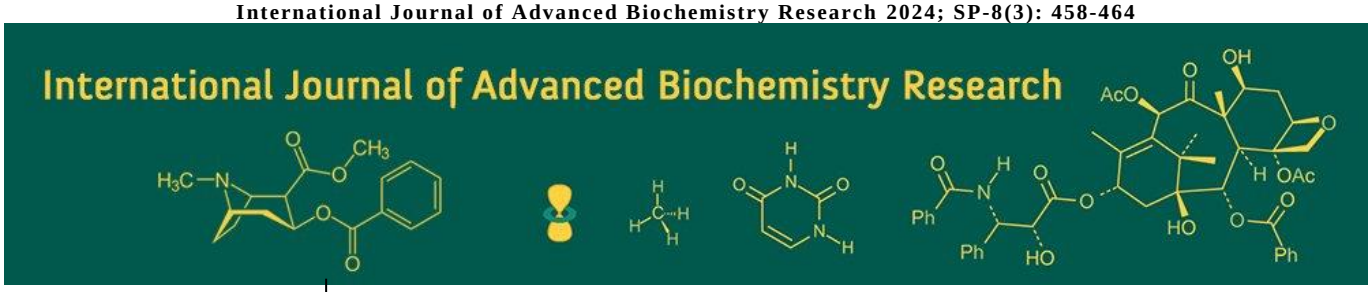

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Evaluation of mineral profile, testosterone, antioxidant enzymes and seminal attributes in infertile male dogs supplemented with certain vitamins and minerals

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Abstract
This study discusses the seminal attributes, serum testosterone levels and serum antioxidants and mineral profile in six infertile male dogs and the effect of dietary supplementing certain minerals and vitamins for 90 days on their subsequent fertility conducted in small animal OPD, Department of Gynaecology and Obstetrics, Veterinary College, Hebbal, Bengaluru. Serum and semen samples were evaluated for ejaculate volume, pH, individual sperm motility, percentage of live sperm, sperm cell concentration (million/mL) and sperm abnormalities. Results revealed that significant improvement (p<0.005) in serum zinc concentration was noticed on day 90 while other minerals were within the normal physiological range post supplementation. Antioxidant, glutathione peroxidase concentration significantly increased by day 90 of supplementation, while the levels remained unaltered for superoxide dismutase and catalase. Significant improvement (p<0.005) in seminal attributes *viz.* sperm concentration, sperm motility sperm viability and plasma membrane integrity noticed on day 90 whereas, reduction in sperm abnormality on day 60 and day 90 which corresponds to the normal physiological length of total duration of spermatogenesis in dogs. Serum testosterone concentration did not improve significantly after 90 days of supplementation. In conclusion, supplementation with minerals and vitamins for 60 days and beyond can improve the quality of semen in dogs with lowered fertility.

Keywords: Fertility, reproductive performance, supplementation, male dogs

Introduction
Presently, the diminishing reproductive performance in the canine species is one of the main concerns involving breeders and pet practitioners, who look for specific treatments to face it. Several female and male factors are responsible for temporary reduction in fertility and the involvement of male counterpart can be suspected with an altered semen picture. Male infertility is the male's inability to achieve pregnancy in a fertile female [1]. Infertility in male dogs was defined as conception failure at least 3 consecutive matings with different bitches in oestrus [2]. Lopes-Santiago [3] stated that dogs that had low sperm count < 20×10^6 spermatozoa per ml and more than 30% sperm abnormality were said to be infertile. The various factors that contribute to male dog infertility have been the subject of numerous studies. The causes of infertility can be broadly divided into three main classes: infertility due to a. specific lesion - orchitis, epididymitis, prostatitis, prostatic hyperplasia, balanitis, testicular tumours; b. functional infertility - cryptorchidism, hormonal imbalance, nutritional deficiencies (Macro-mineral and micromineral), hereditary or genetic factors, psychological causes; c. infertility of unknown origin [4, 5, 6]. A variety of etiological factors such as hormonal imbalance, heat stress, nutritional (Macro, micromineral and vitamins) deficiencies, toxins and autoimmune conditions have been reported but, in most cases, the cause of poor semen quality remains idiopathic in nature [7, 8].
Seminal attributes such as semen volume, semen pH, sperm concentration, total motility, sperm viability, sperm abnormality and sperm plasma membrane integrity in the whole ejaculate are commonly examined to evaluate the quality of semen [9].

Estimation of antioxidant enzymes concentration like superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) in serum is also important tool to assess the quality of semen [10]. Assessment of mineral profile in serum of hypofertile dogs help to rule out nutrient deficiency. Nutrient deficiencies of these mineral and vitamins can result in serious impairment of sperm potential [11]. Determination of testosterone concentration in blood serum help to know the reproductive status of animal. Instead of culling such hypofertile valuable male dogs, dietary supplementation of certain elements (Minerals and vitamins) which improve the spermatogenic cycle can be the crucial to improve seminogram, especially in conditions of deficiency. Furthermore, very minimal data is available for dogs, concerning the effect of a daily supplementation with a complex of minerals and vitamins on semen quality. Thus, the present study investigated this effect on semen attributes, mineral profile and anti-oxidant capacity in healthy but infertile male dogs.

Material and Methods

Animals, Supplementation of minerals and vitamins, Clinico-andrological examination

The study was conducted on client owned male dogs aged between 2 - 8 years presented to the small animal OPD, Department of Gynaecology and Obstetrics, Veterinary College, Hebbal, Bengaluru with complaint/history of infertility where the dogs fail to impregnate different female dogs within three matings. All the infertile dogs irrespective of the breed were subjected for physical examination, testicular and prostate echography and seminogram (Semen volume, sperm motility, sperm live and dead percentage, plasma membrane integrity, sperm morphological studies). Based on the preliminary examination findings, the male dogs found to be subfertile/ infertile were considered for the study. All the dogs included in the study were supplemented with two tablets (large breeds) and one tablet (Small breeds) containing minerals and vitamins for 90 days. Each tablet contains (Ca, P, Mg, Fe, Zn, Cu, Se, A, D3, E, K, B complex, C, choline, nicotinamide). Dogs considered in the present study were subjected to Clinico-andrological examination of penis, prepuce and scrotum followed by ultrasonographic examination of testes and prostate to know variation if any in the parenchymal consistency and echogenicity. Real time, B-mode ultrasound scanner with linear transducer of 5 MHz (PROSOUND ALPHA) was used for ultrasonographic examination of testes and prostate.

Collection of blood sample and semen sample

Blood sample collected from all six healthy infertile male dogs from the cephalic vein or saphenous vein. Semen was collected on day 0, 30, 60 and 90 by digital manipulation method.

Macroscopic evaluation of semen

The semen volume was directly collected and recorded graduation based pre warmed polyvinyl container. The pH of the semen sample was estimated using a digital pH meter

Microscopic semen evaluation

Sperm viability was evaluated using Eosin-Nigrosine staining technique. Sperm concentration was assessed using a Neubauer's haemocytometer by direct sperm counting of semen. Morphologically abnormal sperm in the semen sample was assessed using eosin and nigrosine staining method. Plasma membrane integrity evaluated using HOST (Hypo-osmotic sperm swelling test).

Mineral profile estimation

Mineral profile estimation of Zinc, Iron, Calcium, Magnesium, Phosphorus done using calorimetric method.

Testosterone estimation

Testosterone concentration was estimated in blood serum sample by electrochemiluminescence immunoassay "ECLIA"

Antioxidant enzymes estimation

The activity of SOD and GPX was determined using Canine Superoxide Dismutase ELISA kit and Canine glutathione peroxidase ELISA kit (PUREGENE Laboratories, Delhi). The activity of catalase assayed spectrophotometrically according to the method described by Aebi (1983).

Results

Complete blood count and hematobiochemical parameters such as total erythrocyte count, total leukocyte count, platelets count, packed cell volume, haemoglobin value, serum SGPT value, serum creatinine value remained same before and after supplementation of vitamins and minerals.

Effect of supplementation of minerals and vitamins on seminal attributes

The semen volume and semen pH remained constant throughout the study period. Total sperm motility in per cent was found to increase significantly from day 30 day onwards and throughout the study period. Significant improvement in sperm concentration, sperm viability and sperm membrane integrity was recorded from day 90 post supplementation. Sperm abnormality reduced from day 60 significantly in infertile male dogs supplemented with certain minerals and vitamins (Table. 1).

Effect of supplementation of minerals and vitamins on serum mineral profile

The mean serum zinc concentration (µ/dL) on day 90 was (93.3±2.37) which was significantly ($P = 0.026$) increased after supplementation of vitamins and minerals. Whereas serum concentration of iron, calcium, phosphorus, magnesium remained within the normal range throughout the study period (Figure. 1)

Effect of supplementation of mineral and vitamins on antioxidant enzymes

The serum glutathione peroxidase (ng/mL) was found to increase significantly ($p=0.026$) after supplementation of certain minerals and vitamins for 90 days (42.40±6.93). whereas, superoxidise dismutase and catalase levels in serum remained same before and after supplementation of vitamins and minerals (Figure. 2).

Effect of supplementation of mineral and vitamins on testosterone

In present study the mean serum testosterone concentration (ng/mL) recorded before and after supplementation with minerals and vitamins remained low 0.38±0.02 (Figure 3).

Discussion

As the experimental study was carried out on apparently healthy dogs, all the haemato-biochemical parameters were within the normal physiological range which is in accordance with Gradil *et al*. [12] and Ciribe *et al*. [13]. All male dogs included in the study were healthy throughout the study period, and no adverse effects were noticed upon supplementation of minerals and vitamins for 90 days.

Effect of supplementation of mineral and vitamins on seminal attributes

The mean semen volume and semen pH recorded in the present study conducted on dogs before and after supplementation of minerals and vitamins were in congruence with values reported by Pinto and Kozink [14], Domosławska *et al*. [15], Domosławska *et al*. [16] and Domoslawska *et al*. [10] who reported mean semen volume (mL) of 3.40±0.30, 2.98±0.96, 2.90±1.29and 2.63±0.56, respectively. The semen pH recorded in the current study conducted on dogs was within the normal range and is in accordance with results obtained by Gradil *et al*. [12] and Kustritz [17] who have reported pH range of 6.4 to 6.8 and 6.3 to 6.7 respectively.

A significant increase in the sperm concentration was observed in dogs supplemented with certain vitamins and minerals for 90 days which is in accordance with Ebsich *et al*. [18], Domoslawska *et al*. [16], Domoslawska *et al*. [10], Ciribe *et al*. [13], Rodriguez- Martinez and Barth [19] and Alonge *et al*. [5] who reported mean sperm concentration (millions/mL) of 77.50±55.7, 186.82±62.25, 292.07±68.9, 698.9±96.27, 253.4±37.6 and 899.43±150.67, respectively. Natural folate plays an important role in the synthesis of purines and pyrimidines for tRNA and DNA, which are both important in spermatogenesis Ebsich *et al*. [18]. According to Wallock *et al*. [20] men who received supplements in addition to the normal diet and especially antioxidants were associated with a higher number of spermatozoa with better motility. This improvement in sperm concentration in the current study can be attributed to the cumulative effect of minerals and vitamins supplementation.

A significant increase in the sperm viability was observed in male dogs by day 60 post supplementation which is in close agreement with Domoslawska *et al*. [16] Domoslawska *et al*. [10] and Alonge *et al*. [5] who reported 93.06±3.08, 93.06±3.08 and 91.86±3.34, respectively. A significant decrease in the sperm abnormality by day 60 and 90 was observed when compared to day 0 is in line with Domoslawska *et al*. [16] Domoslawska *et al*. [10] who reported mean sperm abnormality of 19.14±7.3 and 20.0±8.36, respectively. Kołodziej and Jacyno [21] and Koziorowska-Gilun and Strzezek [22] reported improvement in the percentage of live spermatozoa is linked to higher antioxidant status of glutathione peroxidase concentration in the spermatozoa which is attributed to increased serum concentration of Zn, which acts a cofactor for glutathione peroxidation. Domosławska *et al*. [10] noticed the significant improvement in the mean morphologically normal spermatozoa and improved fertility after supplementation of vit E and selenium for 90 days. Similarly, in the present work significant increase in Zn and glutathione peroxidise might be responsible in preventing the detrimental effects of ROS on spermatozoa and thus improving the per cent of viability and decreasing the abnormality in spermatozoa.

According to the several research literatures, a change in the structure of the plasma membrane and its functional doings effectively influences the sperm motility, fertilization capacity and embryonic development [23, 24]. Due to the presence of higher concentration of polyunsaturated fatty acids, the plasma membrane of spermatozoa are particularly sensitive to oxidative damage [25] and its avoidance is considered fundamental to keep up the normal fertility [5]. For this reason, it is necessary to enrich the diet with adequate levels of antioxidants, too, in order to protect male germ cells against oxidative damage. In the present study no significant improvement in the sperm membrane integrity was observed till day 60, while significant enhancement was observed after supplementing for 90 days. Improvement in the percentage of swollen sperm indicates the presence functional and intact plasma membrane [26]. In the present study, supplementation of vitamin-E and zinc and significant increase in Glutathione peroxidase might have prevented the lipid peroxidation and neutralize the possible production of reactive oxygen species. Supplementing Zinc has shown to stabilize the cell membrane of spermatozoa by influencing the fluidity of lipids of biological membranes [27]. These trace elements might have played a synergistic role on the structural and functional membrane integrity of spermatozoa.

Effect of supplementation of mineral and vitamins on serum mineral profile

The mean serum zinc concentration of dogs in present study is in accordance with Zentrichova *et al*. [28], Kazmierski *et al*. [29] and Cedeno *et al*. [30]. reported mean serum zinc concentration in healthy dogs as 27.37-110.0 µg/dL, 120.0±0.4 µ/dL and 95.8±3.1µg/dL respectively and were within the physiological range. The mean serum iron concentration (µ/dL) of dogs in the current study is in accordance Kazmierski *et al*. [28] and Cedeno *et al*. [29] 175.1±56.7 and 168.0±60, respectively. The mean serum calcium concentration(mg/dL) of dogs in present investigation is in accordance Aquino-Cortez *et al*. [31] and Mickel *et al*. [32] who reported 3.39±0.60 and 9.0mg/dl to 11mg/dL, respectively and values obtained in the present study were found to be within physiological range.

The mean serum magnesium concentration(mg/dL) of dogs in current study is in congruence with values obtained by Aquino-Cortez *et al*. [31] and Michael *et al*. [32] who reported 2.95±0.54 and 1.82 to 2.31±0.34 mg/dL respectively. The values obtained in present study were found to be within the physiological range. Magnesium has important role in sperm motility and sperm concentration. The energy required for the sperm motility is liberated from the ATP by the Mg^{2+} - dependent ATPase. The mean serum phosphorus concentration (mg/dL) of dogsin present study is in close agreement with the findings ofAquino-Cortez *et al*. [31] and Cortadellas*et al*. [33] who reported 9.14±18.06 and 4.5 - 5.5 respectively. Phosphate ion is crucial to the activities of prostatic acid phosphatase (PAP) which has been associated with liquefaction process of semen and adenyl cyclase; the primary regulator of sperm motility [34].

Effect of supplementation of mineral and vitamins on antioxidant enzymes

Superoxide dismutase and Catalase

In current study the mean serum superoxide dismutase concentration (ng/mL) and catalase (mmoL/min/g protein)

did not improve significantly before and after supplementation of vitamins and minerals. A contrary finding by Michael *et al*. [23] showed that catalase addition in canine semen extenders resulted in a noteworthy increase of the superoxide anion production compared with the controls. This may be explained by the fact that O2 is a primary product then dismutases to H2O2, under the influence of SOD, which is the main goal of catalase.

A significant improvement was observed in the mean serum glutathione peroxidase concentration by day 90 (42.40±6.93) ng/mL. Because Glutathione peroxidase act as co-enzyme protect the sperm from extracellular oxidants are responsible for lipid peroxidation of sperm membrane, impacting sperm motility and morphology. Intracellular ROS can react with proteins, altering the physiological pathways by oxidation of substrates, and DNA, causing sperm DNA fragmentation. Therefore, glutathione peroxidase plays an essential role in balancing the seminal concentration of oxidants in sperm physiology [35].

In the present study, significant increase in the Glutathione peroxidase concentration by day 90 is attributed to the marked reduction in the sperm abnormality, increased number of live spermatozoa and stabilized membrane integrity.

Effect of supplementation of mineral and vitamins on testosterone concentration

In present study no significant improvement was observed in the mean serum testosterone concentration on day 0 before supplementation and after supplementation day 30, day 60 and day 90. The mean serum testosterone concentration of present study at day 30, 60, 90 was in close agreement with the findings of Risso [36] mean testosterone levels on day 30 was (1.31±0.3 ng/ml) and remained low on days 60 (1.84±0.5 ng/ml), 90 (1.20±0.3), and 120 (0.89±0.3) after supplementation fish oil over period of 120 days. The decrease in testosterone concentrations was related to changes in the synthesis of prostaglandin series 2 to series 3 and the inhibition of prostaglandin formation from AA by EPA/ DHA treatment. Differences in the values obtained among studies could be due to the size of the animal, breed, sources of dietary supplements, supplementation period, season, petting practices or semen handling procedures [36].

Table 1: Effect of supplementation of minerals and vitamins on mean macroscopic and microscopic seminal attributes (n=6)

Parameter	Day 0	Day 30	Day 60	Day 90
Volume (mL)	2.70±0.32	2.53±0.32	2.72±0.32	2.77±0.32
pH	6.85±0.10	6.73±0.08	6.62±0.08	6.93±0.05
Total motility (%)	55.33±6.77	61.5±6.00	74.67±4.72	82.67±3.66
Sperm concentration (10^6/mL)	44.97±10.38	46.53±68.00	66.02±14.91	75.53±17.67
Sperm viability (%)	56.67±6.90	57.17±6.47	65.17±7.94	67±8.41
Membrane integrity (%)	53.83±7.32	55.83±6.87	64.83±8.44	66.17±8.63
Sperm abnormality (%)	21.67±2.70	19.5±1.34	14.00±1.44	13.17±0.98

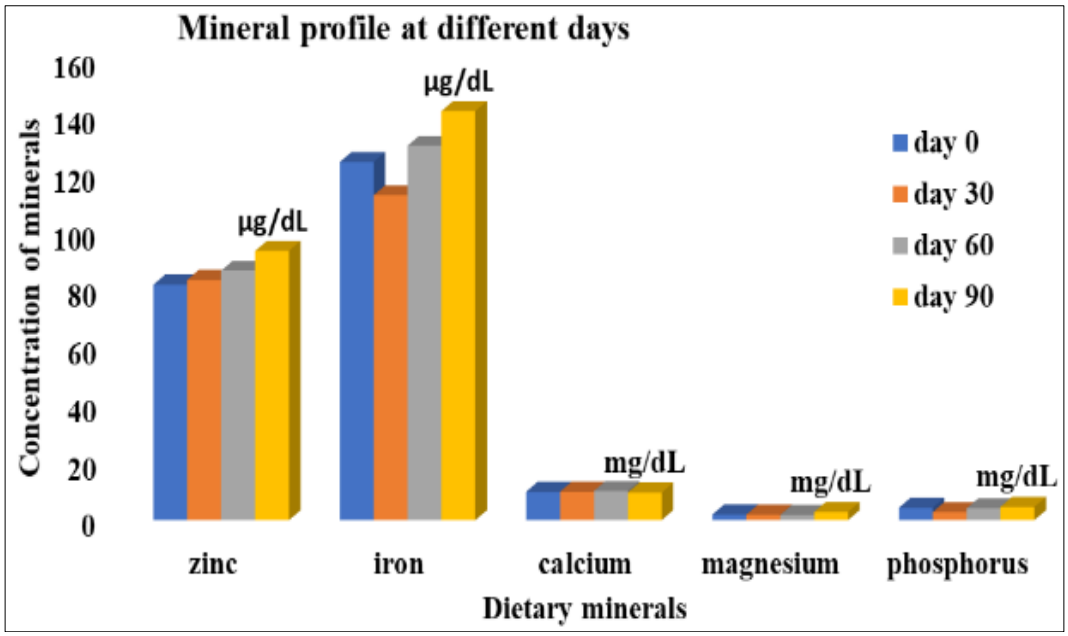

Fig 1: Effect of supplementation of minerals and vitamins on mean mineral profile in dogs

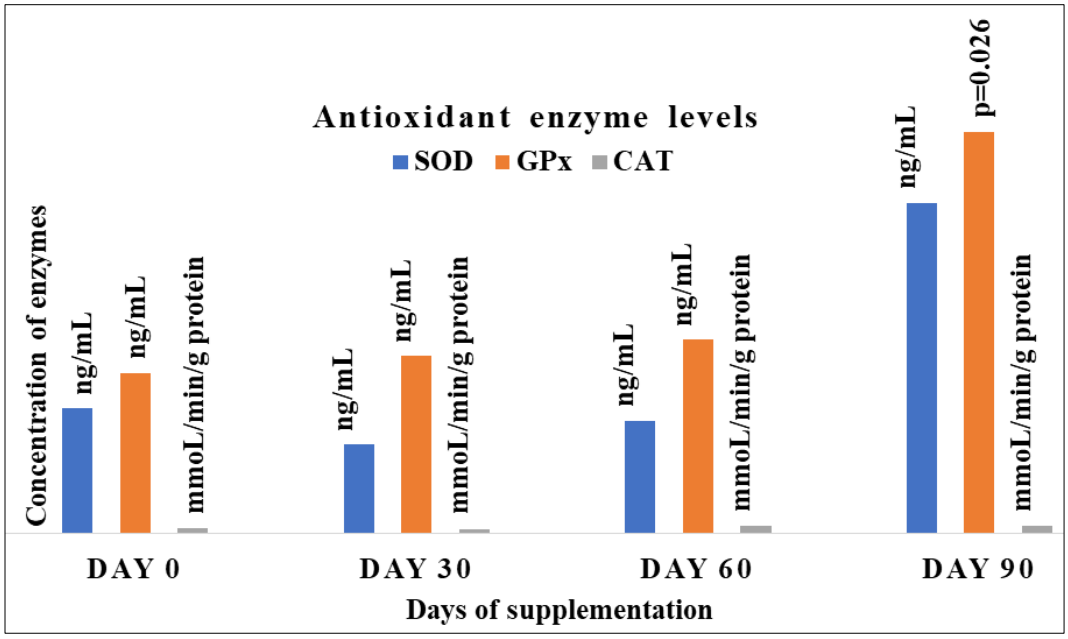

Fig 2: Effect of supplementation of minerals and vitamins on mean antioxidant enzymes status in dog

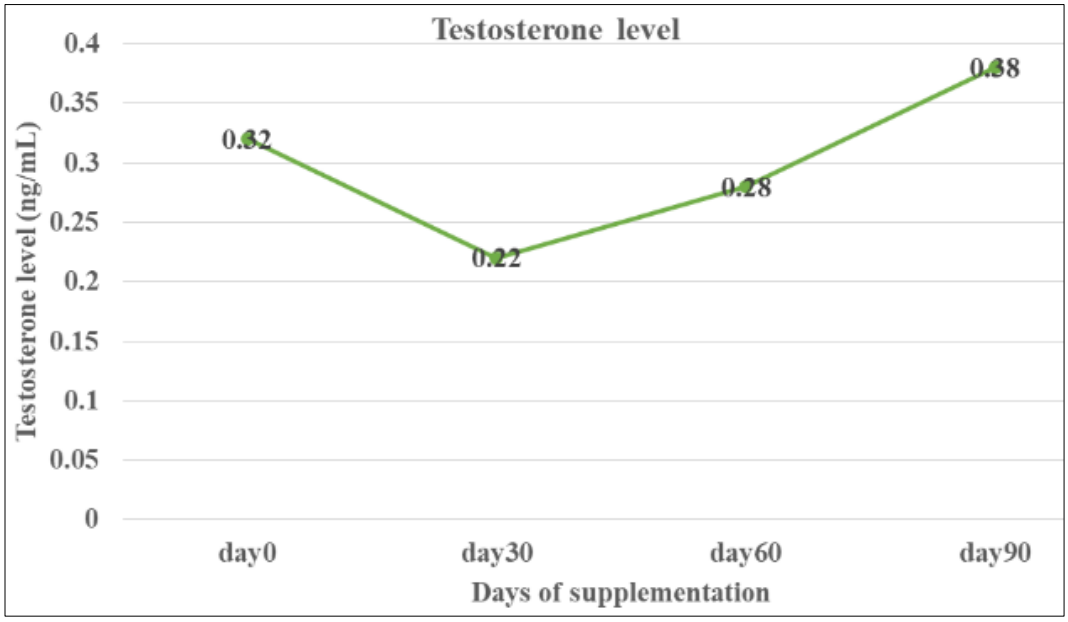

Fig 3: Effect of supplementation of minerals and vitamins on mean serum testosterone concentration in dogs

Conclusion

Supplementation of certain minerals and vitamins (Zinc, vit E, selenium, folic acid) and balanced diet can be pointed as economical and harmless alternatives within an innovative multimodal approach to improve reproductive performance in healthy infertile dogs. Seminal attributes such as sperm concentration (**10^6**/mL), sperm vitality (%) and sperm membrane integrity (%) and showed significant increase by day 90 in dogs supplemented with certain vitamins and minerals Whereas, sperm abnormality (%) reduced by day 60 and day 90 of vitamin and mineral supplementation and sperm total motility increased by day 30 onwards. In minerals zinc concentration increased by day 90 (93.33±2.37 µ/dL) whereas iron, calcium, magnesium and phosphorus concentration did not vary significantly throughout the supplementation duration. Antioxidant enzymes like superoxide dismutase and catalase remained unchanged throughout the supplementation of vitamins and minerals but glutathione peroxidase concentration increased by day 90 of vitamin and minerals supplementation 42.40±6.93 (ng/mL). Testosterone concentration remained low during the supplementation of vitamins and minerals may be due to the size of the animal, breed, sources of dietary supplements, supplementation period, season, petting practices or semen handling procedures.

Based on the findings obtained in current investigation, it could be concluded that dietary supplementation of minerals and vitamins for 60 days and beyond facilitated the improvement in seminal attributes, mineral profile and antioxidant enzymes in infertile male dogs. Supplementation of certain minerals and vitamins improved the reproductive status and is cheap, effective and safe alternative to improve the semen quality, antioxidant enzyme status and mineral profile in male dogs. However, further intensive investigation needs to be done on large population size before treating the infertility in male dogs. Supplementation of zinc, vit E and folic acid benefited augmenting the fertility in sub-fertile / infertile male dogs.

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