1±.33	5.2±.24	4.9±1.2	5.3±.30
Hemoglobin (g/dL)	15±1.1	15±1.1	15±1.2	15±.59	15±1.0	15±.95	15±.93	15±.63	15±.66	15±.78
Hematocrit (%)	44±3.5	44±3.7	44±3.6	45±2.4	43±3.3	46±3.0*	44±2.3*	45±1.9*	46±2.1*	46±2.7*
MCV (fL)	88±2.8	88±2.8	88±2.4	89±2.4	89±2.9	87±2.7	86±2.8	87±2.6	87±2.3	87±2.5
MCH (pg)	30±1.2	30±1.3	30±1.0	30±1.3	30±1.0	29±1.0	29±.95	29±.98	29±1.1	29±.78
MCHC (g/dL)	34±.69	34±.76	34±.64	33±.78	34±.81	33±.60	34±.85	33±.56	33±.61	34±.60
Neutrophils	2.9±1.0	2.5±.43	2.9±1.1	2.8±1.0	2.3±1.1	2.8±.52	2.4±.47	2.6±.66	2.5±.29	2.9±1.1
Lymphocytes	1.7±.51	1.9±.84	1.7±.62	1.8±.87	1.6±.69	2.0±.39	1.6±.39	1.8±.50	1.7±.44	1.8±.44
Monocytes	.45±.12	.39±.12	.44±.12	.36±.07	.37±.14	.43±.14*	.38±.04*	.39±.13*	.40±.09*	.44±.12*
Eosinophils	.12±.06	.13±.08	.11±.05	.12±.06	.12±.06	.14±.08	.14±.09	.16±.10	.16±.09	.16±.07
Basophils	.06±.02	.05±.01	.05±.01	.05±.01	.05±.02	.07±.02*	.06±.02*	.07±.02*	.07±.01*	.06±.01*

Note: * denotes significant main effects for groups

Table 3

Serum Clinical Chemistry Markers

	Group 1 (300 mg)					Group 2 (600 mg)				
Week	0	1	3	8	11	0	1	3	8	11
Triglyceride (mg/dl)	86.8±29.5	98.5±46.1	105.3±29.5	110.3±58.8	85.9±41.9	117.5±60.2	115.5±50.5	116.6±40.5	110.9±43.4	97.8±36.1
Cholesterol (mg/dl)	177.0±38.2	182.1±26.1	173.9±32.2	175.0±22.6	166.1±20.7	194.6±24.9*	185.1±22.3*	201.1±20.8*	201.6±37.8*	199.5±31.0*
HDL (mg/dL)	50.1±12.5	51.3±10.9	53.6±17.6	49.4±7.9	48.3±8.9	53.0±4.7	53.8±9.8	58.5±11.9	53.9±18.7	57.0±18.3
LDL (mg/dL)	107.9±26.9	112.0±18.8	105.4±23.5	104.4±22.8	100.6±16.8	115.9±21.5*	111.4±21.7*	124.8±22.0*	127.1±33.5*	121.4±25.2*
GGT (U/L)	23.9±5.7	23.8±4.6	23.1±4.8	27.3±7.9	26.0±7.0	32.3±9.6*	33.6±11.2*	33.6±7.8*	38.8±11.8*	36.4±8.3*
LDH (U/L)	127.0±19.4	130.6±23.3	125.4±19.6	114.5±25.8	130.0±33.0	125.5±17.4	128.0±18.0	132.8±23.4	132.1±24.6	140.6±32.6
Uric Acid (g/dl)	6.1±1.7	5.8±1.1	5.7±0.9	5.2±1.0	5.5±1.4	6.1±1.3	6.1±1.6	6.5±1.2	5.7±1.0	5.7±1.3
Glucose mg/dl	94.5±9.4	95.8±10.3	97.6±10.4	94.0±8.2	93.9±9.6	95.1±8.4	94.3±6.5	100.5±6.1	105.1±17.1	89.9±14.8
BUN (mg/dL)	16.9±2.9	19.4±5.0	20.6±4.5	18.5±3.0	19.4±3.6	21.1±7.6*	22.3±5.1*	22.5±3.8*	19.8±3.5*	21.3±2.4*
Creatinine (mg/dl)	1.4±0.1	1.4±0.2	1.4±0.2	1.3±0.2	1.3±0.3	1.4±0.2	1.4±0.2	1.5±0.2	1.5±0.2	1.3±0.3
Ca (mg/dl)	12.5±1.7	14.2±3.2	14.8±3.0	14.9±2.1	15.2±3.4	15.6±4.4*	16.4±3.4*	14.9±2.8*	13.7±2.6*	17.0±3.0*
Tot. Prot. g/dL	9.8±0.5	9.9±0.4	9.9±0.6	9.8±0.5	9.5±0.4	10.1±0.6*	10.1±0.7*	10.8±0.7*	10.4±0.7*	10.2±0.9*
Albumin g/dL	7.4±0.6	7.7±0.4	7.8±0.6	7.4±0.5	7.3±0.5	7.9±0.9*	8.0±0.9*	8.5±1.0*	8.3±0.9*	8.1±0.9*
Tot Bil (mg/dl)	4.7±0.5	4.8±0.4	4.9±0.5	4.6±0.5	4.6±0.3	5.0±0.5	5.1±0.5	5.4±0.6	5.3±0.6	5.1±0.6
ALP (U/L)	0.8±0.3	0.8±0.6	1.0±0.4	0.9±0.5	0.9±0.6	0.6±0.3	0.6±0.2	0.9±0.4	0.8±0.3	0.8±0.2
AST (U/L)	73.9±13.3	76.3±12.1	73.1±12.8	70.5±15.7	64.0±19.9	77.1±21.3	76.1±21.0	79.5±19.4	76.4±19.0	63.1±27.8
ALT (U/L)	24.4±9.6	24.5±9.3	21.3±6.8	18.3±6.6	30.3±25.1	22.6±6.5	27.3±5.5	30.0±11.7	24.5±8.0	26.3±10.0
CK (U/L)	28.6±11.5	25.6±5.5	22.4±7.2	28.4±10.8	27.1±5.5	28.1±14.2	25.0±7.2	23.8±6.7	28.9±15.6	32.6±10.7

Note: * denotes significant main effects for groups

toxicity of the supplement. Table 5 contains the mean values ± SD for urine clinical safety markers. Most of the urine safety markers are measured as to whether or not they are present. Therefore a zero reading indicated that there were no traceable amounts of marker of interest present. Readings that were above a zero reading indicated a trace amount or a range of concentrations that were indicated by numbers: 1, 2, and 3. There were no significant differences for urine clinical safety markers ($p>0.01$). This led us to reject the null hypothesis that supplementation adversely affects urine safety markers.

Table 4

Hemodynamic Clinical Safety Markers

Group 1 (300 mg)					
Week	0	1	3	8	11
Heart Rate (bpm)	64±7.6	58±4.7	61±5.1	61±5.6	64±8.7
SBP (mmHg)	116±13.0*	114±9.8*	117±12.3*	117±10.7*	116±8.8*
DBP (mmHg)	109±6.4	107±5.2	113±6.9	115±11.4	114±4.8
Group 2 (600 mg)					
Week	0	1	3	8	11
Heart Rate (bpm)	57±6.2	58±6.7	59±7.2	61±3.0	64±8.6
SBP (mmHg)	75±8.7	74±9.3	73±8.1	70±7.9	77±5.6
DBP (mmHg)	69±8.2	74±4.5	71±9.4	76±9.9	74±6.3

Note: * denotes significant main effects for groups

Anthropometric Measures

At each of the 5 testing session, subjects had anthropometric measures obtained that consisted of BIA and DEXA variables. Table 6 displays the means ± SD values of each anthropometric measure. Results showed that over the course of the study there

Table 5

Urine Clinical Safety Markers

Group 1 (300 mg)					
Week	0	1	3	8	11
Presence in Urine of					
Glucose	0±0	0±0	0±0	0±0	0±0
Ketones	0±0	0±0	0±0	0±0	0±0
Blood	0±0	0±0	0±0	0±0	0±0
Protein	0±0	0±0	0±0	0±0	0±0
Nitrite	0±0	0±0	0±0	0±0	0±0
Bilirubin	0±0	0±0	0±0	0±0	0±0
Leukocytes	0±0	0±0	0±0	0±0	0±0
Other Markers					
Specific Gravity	1.02±0.01	1.02±0.01	1.01±0.01	1.02±0.01	1.02±0.01
pH	5.5±0.76	5.6±0.58	5.9±0.90	5.4±0.68	5.25±0.53
Urobilinogen (E.U./dL)	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0

Group 2 (600 mg)					
Week	0	1	3	8	11
Presence in Urine of					
Glucose	0±0	0±0	0±0	0±0	0±0
Ketones	0±0	0±0	0±0	0±0	0±0
Blood	0±0	0±0	0±0	0±0	0±0
Protein	0±0	0±0	0±0	0±0	0±0
Nitrite	0±0	0±0	0±0	0±0	0±0
Bilirubin	0±0	0±0	0±0	0±0	0±0
Leukocytes	0±0	0±0	0±0	0±0	0±0
Other Markers					
Specific Gravity	1.02±0.01	1.02±0.01	1.02±0.01	1.02±0.01	1.02±0.01
pH	5.8±1.13	5.8±1.13	5.8±1.13	5.8±1.13	5.8±1.13
Urobilinogen (E.U./dL)	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0

Note: * denotes significant main effects for groups

Table 6

Anthropometric Measures

Group 1 (300 mg)					
Week	0	1	3	8	11
BIA					
ECF (L)	19.6±2.5	19.9±3.1	19.6±2.4	19.5±2.3	20.0±2.5
ICF (L)	30.0±5.1	30.3±5.3	30.0±5.0	29.2±4.2	29.9±4.8
TBW (L)	49.5±7.4	50.2±8.1	49.6±7.2	48.7±6.2	49.9±7.0
ECF/ICF	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1
BCM (kg)	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
ECF TBW (L)	42.8±7.3	43.2±7.6	42.8±7.2	41.8±6.1	42.7±6.8
FFM (kg)	67.2±10.3	68.0±11.2	67.3±10.0	66.0±8.6	67.6±9.8
BFP	22.0±4.6	21.1±2.9	21.6±3.7	23.0±5.4	21.2±3.6
DEXA					
BMA (cm2)	2,136.3±138.1	2,174.1±157.7	2,174.6±126.4	2,164.2±127.1	2,162.3±161.8
BMC (g)	2,638.2±285.9	2,657.3±296.2	2,666.6±246.5	2,646.7±258.4	2,670.6±302.2
BMD (g/cm2)	1.2±0.1	1.2±0.1	1.2±0.1	1.2±0.1	1.2±0.1
FM (kg)	12.2±6.3	12.4±6.2	12.5±6.2	13.0±5.5	13.0±5.8
LM (kg)	64.5±7.7	64.6±8.0	64.1±7.5	63.9±8.7	64.0±8.1
FFM (kg)	67.1±7.9	67.2±8.3	66.8±7.7	66.5±8.9	66.4±8.6
Total Mass (kg)	79.3±13.2	79.6±13.7	79.3±12.9	79.5±13.0	78.8±13.8
BFP	14.7±5.4	14.9±5.2	15.1±5.3	15.9±4.7	15.8±4.9

Group 2 (600 mg)					
Week	0	1	3	8	11
BIA					
ECF (L)	19.8±3.1	20.2±3.3	20.0±2.7	20.2±3.3	21.2±3.1
ICF (L)	29.5±6.2	29.7±5.0	29.9±4.5	30.6±6.4	32.5±5.8
TBW (L)	49.3±9.2	49.8±8.2	49.9±7.2	50.8±9.6	53.6±8.7
ECF/ICF	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.1
BCM (kg)	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
ECF TBW (L)	42.1±8.8	42.4±7.1	42.8±6.5	43.7±9.2	46.4±8.3
FFM (kg)	66.7±12.8	67.4±11.1	67.7±9.8	68.9±13.3	72.8±12.0
BFP	24.6±4.8	23.5±3.4	23.6±3.5	22.5±7.6	18.1±6.6
DEXA					
BMA (cm2)	2,129.9±270.8	2,179.6±250.6	2,167.3±240.2	2,172.6±257.8	1,935.1±739.0
BMC (g)	2,845.6±534.0	2,882.8±522.8	2,859.1±500.8	2,866.9±515.4	2,873.8±526.3
BMD (g/cm2)	1.3±0.1*	1.3±0.1*	1.3±0.2*	1.3±0.1*	1.3±0.1*
FM (kg)	12.1±3.5	12.5±3.3	12.4±3.5	12.1±3.2	12.6±3.4
LM (kg)	66.2±11.6	66.1±11.9	66.0±10.7	62.9±13.1	67.2±12.7
FFM (kg)	69.0±12.1	68.9±12.4	68.8±11.1	69.9±12.0	70.1±13.2
Total Mass (kg)	81.1±13.3	81.5±13.8	81.3±12.8	82.0±13.5	82.7±14.7
BFP	15.0±4.2	15.4±4.0	15.3±3.9	14.8±3.6	15.3±3.8

Note: * denotes significant main effects for groups

were no significant changes in any of the measured variables ($p>0.01$). However, the 600 mg group did have significantly higher Bone Mineral Density (BMD) at baseline than the 300 mg group ($p=0.001$) that persisted throughout the study. This led us to accept the null hypothesis.

Serum Hormones

The serum blood samples taken at each testing session were used to measure a panel of hormones to see if any changes occurred with supplementation. The hormones measured were total testosterone (TT), free testosterone (FT), estradiol, estriol, estrone, sex hormone binding globulin (SHBG), leuteinizing hormone (LH), follicle stimulating hormone (FSH), dihydrotestosterone (DHT), cortisol, and growth hormone (GH). The two groups consisted of two dosage amounts to observe whether or not 6OXO™ had dosage dependent effects on hormone levels. Table 7 shows the mean values ± SD of all the hormones over the testing sessions. There were significant differences seen between dosage groups ($p=0.000$) and between testing sessions ($p=0.023$). There were no significant differences, however, for the interaction of dosage and testing session ($p>0.05$). It was observed that the 600 mg group had lower levels of SHBG ($p=0.022$) and LH ($p=0.000$), and higher levels of FSH levels ($p=0.014$) with a possible trend towards higher Cortisol levels ($p=0.053$).

The testing sessions themselves showed trends and significant differences. Total testosterone showed a possible trend towards higher total testosterone concentration in testing session 1 compared to baseline ($p=0.078$) with another possible trend towards a higher testosterone concentration in testing session 2 compared to baseline ($p=0.069$) (Figure 9). Free testosterone showed a possible trend in which testing sessions 1 and 2

had higher free testosterone concentrations than baseline (p=0.084 and 0.079, respectively) (Figure 10). Estrone was significantly higher at testing session 1 compared to baseline (p=0.027) and a trend towards being higher at testing session 2 compared to baseline (p=0.053) (Figure 11). DHT concentrations showed significant differences from baseline in testing session 1 (p=0.000), testing session 2 (p=0.017), and in testing session 3 (p=0.004) (Figure 12).

Changes in Serum Hormones After Baseline Subtraction

In order to account for any effects of difference in baseline, the testing sessions were adjusted by subtracting baseline hormone concentration from testing session hormone concentrations. The differences from baseline were then analyzed for significant differences and labeled as time points relative to baseline (baseline as time point 0). Table 8 contains the mean values ± SD of the change in hormone levels from baseline. When expressing the serum hormone values relative to baseline, it was observed that the main effects for dosage group (p=0.007) and testing session (p=0.009) were significantly different. However, there were no significant differences for the interaction of the two independent variables (p>0.05). When testing dosage group main effects, it was found that the 600 mg group had higher Estrone levels (p=0.018), but lower SHBG levels (p=0.001) than the 300 mg group. When testing session main effects were analyzed it was seen that for change in total testosterone levels from baseline, time point 4 levels were significantly lower than time points 1 and 2 (p=0.041 and p=0.034, respectively) (Figure 13). Change in free testosterone concentrations from baseline was shown to be significantly lower for time point 4 when compared to time points 1 and 2 (p=0.016 and p=0.041, respectively), and was seen to have a possible trend of being

Table 7

Serum Hormone Levels

Group 1 (300 mg)					
Week	0	1	3	8	11
TT (ng/mL)	4.72±2.14	6.15±1.38	5.95±2.04	5.82±2.13	5.00±1.00
FT (pg/mL)	17.16±8.80	37.07±33.03	30.13±17.28	30.53±16.78	18.21±10.68
Estradiol (pg/mL)	89.64±86.19	121.20±125.60	112.34±124.22	107.12±106.63	101.77±103.78
Estriol (ng/mL)	0.04±0.03	0.04±0.04	0.05±0.07	0.05±0.06	0.06±0.05
Estrone (pg/mL)	334.10±89.50	428.79±122.41	400.86±135.43	397.19±166.06	323.45±89.27
SHBG (nmol/L)	128.96±36.68	135.72±33.53	127.39±37.26	137.04±42.54	132.45±43.52
LH(mIU/mL)	6.64±4.22	8.97±5.61	6.02±3.18	6.26±3.02	5.43±2.39
FSH (mIU/mL)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DHT (ng/mL)	0.94±0.38	3.12±1.75	2.76±1.44	2.39±1.33	1.00±0.32
Cortisol (ug/dL)	23.04±7.06	24.33±7.31	23.59±4.06	19.48±8.95	22.42±5.00
GH (pg/mL)	6.94±9.65	59.71±150.74	2.42±4.07	12.20±14.22	107.61±284.38
Group 2 (600 mg)					
Week	0	1	3	8	11
TT (ng/mL)	3.98±1.24	5.58±1.50	5.84±1.30	5.44±1.97	4.49±1.06
FT (pg/mL)	22.66±13.00	39.16±13.18	45.05±27.00	40.61±36.11	22.43±9.82
Estradiol (pg/mL)	49.12±23.64	71.38±35.88	145.51±219.39	75.76±47.99	78.30±54.54
Estriol (ng/mL)	0.03±0.04	0.04±0.05	0.04±0.03	0.05±0.03	0.04±0.02
Estrone (pg/mL)	268.78±43.47	425.28±137.04	431.69±141.66	365.56±92.00	308.75±101.61
SHBG (nmol/L)	130.57±26.61*	119.51±32.57*	106.87±17.64*	101.40±29.48*	112.92±35.81*
LH(mIU/mL)	3.22±1.77*	4.48±2.07*	4.27±1.97*	4.10±2.55*	3.85±2.58*
FSH (mIU/mL)	0.12±0.22*	0.09±0.25*	0.03±0.07*	0.08±0.23*	0.19±0.39*
DHT (ng/mL)	0.93±0.20	3.01±1.34	4.37±3.75	2.79±1.72	1.68±1.15
Cortisol (ug/dL)	24.47±6.47	25.39±4.08	25.51±3.56	26.32±8.14	24.85±4.96
GH (pg/mL)	56.77±114.14	49.66±72.05	119.42±306.23	33.39±43.73	165.83±254.07

Note: * denotes significant main effects for groups

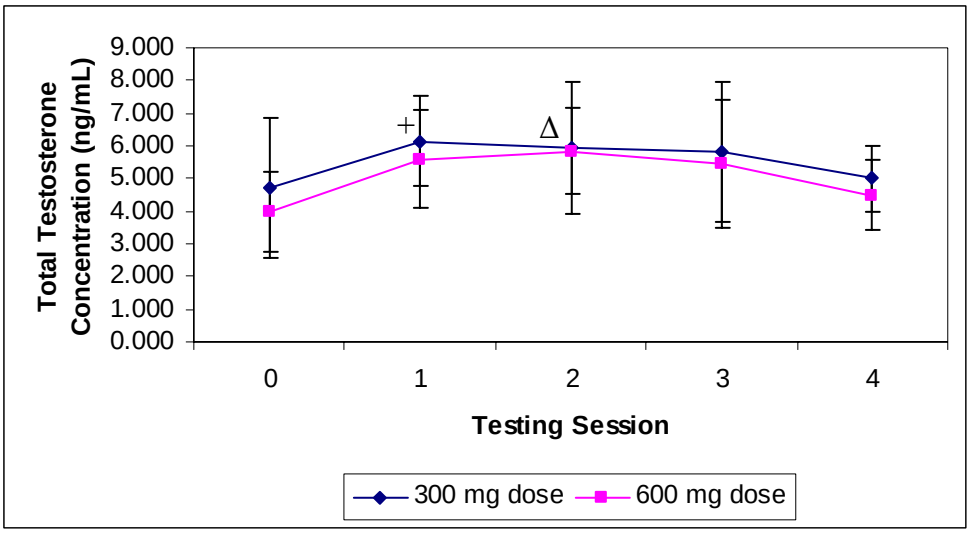

Figure 9. Serum Total Testosterone Concentration Progression. Total testosterone was measured by enzyme immunoassay from serum samples obtained. Testing session 1 when compared to baseline showed a possible trend of having higher concentrations (p=0.078), denoted by a +, that were independent of any group effects. Testing session 2 compared to baseline showed a possible trend with higher testosterone concentrations (p=0.069), denoted by a Δ, that was independent of any group effects.

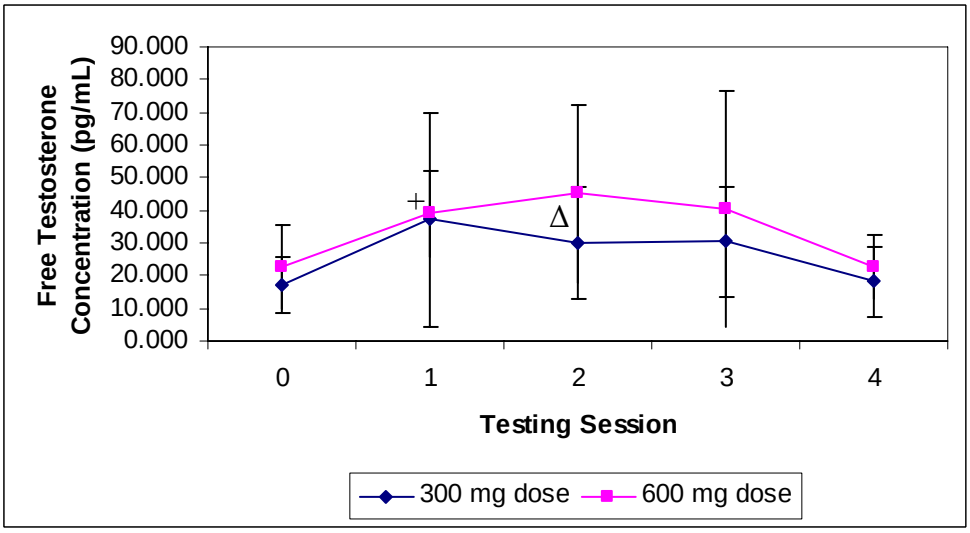

Figure 10. Serum Free Testosterone Concentration Progression. Free testosterone was measured by enzyme immunoassay from serum samples obtained. Testing session 1 when compared to baseline showed a possible trend of having higher concentrations (p=0.084), denoted by a +, that were independent of any group effects. Testing session 2 compared to baseline showed a possible trend with higher free testosterone concentrations (p=0.079), denoted by a Δ, that was independent of any group effects.

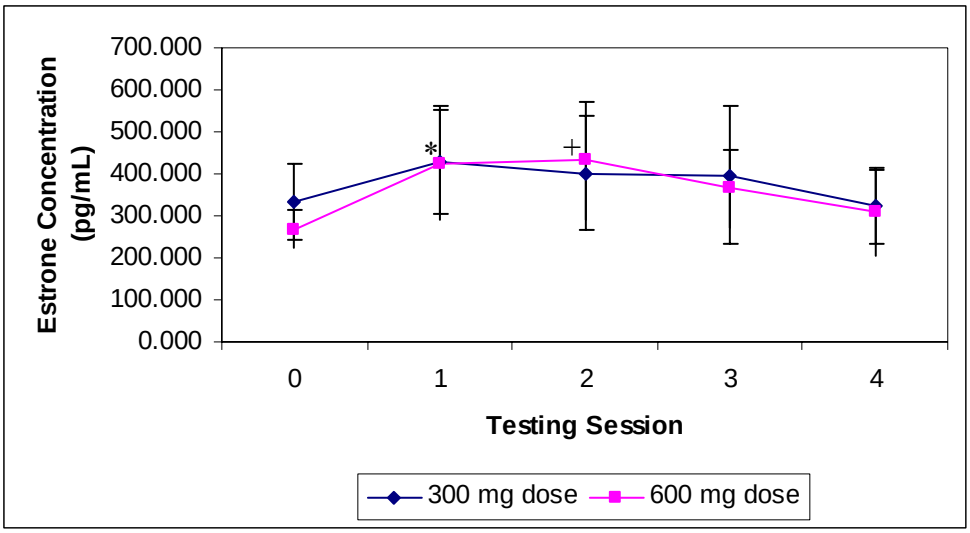

Figure 11. Serum Estrone Concentration Progression. Estrone was measured by enzyme immunoassay from serum samples taken at each testing session. Testing session 1 was significantly higher in estrone concentrations than baseline (p=0.0027), denoted by an *, which were independent of any group effects. Testing session 2 compared to baseline showed a possible trend with higher estrone concentrations (p=0.079), denoted by a +, that was independent of any group effects.

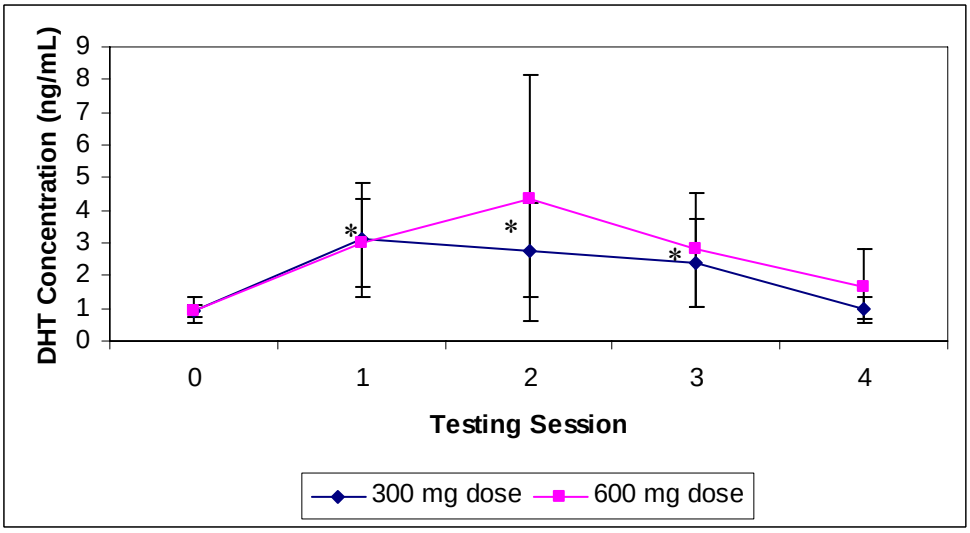

Figure 12. Serum DHT Concentration Progression. DHT was measured by enzyme immunoassay from serum samples taken at each testing session. Testing session 1, 2, and 3 were significantly higher in DHT concentrations than baseline (p=0.000 and p=0.017 and p=0.004, respectively), denoted by an *, which were independent of any group effects.

lower than time point 3 (p=0.051) (Figure 14). Estrone concentrations were seen to be statistically higher in testing session time point 1 (p=0.014) and time point 2 (p=0.031) as compared to time point 4 (Figure 15). There were significantly lower levels of DHT concentrations for time point 4 when compared to time points 1 and 2 (p=0.048 and p=0.006, respectively) (Figure 16). This led us to accept our null hypothesis.

Supplement Serum Hormone Changes versus a Control Group

These changes and trends that were observed during the supplement protocol and washout period were not easily elucidated, and the question arises as to whether or not these changes can be attributed to solely the 6OXO^{TM} regimen. To piece out this hypothesis a small sample (n=8), whom had participated in the same exercise training protocol for the same length as the supplement regimen (8 weeks) had their serum concentrations of certain hormones measured to see if they were significantly different from the supplemental groups. Preliminary statistical analysis comparing the hormone panel of total testosterone, free testosterone, estriol, SHBG, LH, and cortisol had some baseline measures that were statistically different from the other baseline measures in the supplement groups (data not shown). Therefore baseline measures were subtracted from the other measures to account for this difference, and the changes from baseline were than analyzed. Testing sessions have been relabeled as time points 1 and 2 with baseline representing time point 0. Table 9 contains the mean concentrations of each hormone measure ± SD.

The results showed that there was a significant difference in hormone levels between groups (p=0.001), but there was no difference over time points (p>0.05). Total testosterone was seen to be significantly higher in both dosage groups (300 mg p=0.002, 600 mg p=0.000) as compared to control (Figure 17). The serum concentrations of free

Table 8

Serum Hormones Concentrations After Baseline Subtraction

	Group 1 (300 mg)				Group 2 (600 mg)			
Time Point	1	3	3	4	1	3	3	4
TT (ng/mL)	1.43±1.56	1.23±1.59	1.09±0.93	0.28±1.40	1.60±0.56	1.86±0.82	1.46±1.06	0.51±0.92
FT (pg/mL)	19.91±25.85	12.97±9.89	13.37±9.73	1.05±4.07	16.49±11.59	22.39±29.87	17.95±27.46	-0.23±12.45
Estradiol (pg/mL)	31.57±46.95	22.70±45.51	17.48±27.12	12.13±24.55	22.26±22.74	96.39±216.81	26.64±32.44	29.18±47.77
Estriol (ng/mL)	-0.01±0.02	0.00±0.05	0.01±0.04	0.01±0.04	0.00±0.04	0.00±0.03	0.01±0.03	0.01±0.04
Estrone (pg/mL)	94.7±75.52	66.8±84.45	63.1±124.46	-10.7±74.25	156.5±112.80*	162.9±128.93*	96.8±80.72*	40.0±99.99*
SHBG (nmol/L)	6.8±18.12	-1.6±36.12	8.1±39.91	3.5±38.04	-11.1±25.23*	-23.7±22.63*	-29.2±26.49*	-17.7±17.62*
LH(mIU/mL)	2.33±4.43	-0.62±4.19	-0.39±3.77	-1.22±2.99	1.26±1.87	1.05±1.78	0.88±2.07	0.63±2.41
FSH (mIU/mL)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-0.03±0.20	-0.09±0.25	-0.04±0.35	0.07±0.50
DHT (ng/mL)	2.18±1.84	1.82±1.42	1.45±1.21	0.06±0.26	2.09±1.31	3.44±3.75	1.87±1.70	0.76±1.12
Cortisol (ug/dL)	1.30±4.32	0.55±6.50	-3.55±8.04	-0.62±8.12	0.92±6.28	1.04±6.70	1.85±7.92	0.37±4.68
GH (pg/mL)	52.77±145.7	-4.51±7.1	5.26±17.2	100.68±279.1	-7.12±84.5	62.65±327.2	-23.39±119.7	109.06±246.6

Note: * denotes significant main effects for groups

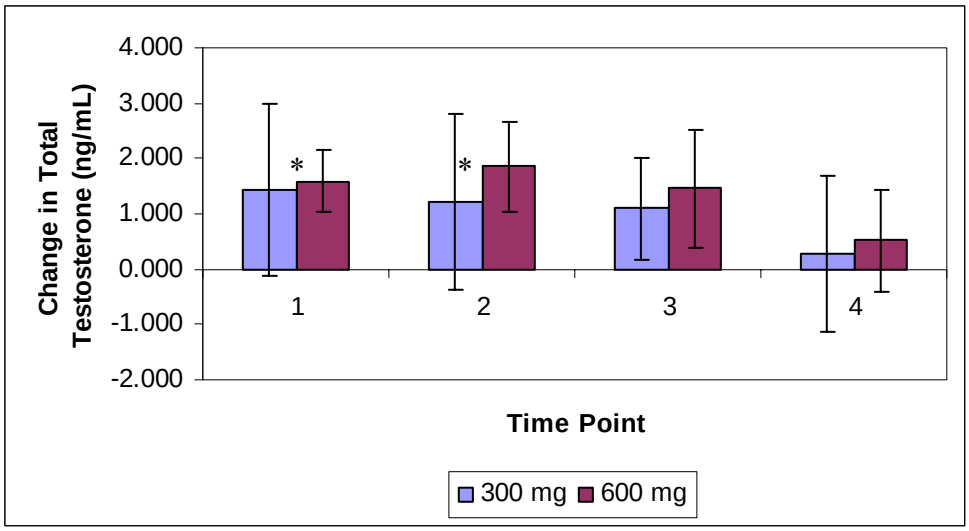

Figure 13. Change in Total Testosterone Concentrations. Change in total testosterone concentration was found by subtracting the baseline total testosterone concentration from each of the 4 testing session concentrations to yield time points 1-4. Time points 1 and 2 were found to be significantly higher concentrations than time point 4 (p=0.041 and p=0.034, respectively), denoted by an *, that was independent of any group effects.

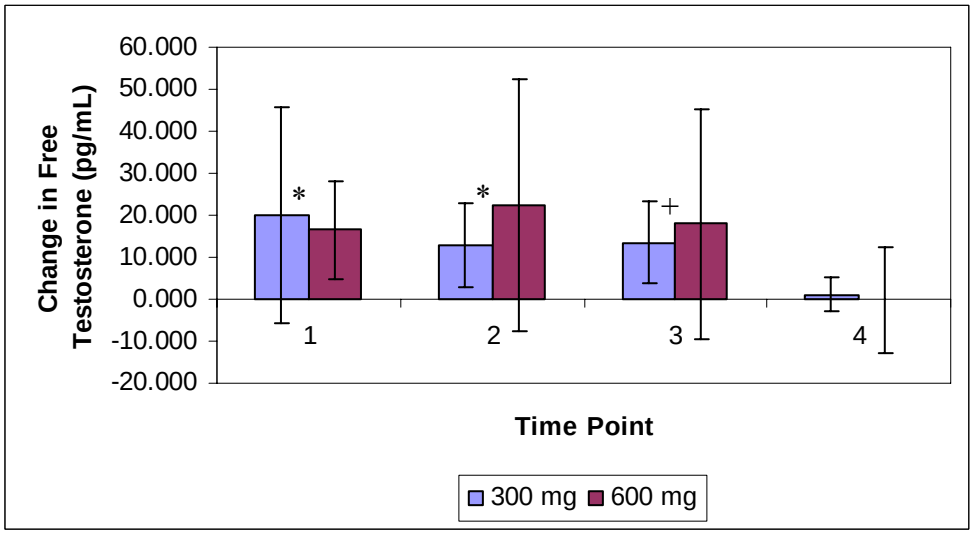

Figure 14. Change in Free Testosterone Concentrations. Change in free testosterone concentration was found by subtracting the baseline free testosterone concentration from each of the 4 testing session concentrations to yield time points 1-4. Time points 1 and 2 were found to be significantly higher concentrations than time point 4 (p=0.016 and p=0.041, respectively), denoted by an *, that was independent of any group effects. It was also observed that time point 3 had a possible trend of higher concentrations as compared to time point 4 (p=0.051), denoted by a +, which was independent of any group effects.

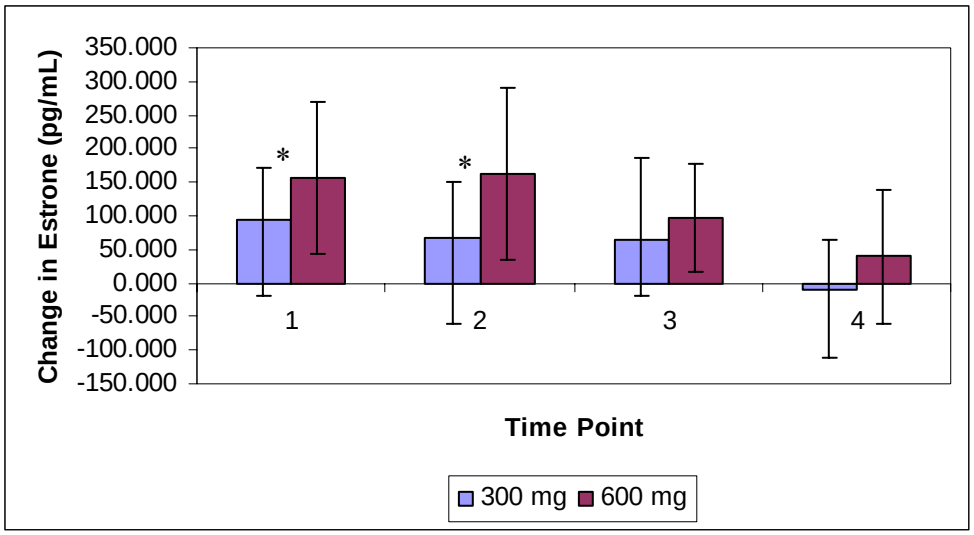

Figure 15. Change in Estrone Concentrations. Change in estrone concentration was found by subtracting the baseline estrone concentration from each of the 4 testing session concentrations to yield time points 1-4. Time points 1 and 2 were found to be significantly higher in estrone concentrations than time point 4 (p=0.014 and p=0.031, respectively), denoted by an *, that was independent of any group effects.

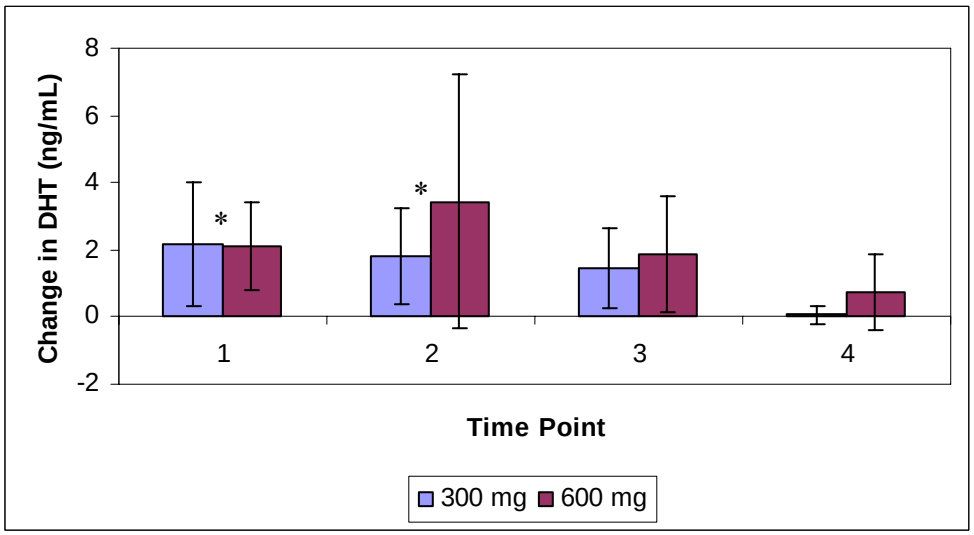

Figure 16. Change in DHT Concentrations. Change in DHT concentration was found by subtracting the baseline DHT concentration from each of the 4 testing session concentrations to yield time points 1-4. Time points 1 and 2 were found to be significantly higher in DHT concentrations than time point 4 (p=0.014 and p=0.031, respectively), denoted by an *, that was independent of any group effects.

testosterone in the 300 mg group was significantly higher than the control group (p=0.002) as was the 600 mg group when compared to the control (p=0.037) (Figure 18). The 600 mg dosage group had a significantly lower serum concentration of SHBG than

Table 9

Supplement Serum Hormone Changes versus Control Group

	00 mg		300 mg		600 mg	
Time Point	1	2	1	2	1	2
TT (ng/dL)	-0.15±0.96	-0.25±0.84	1.23±1.59*	1.09±0.93*	1.86±0.82*	1.46±1.06*
FT (ng/dL)	0.4±12.16	0.3±7.97	13.0±9.89*	13.4±9.73*	22.4±29.87*	18.0±27.46*
Estriol (ng/mL)	0.01±0.05	-0.01±0.03	0.00±0.05	0.01±0.04	0.00±0.03	0.01±0.03
SHBG (nmol/L)	-1.0±17.9	5.2±33.6	-1.6±36.1	8.1±39.9	-23.7±22.6*	-29.2±26.5*
LH(mIU/mL)	-0.50±1.89	-0.21±1.34	-0.62±4.19	-0.39±3.77	1.05±1.78	0.88±2.07
Cortisol (ug/dL)	-2.47±4.98	-2.50±2.13	0.55±6.50	-3.55±8.04	1.04±6.70	1.85±7.92

Note: * denotes significant main effects for groups

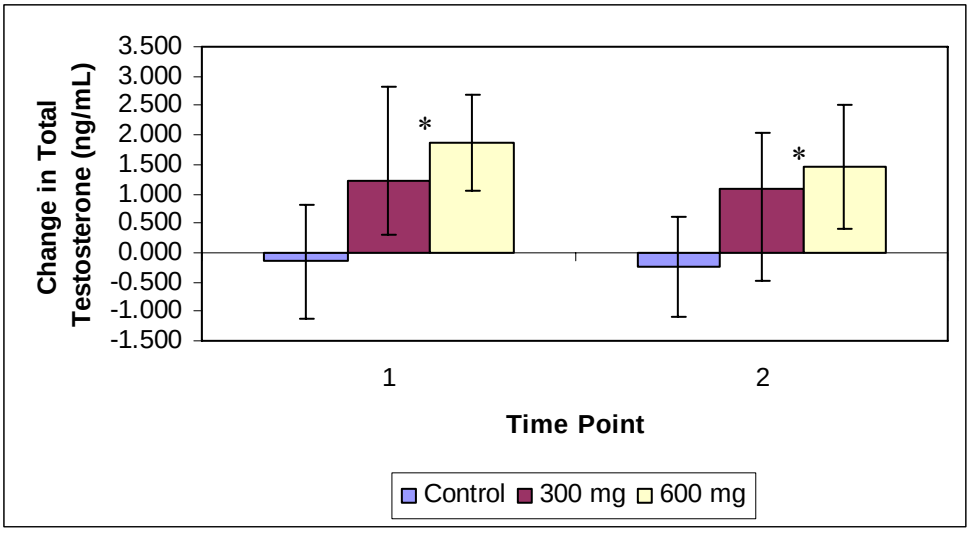

Figure 17. Comparison of Supplement Dosage Group Change in Total Testosterone Levels to that of Controls. Change in total testosterone was measured by subtracting baseline testosterone concentrations from each of the 2 testing session concentrations to yield time points 1 and 2. Total testosterone concentrations were statistically higher in the supplement groups as compared to controls (300 mg p=0.002 and 600 mg p=0.000). Significance is denoted by an *.

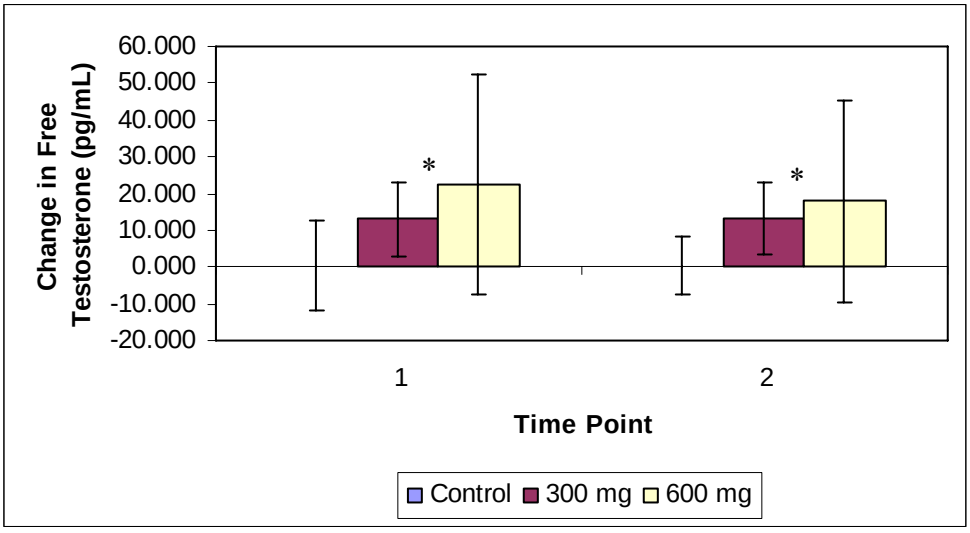

Figure 18. Comparison of Supplement Dosage Group Change in Free Testosterone Levels to that of Controls. Change in free testosterone was measured by subtracting baseline free testosterone concentrations from each of the 2 testing session concentrations to yield time points 1 and 2. Free testosterone concentrations were statistically higher in the supplement groups as compared to controls (300 mg p=0.002 and 600 mg p=0.037). Significance is denoted by an *.

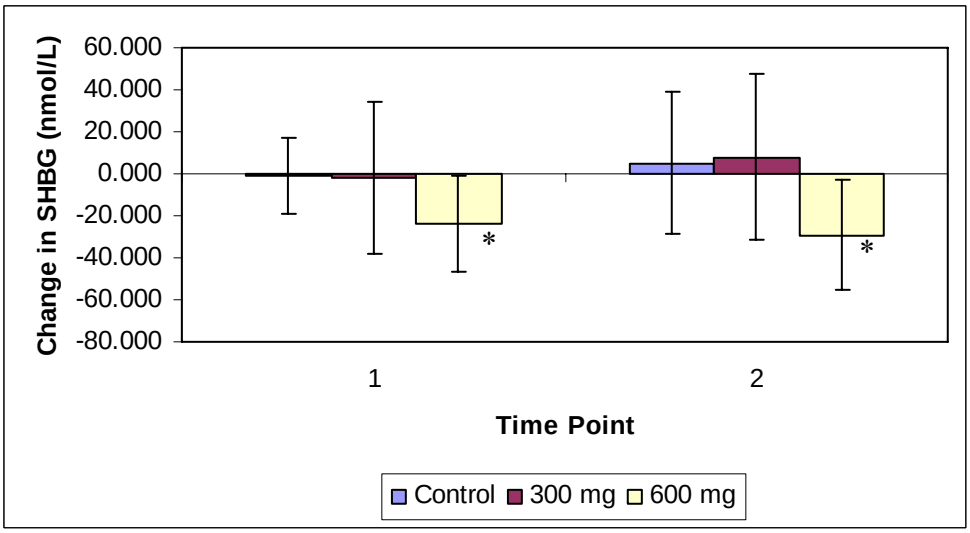

Figure 19. Comparison of Supplement Dosage Group Change in SHBG Levels to that of Controls. Change in SHBG concentration was measured by subtracting baseline SHBG concentrations from each of the 2 testing session concentrations to yield time points 1 and 2. SHBG concentrations were found to be significantly lower in the 600 mg dosage group as compared to the 300 mg group (p=0.030) and the control group (p=0.023). Significance is denoted by an *.

both the control and 300 mg group ($p=0.030$ and $p=0.023$, respectively) (Figure 19). When the rest of the hormone panel was analyzed it was seen that cortisol, LH, and estriol were not significantly different between the three groups ($p>0.05$). This led us to reject our null hypothesis that the supplement groups will not differ in hormone concentrations from a control group.

CHAPTER FIVE

Discussion

There were no significant changes in clinical chemistry markers measured in whole blood, serum, or urine during the course of the study. Since safety markers did not change significantly from baseline with ingestion of 6OXO™ and discontinuation of ingestion, it can be concluded that 6OXO™ does not adversely affect these markers. Even though there were significant differences seen between the two dosage groups these differences persisted through out the study starting with their respective baseline values. These differences were not important because it is just variation between sampling of the groups. The groups were matched for age and weight to create a random sample and any group differences seen through out the entire study would just be because of how the group was set up. There were also no changes in diet over the course of the study. Therefore it can be inferred that 6OXO™ is safe to ingest over an 8 week span.

Anthropometric measures were taken to see if any body composition changes were occurring with supplementation of 6OXO™. There were no changes over the course of the study and the difference that was observed was due to one group having a higher baseline level that persisted over the course of the study. This suggests that 6OXO™ supplementation does not appear to affect body variables such as FFM and FM related to body composition. Supplementation with 6-OXO™ did not cause an anabolic response by changing fat free mass, lean mass, or total mass. The possible reason for this even with the increase in testosterone could be due to high dose concentrations. It may be that at such a dose as was looked at in this study that the body is inundated with 6-

OXO™ that it is competing at the androgen receptor not allowing testosterone to bind and have its anabolic effect. If a lower doses were studied it might be seen that there is an increase in testosterone and because there would be much less 6-OXO™ to compete at the androgen receptor leading to an increase in fat free mass and possibly a decrease in fat mass.

In looking at serum hormone levels, possible trends and some significant differences arose between testing sessions. To elucidate whether these possible trends where actually significant changes, each subject's baseline concentrations were subtracted from the rest of the testing session concentrations to moderate any baseline effects. These changes in baseline were then compared to testing session 4, which was the wash out testing session. This testing session was not significantly different from baseline and had relative small concentrations when baseline was subtracted. Therefore the hormone concentrations at testing session 4 worked in a two fold manner. First, it allowed for estimation of long term effects of 6OXO™. Any residual effects of 6OXO™ would show up as a significant change in between baseline and testing session 4. Since there were no changes from baseline, this suggests that subjects' hormone levels were returning back to pre-supplement concentrations. Because testing session 4 was not significantly different from baseline any significant differences seen in change of concentration from baseline when compared to the change in testing session 4 from baseline would mean higher hormone levels caused by supplementation.

When the change from baseline was analyzed it was perceived that total testosterone levels were found to be higher during supplementation with 6OXO™. This is in agreement with Mauras et al. (2003), Leder et al. (2004), and Harder et al. (2004)

whom all saw an increase in total testosterone with ingestion of an aromatase inhibitor. Subsequently the increase seen in free testosterone was not unexpected. Free testosterone is derived from total testosterone by being the bioavailable fraction. Therefore it seems logical that if the total amount increased that the amount that is free should also increase. The interesting observation is that if free testosterone increases what besides an increase in total testosterone would lead to free testosterone's increase. It is known that the majority of testosterone is bond in serum so maybe an increase in free testosterone could also be attributated to a decrease in SHBG concentration which is one of the proteins responsible for binding the majority of testosterone. This was not seen and instead SHBG concentration did not change over the course of the study. In fact, the 600 mg group had lower SHBG concentrations throughout the entire study had only a peak change from baseline in their free testosterone of 99% while the 300 mg group, with their higher SHBG concentrations, had a peak change from baseline of 116%. Yet, because SHBG concentration did not significantly change over the course of the study this suggests that that 6OXO™ might contribute to keeping SHBG levels constant while increasing testosterone.

The purported mechanism for an increase in testosterone with inhibition of testosterone has been reported as when there is a decrease in estrodiol levels this leads to feedback to the hypothalamus stimulated testosterone production to increase estradiol concentration, (Hayes et al., 2000; Schnorr et al., 2001). This would infer that in order for an increase in testosterone to occur, a decrease in estradiol would have to be seen. This is not what happened in this study. It was seen that estradiol concentrations did not change over the course of the study. The reason that these changes were not seen is probably due

to the time frame of the measurements. If acute estradiol changes stimulated an increase in testosterone production and aromatase synthesis so that the body can maintain its homeostatic levels of estradiol, then a week difference might have given the body a chance to regain its levels. Also, in order for the body to maintain the homeostatic level of estradiol required, testosterone concentration would need to remain high in order to compete for the active site of aromatase in the presence of an inhibitor of aromatase. This would lead to the observation of testosterone concentration being elevated while estradiol concentration remains unchanged.

Changes in hormone concentration that were also significant were DHT and estrone. These two showed significant increases in hormone concentration over the course of the study. DHT and estrone had peak percentage changes from baseline of 50% and 28% in the 300 mg group respectively and 372% and 61% in the 600 mg group, respectively. While these percents seem impressive for DHT, concentration was not significantly different for group main effects. It seems logical that DHT concentration would increase with increasing testosterone concentration because DHT is a metabolite of testosterone. Therefore, as testosterone concentration increases so should DHT concentrations. This would suggest that testosterone concentration is indeed increasing. With increasing testosterone, the hormones directly correlated to testosterone are being changed, i.e. free testosterone concentrations and DHT concentrations. This also implies that if 6OXO™ would indeed be working as it is advertised to do these changes would be seen without a significant rise in estradiol, which is indeed what is seen.

Yet, what is seen is a significant increase in estrone concentration with supplementation. Estone is synthesized from androstenedione, a precursor of testosterone

by aromatase. One explanation for this increase seen in the hormone that is synthesized from androstenedione, is that in order to increase testosterone concentration androstenedione, a precursor for testosterone, needs to be either converted much more readily to testosterone or production of androstenedione needs to increase. Since androstenedione concentrations were not looked at in this study it is hard to say whether the former, the latter, or a combination of both occurred. However, an increase in androstenedione could lead to competition with the aromatase inhibitor causing an increase in estrone concentration. Another plausible explanation could be the derivatization of 6OXO™ by enzymes located in the body. 6OXO™ is not protected form modification in the human body and any changes in structure or even 6OXO™ itself could be sensed in the assays used to measure for estrone levels. The second explanation seems more feasible because there was no increase in estradiol and estriol observed, which would have been seen because these three compounds are readily converted to one another. An increase in one should lead to an increase in the others.

Because 6OXO™ is a type I steroidal aromatase inhibitor, indicating that is similar in structure to testosterone. The manner of analysis for serum hormone analysis is usually done by hormone ELISA's and EIA's. These kits use antibodies to the hormone of interest, and they recognize the hormone of interest by certain structure features. Therefore, if a compound that is similar in structure to the hormone of interest is identical or close to it in the region that the antibody recognizes this could lead to a higher signal because the antibody is now detecting the compound. This was shown to occur for testolactone by Cummings and colleagues (1998). This would lead to the large differences that were not correlated with related concentrations. For example total

testosterone, free testosterone, and DHT all increased and they are related to one another, but estrone increased while its sister hormones estriol and estradiol remained unchanged. If this manner of analytic hormone analysis was not extremely specific to testosterone and other like hormones, it might exaggerate the signal due to the presence of 6OXO™ in the blood.

To see if our experiments fell into this conundrum, we tried to test the specificity of our ELISA and EIA kits, but due to the inability to obtain a pure form of the compound we were only able to estimate if there were any interactions. When aqueous suspensions of 6OXO™ (including all fillers present in the encapsulated form) was mixed with BSA (for any nonspecific binding) and it was found to cause a dramatic increase in signal for the estrone kit (data unpublished). This brings the changes that were seen in estrone into question. But we cannot be sure that this signal is not actually due to a supra-concentrated 6OXO™ solution. If the kit did have a small affinity for 6OXO™ but had much higher affinity for the hormones of interest then it can be reasonably assumed that in the presence of the two, one a hormone, one a compound, the one with the higher affinity will bind more readily and the lower affinity will probably not bind. But if the 6OXO™ is at extremely high concentrations, it will by sheer number of molecules force the hormone of interest out of the way and bind with the antibody. This would lead to a very large signal similar to what was seen in our estimated experiments.

To also see if these changes were more than just random variations that would be seen with exercise we looked at a control group that had participated in the exact resistance training protocol for the same amount of time. Because of differences seen in

baseline levels, all analyses were done with the baseline subtracted. It was seen that both supplement groups had higher testosterone and free testosterone levels than the control group. The control group's hormone levels hardly changed over the course of the workout period while the supplement groups had peak increases of 30% (300 mg) and 47% (600 mg) in total testosterone concentrations and 116% (300 mg) and 99% (600 mg) increase in free testosterone concentrations. These changes were similar to the work done by Incledon (unpublished observations). This proves that 6OXO™ does indeed increase testosterone and free testosterone concentrations. While this might suggest that 6OXO™ supplementation would lead to increased muscle mass because of the anabolic effect of higher testosterone levels, this is not necessarily true because in our experiments it was seen that there were no increases in lean muscle mass or improvements in body composition. This lack of change even at higher testosterone levels can probably be attributed to 6OXO™'s chemical structure. By being chemically similar to testosterone it could interact with testosterone in a competitive fashion not only at the aromatase enzyme but also at the androgen receptor in muscles. Competition at the androgen receptor would decrease testosterone's ability to bind to the receptor and stimulate muscle growth. This would explain the lack of increased muscle mass with increased testosterone concentrations.

Therefore we can conclude that 6OXO™ increases total testosterone, free testosterone, and DHT by inhibition of aromatase in a manner that does not depend on either the 300 mg dose or the 600 mg dose. These increases are transient and do not adversely affect safety markers, nor adversely affect hormone levels from returning to baseline.

Conclusion

6OXO™ supplementation is well tolerated by subjects and does not adversely affect the body as indicated by clinical safety markers. Supplementation with 6OXO™ did not increase body mass nor improve body composition which leads us to believe that 6OXO™ is not an anabolic supplement.

Both 300 mg and 600 mg dosages of 6OXO™ were able to increase total testosterone, free testosterone, and DHT with supplementation independent of dose. Concentrations of estrone increased, but in a manner that might be due to cross reactivity of 6OXO™ and the antibody used to measure those concentrations. Further research should be done to make the tests for hormone concentration more specific for that hormone and to make sure that similar compounds are not giving a false positive. More research should be done on the actual dosing regimen and whether or not a smaller dose can be used since there were no significant differences between taking the higher dose and the lower dose. Another aspect might be to have a complete cross over study in order to see the effects of each dose on a specific person. This would allow the researcher to see if some subjects respond better to a smaller dose while others respond better to a higher dose. While this study has answered some questions into the supplementation of aromatase inhibitors, it has left many more unanswered.

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