

## RESEARCH PAPER

# Impact of vitamin D3 Nanoemulsion on spermatogenesis and antioxidant enzymes in Vitamin D deficient induced albino male rats.

Diyar Hamid Karim<sup>1</sup>, Sulaf Mustafa Mohammed<sup>1</sup>, Hoshyar Abdullah Azeez<sup>2</sup>

<sup>1</sup> Department of Biology, college of Science, University of Sulaimani, As-Sulaymaniyah, Kurdistan Region, Iraq.

<sup>2</sup> College of pharmacy, University of Sulaimani, As-Sulaymaniyah, Kurdistan Region, Iraq.

### ABSTRACT:

Vitamin D deficiency is common with several effects that include nonskeletal impacts. Many studies showed associations between Vitamin D deficiency and oxidative statuses with disturbed testicles in male rats (*Rattus norvegicus*) which might be improved by vitamin D. The aim of this work is to find the effects of nanoemulsion pea protein isolates and novel vitamin D carrier, developed by sonication and pH shifting of pea protein isolate nanoemulsion on the spermatogenesis in induced vitamin D deficient male rats. Thirty male albino rats distributed into 5 groups, sufficient control was fed with normal diet, deficient control was treated with 100 mg/kg Rimfapicin and 50 mg/kg Isoniazid for three weeks, pea protein isolate group: vitamin D deficient rats treated daily with 60 mg/ml/kg nanoemulsion pea protean isolate, Vitamin D deficient rats were treated with vitamin D (54 mcg/ml/ kg), and pea protean isolate + vitamin D group: Vitamin D deficient rats were treated by nanoemulsion pea protein isolate (60 mg/dl/kg) plus Vitamin D (54 mcg/ml/ kg) for four weeks. The serum used for Vitamin D and total testosterone assay, after estimation of sperm count and morphology. Homogenized testes used for determination of Catalase and Glutathione peroxidase. The results revealed that vitamin D and supplementations each one alone have a positive significant effects on spermatogenesis. The nanoemulsion pea protein isolate +vitamin D resulted in a significant increase in the levels of vitamin D of total testosterone more than the supplementation of vitamin D and nanoemulsion pea protein isolate alone. Also sperm count, normal sperm morphology, as well as catalase and Glutathione peroxidase improved significantly in this group. In conclusion we demonstrated vitamin D nanoemulsion as more efficient formulation with more prominent effects on spermatogenesis in induced vitamin D deficient rats.

KEY WORDS: Vitamin D, Testosterone, Nanoemulsion, Catalase, Glutathione Peroxidase, Sperm morphology, Sperm count.

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## 1. INTRODUCTION:

The function of Vitamin D is not restricted in calcium metabolism homeostasis in skeletal and muscular system but vitamin D have many important roles in non-skeletomuscular system for example fertility (Spiro & Buttriss, 2014), (Farhangi et al., 2017), (Caprio et al., 2017). Many research findings have detected that vitamin D have beneficial effects on extraskeletal tissues as well (Forrest & Stuhldreher, 2011).

most common function of vitamin D proceeds many systems and organs which are include sexual reproductions, cardiovascular system, respiratory system, some immunological reactions, common health, as well as regulation of gene expression. (Jameson, 2018), (Prusik et al., 2018), (Pludowski et al., 2018), and (Karefylakis et al., 2018). Vitamin D receptors locate inside of nucleus, mostly abundant in more than 38 types of tissue, There are more than 200 genes have been found that vitamin D could affect them (Haussler et al., 2011) and (Sung et al., 2012). Leydig cells and Sertoli cells are two main endocrine cells in

### \* Corresponding Author:

Diyar Hamid Karim

E-mail: [diyari\\_hami@gmail.com](mailto:diyari_hami@gmail.com)

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testes that secrete and produce many sex hormones (O'Donnell et al., 2017). Many hormones and vitamin directly or indirectly interacts the normal function of testes (Mortimer et al., 2013). vitamin D receptor and vitamin D metabolizing enzymes have been existed in the testes this is the main evidence of effectiveness of vitamin D in male fertility (Jensen, 2014). Vitamin D deficiency implicates in various physiological abnormalities such as: asthma, impaired pregnancy consequence, osteoporosis, diabetes mellitus type two, cancer and cardiovascular disorder. In addition, vitamin deficiency could affect male reproductive system in many ways such as: deterioration growth of seminiferous tubules and decreased testicular weight, drops sperm count in caudal epididymis, damage testicular role, and disrupt Sertoli and Leydig cells. (Chen & Zhi, 2020) and (Sharma & Sharma, 1997). The reason behind the idea that vitamin D is close relationship with male fertility return to most reproductive parts of male and spermatozoa contain vitamin D receptor as well as vitamin metabolite enzymes. (Martin Blomberg Jensen et al., 2010). Moreover, some data showed that spermatogenesis is positively affected by Vit.D deficiency (Zanatta et al., 2011) and (O'sullivan et al., 2016)

Nanoemulsion acts as active system to carry of hydrophobic drug that have low solubility and poor absorption by gastrointestinal tract and keep them from low pH which increase cellular uptake. (Liu et al., 2019). Nanoemulsions can increase cellular absorption of the encapsulated drugs the reason behind this is micro sized and high stabilized. (He et al., 2011). In the last few years, because of less allergy, and good food make pea protein as a novel food ingredient has increasing attraction among researcher. Pea protein isolate has been modified and ameliorated in structure, physical and chemical characteristics by the combination of pH-shifting and ultrasonication. (Almajwal et al., 2019). In the last few years, authors expansively investigated that lipid and polymers act as a chemical carrier and deliver system for Vit.D (Park et al., 2017) (Hasanvand et al., 2018). Here are many problems related with these delivery systems for example short half-life, oxidation exposure, perhaps it will hydrolysis, allergy and others. (Sharma & Sharma, 1997) (Almajwal et al., 2019) improved serum Vit.D in vitamin D deficient rats by pea protein

isolate based vitamin D nanoemulsion. The aim of this study to know the effectiveness of nanoemulsion for bioavailability of Vit.D and increase oxidative enzymes activity in tests of male rats.

## 2. MATERIALS AND METHODS

### 2.1. Experimental Conditions

All Thirty male albino rats their weight were (300-350 g). The animals were kept in stainless steel cages with constant humidity, in a silent animal house with 12-h light /12-dark cycles; range of temperature was  $22\pm 4$  °C The rats obtain a typical diet with tap water ad libitum during all experimental time, before the start of the experiment; rats were permitted to acclimate one week to their situation. The animals were kept in animal house, after one week divided rats to five groups each group with 6 rats. During three days for each week, we monitored the rats both food stuff and their weight.

### 2.2. Preparation of pea protein isolates

The protein content of pea is 18-30%, which include albumins, globulins and prolamins, and rich in profile amino acids. (Shand et al., 2007). Ultrasound is a kind of mechanical wave that is used for formation of nanoemulsion (Kentish & Feng, 2014). Nanoemulsions are submicron sized colloidal particulate system (5-200 nm), thermodynamically unchangeable, transparent, and isotropic system, by mean an emulsifying factor both non-miscible fluids are integrated to form one phase. Biologically active substances such as drugs are carried and delivered by nanoemulsions and enhance the therapeutic efficacy of the drug. (Çımar, 2017), (Solans & Solé, 2012). (Jaiswal et al., 2015).

Pea protein isolate has been prepared according to (Boye et al., 2010), (Liang & Tang, 2013) and (Stone et al., 2015) methods, based on alkaline extraction and precipitation of acid procedure.

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### 2.4. Preparation of nanoemulsion of pea protein isolate:

Ultrasound application was utilized using a Qsonica Q700 Sonicator processor at frequency 20 kHz (QSONICA,LLC 53 CHURCH RD.NEWTOWN,CT, USA). The sample has been exposed to acoustic energy, the acoustic power density (APD) was adjusted at 68 W/100 mL by using an ultrasonic probe the diameter was (13 mm). Snow bath has been applied to prevent protein denaturation by high temperature.

Three gram of pea protein isolate (PPI) was dissolved in (100 ml of deionized water) (30 mg/mL (w/v), pH 7.0. The dispersion was stirred at room temperature for 30 min then adjusted to pH 12.0 at 25 °C. Immediately, the protein dispersion was exposed to 5 min ultrasonication, followed by adjusting pH back to 7.0 for the treated solution and put at 25°C about one hour, then centrifuged at 8610 rpm for 15 min at 15 °C and collected supernatant then kept at 4 °C in a refrigerator.(S. Jiang, 2015)

### 2.5. Induction of vitamin D deficiency:

Vitamin D deficiency was induced experimentally by gavage of rats about three weeks with **Rifampicin capsules** (RIF) (100

mg/kg b.w.) and **Isoniazid** (INH) (50 mg/kg b.w.) (Brodie et al., 1982)

### 2.6. Experimental design

The rats were divided into five experimental groups, six rats for each one as following. sufficient control rats this group received 2 ml of 0.1N of HCl plus regular diet; the remaining vitamin D deficient rats were subdivided in to four groups; deficient control without treatment ; group pea protein isolate they received regular diet with nanoemulsion pea protein isolate (60 mg/ml/kg b.w. orally); group vitamin D they received regular diet with vitamin D was dissolved in olive oil (2160 IU/ml/ kg b.w. orally); group pea protein isolate +vitamin D they received regular diet plus vitamin D dissolved in olive oil (2160 IU/ml/ kg b.w. orally) with nanoemulsion pea protein isolation (60 mg/ml/kg b.w. orally). About four weeks for each group. Entire processes were done by involving sterilized laboratory condition.

### 2.7. Blood sample collection and biochemical analysis

All the rats has been fasted during the night at the end of four weeks, the experiment duration and anesthetized by using ketamine 10% (50 mg/kg/b.w) and xylazine 2% (10 mg/kg/b.w) via intraperitoneal. Blood samples were collected by heart puncture vacutainer tubes with silica clot activator. The samples were centrifuged at 4000 rpm for 10 min at 4 °C. The separated serum samples were frozen and kept at -80 °C until later analysis.

### 2.8. Testicular homogenization:

The testes have been removed surgically and washed by 1.15% KCL and homogenized in 0.01 M potassium buffer (pH 7.4), followed by centrifugation 13000x g for 10 min at -4 °C then supernatant of homogenized testes was collected and stored in refrigerator at -4 °C. Olayinka, E. T., & Ore, A. (2015)

### 2.9. Sperm count

200 mg of caudal epididymis mixed with NaCl till (2ml) in a Perti dish (30 mm). The

Macerated caudal epididymis has been divided by scissor into four parts then left the tissue for 30-60 seconds to let sperms leak from the tubules (the solution was tint whitish/grayish, similar to the diluted semen), and the resulted fluid will be handled exactly as the semen. Collected the fluid into an Eppendorf tube (1.5ml) for microscopical examination of sperm morphology and sperm cell count. The number of sperms was determined with an improved neubaure hemocytometer with a cover glass. The measured sperm number was multiplied by the dilution factor to yield the total sperm count. The tissue suspension is diluted 10 times, and then it was further diluted in a NaCl + formalin (20 microL semen + 180 microL NaCl + formalin) to kill the sperms. So consider that two dilution factors ( $\times 10^6$ ) in final calculation using the hemocytometer chamber, all steps have been done on hot stage at 37°C.

### 2.10. Sperm morphology:

Karanawska (1976) method has been used with few modifications to prepare sperms from epididymis and Vas deferens. The epididymis and Vas deferens were put in small Petri-dish containing 0.9% normal saline. The two organs have been placed on the glass and by press small needle slightly over them; the sperm has been extracted, then together with a drop of the normal saline has been smeared on the slide. The slide has been stained with 1% Eosin for 10 min, next it has been washed with tap water, later stained with Hematoxylin for 15 min, then it has been washed by tap water (Fattah & Mustafa, 2012). Smears were examined under an oil-immersion objective at  $\times 100$ . Four hundred sperm cells were examined per animal to determine the morphological abnormalities. Any disorders in the morphology and structure of either head or tail or both were considered as abnormal. Number of abnormal sperm divided to total sperm was defined as percentage of abnormal sperm (Mohammed, 2015). The presence of sperm without head, sperm without tail, abnormal neck, abnormal hook and abnormal head was assessed. We examined five different fields for each sample.

### 2.11. Statistical analysis

All results were expressed as mean  $\pm$  SEM using Graph Pad Prism software (version 2019) and then analyzed by one-way ANOVA and then

by Tukey's test.  $P < 0.05$  was considered as statistically significance level.

## 3. RESULTS AND DISCUSSION

### 3.1. Serum Vitamin D3

Isoniazid and Rifampicin induced vitamin D deficient rats showed significant reduced of serum vitamin D ( $11.3 \pm 0.7162$  ng/ml) ( $P < 0.05$ ) when compared with sufficient rats ( $17.5 \pm 0.7662$  ng/ml). Similar results were obtained by previous experimental vitamin D deficient study (Brodie et al., 1982), which revealed that oral supplementation of anti-TB drugs, such as isoniazid and rifampicin; decrease the level of serum Vit.D via various kinds of mechanisms. However, serum Vit.D level in sufficient control rats ( $17.5 \pm 0.7662$  ng/ml) and deficient control ( $11.3$  ng/ml) showed significantly ( $P < 0.05$ ) differences in comparison to Vit.D deficient treated with Vit.D and nanoemulsion PPI+ Vit.D ( $24.14 \pm 0.9869$ ,  $29.84 \pm 2.045$  ng/ml) respectively. Similar results were obtained by (Kadappan et al., 2018). Also (Grossmann & Tangpricha, 2010) showed that the nanoemulsion significantly increased the serum 25(OH)D3 by 73% when compared to the vehicle nanoemulsion without Vit.D. (S. Jiang et al., 2019) showed in their review Vit.D in an oil vehicle produced a greater 25(OH)D response than Vit.D in a powder or an ethanol vehicle in healthy subjects. Increased restoration of Vit.D3 in micelles via in vitro digestion significantly was displayed by modified PPI-prepared nanoemulsions. So they could use nanoemulsion PPI as good choice for sending and keeping medical substances. **Figure 1 and Table 1**

### 3.2. Serum total testosterone

Serum total testosterone level showed significantly ( $P < 0.05$ ) reduction in Vit.D deficient rats ( $2.080 \pm 0.1934$  ng/ml) when compared with sufficient rat group ( $3.520 \pm 0.2267$  ng/ml). Similar result was supported by both (N.W. et al., 2017)(Guttoff et al., 2015) (Fu et al., 2017) separately they revealed that the level of testosterone decreased significantly in Vit.D deficient animals comparing to normal laboratory animals. The reason could be due to the expression of Vitamin D receptor in many reproductive organs. (Martin Blomberg Jensen et

al., 2010) found Vit.D receptor in testis, epididymis, and they suggested that the activity and survival of mature sperm depend on Vit.D, and (Elisabeth Lerchbaum & Obermayer-Pietsch, 2012), these led researchers to point of view of positive effects of Vit.D on spermatogenesis. Vit.D receptor is also exists on sperm head. However, it is lack in the midpiece and flagellum of human sperm that may be evidence about the effect of Vit.D on sperm quality (Aquila et al., 2009). In an observational study, (Menegaz et al., 2009) showed that patients suffered from azoospermic have lower serum Vit.D compared to those have normal sperm count. However, total testosterone level in serum in both Vit.D deficient rats treated with Vit.D ( $4.940 \pm 0.3124$  ng/mL) and nanoemulsion PPI+ Vit.D ( $6.470 \pm 0.2278$  ng/mL) was significantly ( $P < 0.05$ ) increased rats in comparison with Vit.D deficient ( $2.080 \pm 0.1934$  ng/mL) and sufficient control ( $3.520 \pm 0.2267$  ng/mL). Similar results were obtained by previous experimental Vit.D deficient study. (N.W. et al., 2017). Vit.D may also affect spermatogenesis before sperm release (Kinuta et al., 2000). As well as the (Pilz et al., 2011) showed that Vit.D supplementation might increase testosterone levels. However, others could not establish this association between Vit.D and serum testosterone. (Yang et al., 2012) and (E Lerchbaum et al., 2014). **Figure and 2 and Table 1.**

### 3.3. Sperm count and sperm morphology

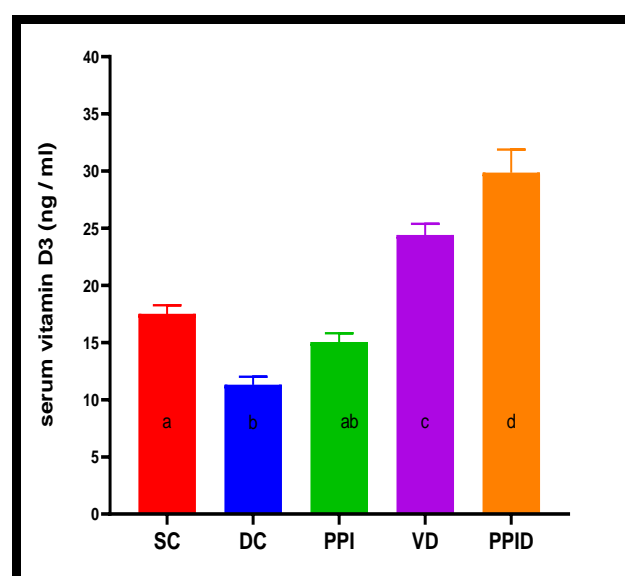
Vit.D deficient rats showed significant ( $P < 0.05$ ) dropped in the percentage sperm count ( $9.648 \pm 2.055 \times 10^6$ /ml), highly significant in the normal sperm morphology ( $P < 0.05$ ) ( $29.38 \pm 9.085$  %) when compared to sufficient control rat group. Similar results were obtained by previous study (Kwiecinski et al., 1989), (Uhland et al., 1992) (Sood et al., 1995), and (Fu et al., 2017). Furthermore (Tartagni et al., 2015). And (Zhu et al., 2016) observed that Vit.D deficient rats suffered from default of spermatogenesis due to trouble in role of Leydig and Sertoli cell the current results are closely in accordance with two previous works. (Johnson et al., 1996) and (Merke et al., 1985) revealed that Vit.D have role spermatogenesis and mature sperm due existence of Vit.D receptor in epididymis, Seroli cell and

spermatogonia of male rodents.

**Table (1) Effect of nanoemulsion PPI, vitamin-D3, nanoemulsion PPI plus and Vit.D3 in vitamin deficient rats on sex hormones (Vit.D and total testosterone) concentrations in Vit.D deficient male rats.**

Groups	Parameters (ng/ml)	
	Serum Vit. D	Serum total Testosterones
SC	$17.5 \pm 0.7662^a$	$3.520 \pm 0.2267^a$
DC	$11.3 \pm 0.7162^b$	$2.080 \pm 0.1934^b$
PPI	$15.04 \pm 0.7692^{ab}$	$2.520 \pm 0.4128^{ab}$
VD	$24.14 \pm 0.9869^c$	$4.940 \pm 0.3124^c$
PPID	$29.84 \pm 2.045^d$	$6.470 \pm 0.2278^d$

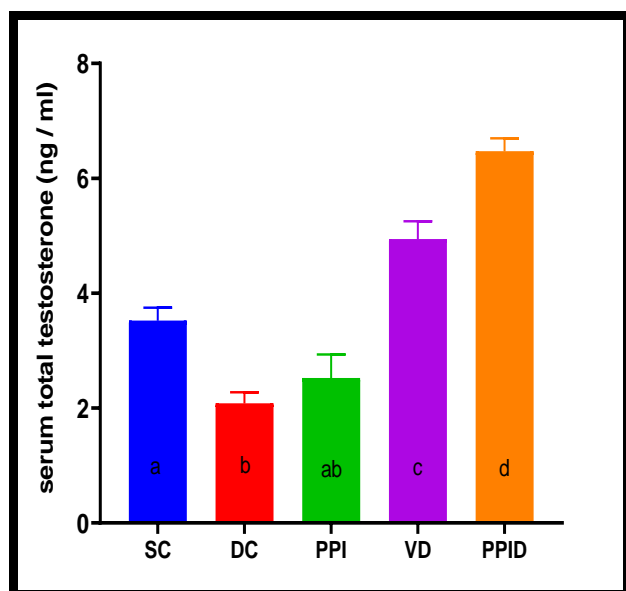
Values expressed as mean  $\pm$  S.E. The different letters mean significant differences \*  $= p < 0.05$ .



**Figure (1) Effect of nanoemulsion PPI, Vit.D and Vit.D nanoemulsion on serum Vit.D (mean  $\pm$  S.E) ng/ml in Vit.D deficient male rats.**

The different letters mean significant differences \*  $= p < 0.05$ .

Furthermore, some medical research showed the relationship between Vit.D and sperm (Zhu et al., 2016) (Hammoud et al., 2012), (Martin Blomberg Jensen et al., 2011), (Ramlau-Hansen et al., 2011), (Dong et al., 2016), (Mahmoudi et al., 2013) (Schachter et al., 1973) and (Macchia et al., 2010)



**Figure (2) Effect of nanoemulsion PPI and Vit.D and Vit.D nanoemulsion on serum total testosterone (mean  $\pm$  S.E) ng/ml in Vit.D deficient male rats.**

The different letters mean significant differences \*  $=p < 0.05$ .

Eventually, (Martin Blomberg Jensen et al., 2011) and in (Yang et al., 2012), they demonstrated separately that Vit.D was an independent factor for sperm activity and normal feature of sperm in 300 and 559 fertile and infertile men respectively. In contrast, in this consideration, paradoxical results are exist.(Yang et al., 2012) and (M. Blomberg Jensen et al., 2012).

The treatment of Vit.D deficient rats with: Vit.D and nanoemulsion PPI + Vit.D showed significant ( $P < 0.05$ ) increased in the percentage of sperm count ( $38.70 \pm 5.490 \times 10^6/\text{ml}$ ) ( $49.21 \pm 4.150 \times 10^6/\text{ml}$ ), normal sperm morphology ( $92.95 \pm 1.598 \%$ ) ( $92.95 \pm 1.598 \%$ ) in comparison with Vit.D deficient rats and ( $9.648 \pm 2.055 \times 10^6/\text{ml}$ ), ( $29.38 \pm 9.085 \%$ ). Similar result in different studies showed the same results like study of (Fu et al., 2017) who showed that the sperm count in the cauda epididymidis was significantly diminished in Vit.D deficient mice (from 24.75 million to 19.21 million).(Sood et al., 1995) showed that number of sperm was elevated significantly from 49.20 to 82.30 millions) in Vit.D deficient rats by injection of Vit.D. Sperm count and morphology in turn ability of fertilization have been rose by improving and adding amino acids to diets (Dong et al., 2016). (Mahmoudi et al., 2013) suggested that Vit.D has great role for spermatogenesis and

sperm development. Vit.D signaling has a positive increase on amount of semen(Jensen, 2014). Interestingly, the treatment of Vit.D deficient rats with nanoemulsion PPI showed significant ( $P < 0.05$ ) elevation in the percentage of sperm count and normal sperm morphology ( $49.21 \pm 4.150 \times 10^6/\text{ml}$ ) ( $94.86 \pm 0.9863 \%$ ) respectively in comparison with Vit.D deficient rats and ( $9.648 \pm 2.055 \times 10^6/\text{ml}$ ) ( $29.38\%$ ). The mechanism of the role of PPI on spermatogenesis, production normal sperm morphology and sex hormones is still not clear. However, PPI contains many types of amino acids (Overduin et al., 2015), some of these amino acids have positive effect on spermatogenesis. One of these amino acids is arginine. (Schachter et al., 1973) showed administration of Arginine to patients who suffered from deficiency of sperm in semen and low count with poor motility have been treated.

(Macchia et al., 2010) suggested that DL-aspartic acid integrates sperm production and development they showed that supplementation of DL-aspartic acid in bucks number of epididymis sperm have been increased. D-aspartic acid stimulates Leydig cell for making steroid hormone binding protein, in turn D-aspartic acid induces sperm motion (Sharpe et al., 1992). In addition the D-aspartic acid trigger cells of hypothalamus and pituitary gland to secret gonadotropic and FSH and LH hormones (Wang et al., 2002). A study revealed that testicle testosterone in rat has been increased by different concentration of d-aspartic acid (D'Aniello et al., 1996). D-aspartic acid is one of the most important components of pea protein (Overduin et al., 2015). Dietary that rich with high amount lysine contents increase live sperm concentration from 77.1 to 78.2% and methionine elevated the motility (72.7 vs 71.2%) in the second ejaculate of male rabbits(Nizza et al., 2000), (Luzi et al., 1996) showed no significant change in amount and concentration of semen from rabbits 19.7% and 14.5% crude protein.

It is difficult to comment on these results without further research on the effect of dilatory protein and amino acid. The protein level seems to have little effect on sperm quality. **Table 2, figure 3 and 4**

### 3.4. Catalase and GSH-PX activity:

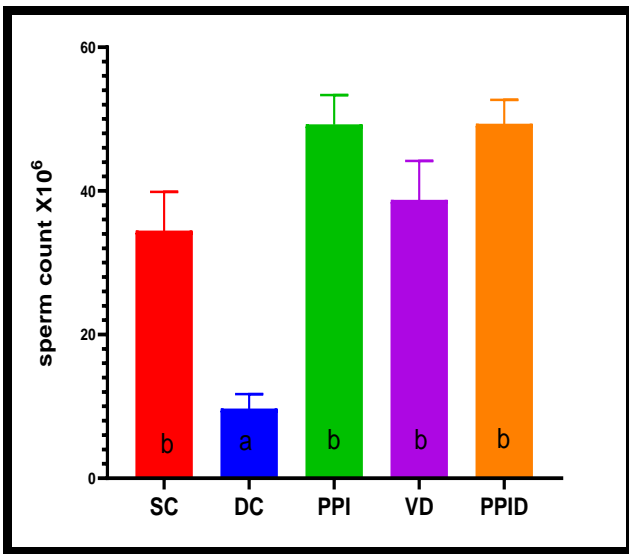
The activities of Catalase and GSH-PX in homogenized testes of Vit.D deficient rats ( $14.96 \pm 0.6849$ ) ( $520.6 \pm 46.96$ ) were not significantly

( $P < 0.05$ ) changed when compared to sufficient control rats ( $20.00 \pm 1.751$ ) ( $747.1 \pm 97.90$ ) respectively.

**Table (2) Effect of nanoemulsion PPI, vitamin-D3, nanoemulsion and PPI plus Vit.D in vitamin deficient rats on sperm count and normal sperm morphology.**

Groups	Parameters	
	Sperm count $\times 10^6$	Normal sperm morphology * %
SC	$34.43 \pm 5.428^b$	$82.92 \pm 2.939^b$
DC	$9.648 \pm 2.055^a$	$29.38 \pm 9.085^a$
PPI	$49.21 \pm 4.150^b$	$94.86 \pm 0.9863^b$
VD	$38.70 \pm 5.490^b$	$92.95 \pm 1.598^b$
PPID	$49.29 \pm 3.377^b$	$92.95 \pm 1.598^b$

Values expressed as mean  $\pm$  S.E. The different letters mean significant differences \* =  $p < 0.05$ .

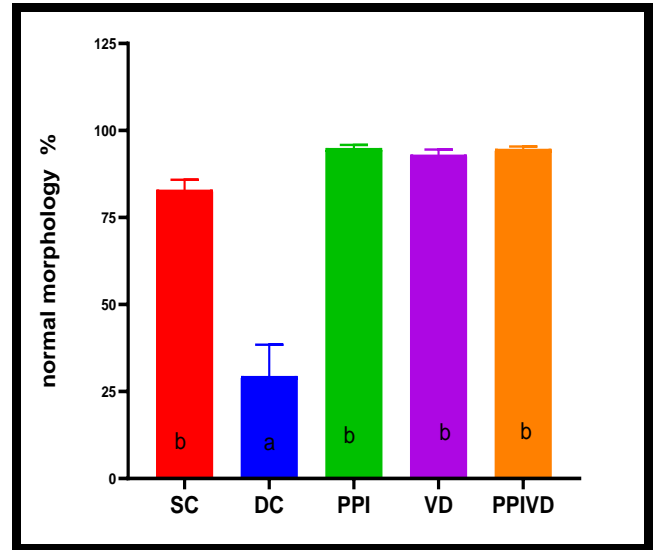


**Figure (3) Effect of nanoemulsion PPI, Vit.D and Vit.D nanoemulsion on sperm count (mean  $\pm$  S.E)  $\times 10^6$  in Vit.D deficient male rats.**

The different letters mean significant differences \* =  $p < 0.05$ .

Moreover, catalase and GSH-PX activities in homogenized testes of Vit.D deficient rats treated with nanoemulsion PPI ( $22.62 \pm 1.271$ ) ( $1053 \pm 209.2$ ), showed significantly ( $P < 0.05$ ) increased in comparison to Vit.D deficient rats ( $14.96 \pm 0.6849$ ) ( $520.6 \pm 46.96$ ) respectively. Interestingly, there are similar significant differences in GSH-PX and catalase activities in

homogenized testes of Vit.D deficient rats treated with Vit. D



**Figure (4) Effect of nanoemulsion PPI, Vit.D and Vit.D nanoemulsion on normal sperm morphology (mean  $\pm$  S.E) % in Vit.D deficient male rats.**

The different letters mean significant differences \* =  $p < 0.05$ .

Nanoemulsion PPI+ Vit.D ( $23.58 \pm 2.488$ ) ( $1023 \pm 61.34$ ), they are significantly ( $P < 0.05$ ) increased in comparison to those of Vit.D deficient rats ( $14.96 \pm 0.6849$ ) ( $520.6 \pm 46.96$ ) respectively. However, catalase and GSH-PX activities in homogenized testes of sufficient control ( $20.00 \pm 1.751$ ) ( $747.1 \pm 97.90$ ) rats showed no significant ( $P < 0.05$ ) changes in comparison to all treated groups in Vit.D deficient rats.

Many studies showed that Nanoemulsion PPI is a good diet and has high efficiency against oxidative stress. Hydrophobic and aromatic amino acids are high abundant in pea protein isolate and have antioxidant activity. furthermore PPI and look like to the modified PPI that have radical scavenging activities (Pownall et al., 2010), (S. Jiang et al., 2019), (J. Jiang et al., 2014), and (Dahl et al., 2012).

Arginine is one of the main amino acid that exists in PPI, which comprise about (4.3%) (Overduin et al., 2015). Many studies reported clearly that arginine has antioxidant activity due to a chemical moiety different from that serving as the substrate for NO biosynthesis, may lower the

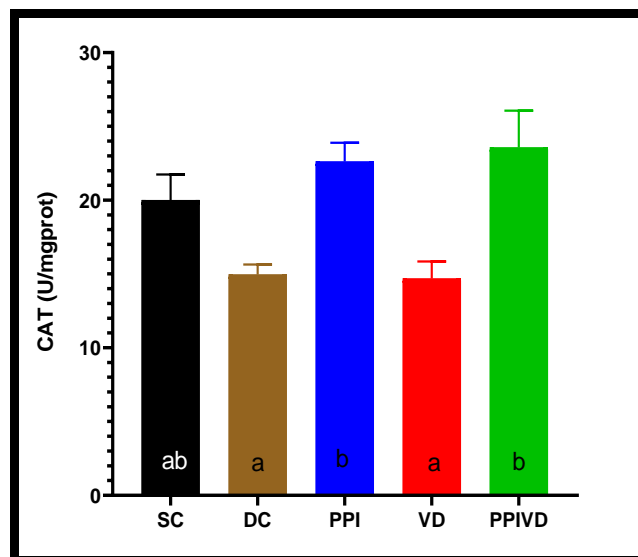
amount of free oxygen radicals and reduce superoxide anion as well as drooping intensity of radical reactions or of antioxidative–enzyme. Thus affect the lowering of oxidative stress (Wallner et al., 2001), (Hosseini et al., 2012),(Lucotti et al., 2009) and(Korish, 2010). Lysine may have ability to keep cell membrane and proteins thus Lysine has efficient in stress state. Lysine suggested that an important role in antioxidant (Seminotti et al., 2008). Even though the PPI contain little amount of tryptophan (1.0%) (Overduin et al., 2015). Tryptophan has been reported to play an important role in the DPPH radical scavenging activity of purified patatin, perhaps as a hydrogen donor (Pownall et al., 2010).

Pea protein is rich in sulfhydryl amino acid cysteine; cysteine an effective cellular antioxidant, thus extracellular cysteine is the primary source of intracellular cysteine, which is necessary for GSH synthesis. (BELL, 2000) , (Atmaca, 2004), (Han et al., 1997).(Korhonen & Pihlanto, 2005) showed that protein hydrolysates possess antioxidant activity. The current results supported by the fact that PPI have antioxidant activity because it contains many other amino acids such as histidine, cysteine, tyrosine, phenylalanine and tryptophan, that make PPI have great antioxidant capacity (Pownall et al., 2011), (Peña-Ramos et al., 2004) and (Erdmann et al., 2008). **Table 3, figure 5 and 6.**

**Table (3) Effect of nanoemulsion PPI, vitamin-D3, nanoemulsion and PPI plus Vit.D in vitamin deficient rats on the antioxidant parameters (CAT and GSH-PX activities)**

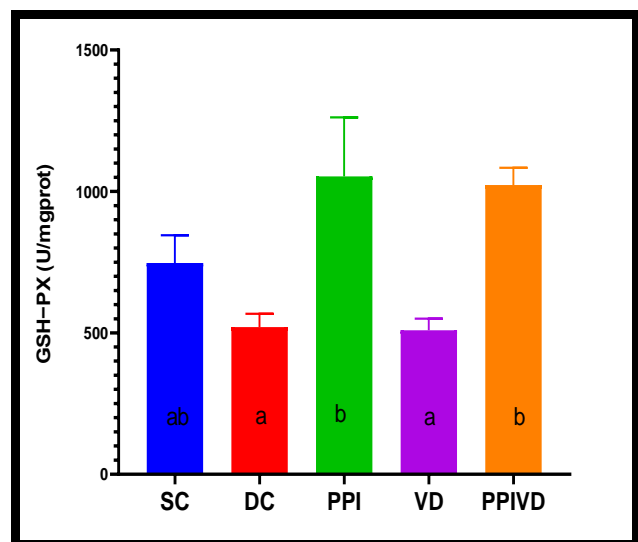
Groups	(U/mgprot)*	
	CAT activity	CAT activity
SC	20.00 ± 1.751 <sup>ab</sup>	747.1± 97.90 <sup>ab</sup>
DC	14.96 ± 0.6849 <sup>a</sup>	520.6± 46.96 <sup>a</sup>
PPI	22.62 ± 1.271 <sup>b</sup>	1053± 209.2 <sup>b</sup>
VD	14.69 ±1.165 <sup>a</sup>	509.0 ± 41.21 <sup>a</sup>
PPID	23.58 ± 2.488 <sup>b</sup>	1023 ± 61.34 <sup>b</sup>

Values expressed as mean ± S.E. The differences letters mean significant differences \* =p<0.05.



**Figure (5) Effect of nanoemulsion PPI, Vit.D and Vit.D nanoemulsion on CAT activity (mean ± S.E) U/mg protein in Vit.D deficient male rats.**

The different letters mean significant differences \* =p<0.05.



**Figure (6) Effect of nanoemulsion PPI, Vit.D and Vit.D nanoemulsion on GSH–PX activity (mean ± S.E) U/mg protein in Vit.D deficient male rats.**

The different letters mean significant differences \* =p<0.05.



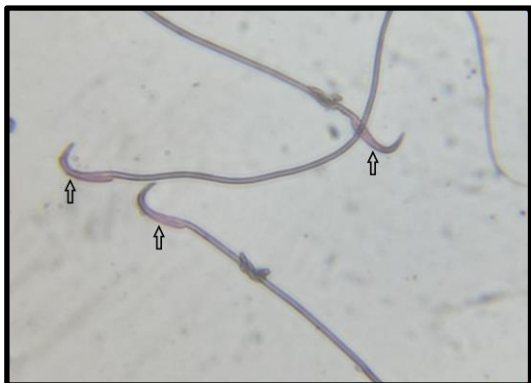


Figure 7: normal sperm morphology.



Figure 8: sperm abnormal head.



Figure 9: sperm without tail.



Figure 10: sperm without head.



Figure 11: sperm with abnormal neck.



Figure 12: sperm with abnormal hook

#### 4. CONCLUSIONS

The results of this study demonstrate that the supplementation of Vit.D deficient rats with Vit.D and nanoemulsion PPI may improve semen analysis, total testosterone and scavenger activity of testes. These findings show that Vit.D nanoemulsion supplementation resulted in a significant increase in the levels of sperm count, normal sperm morphology, as well as catalase and GSH-PX. The result showed that Vit.D nanoemulsion supplementation resulted in a significant increase in the levels of Total testosterone, sperm count, normal sperm morphology, as well as catalase and GSH-PX. Vitamin D nanoemulsion has a powerful effect on spermatogenesis in induced Vit.D deficient rats.

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