

Evaluation of Nano Selenium on Rabbit growth and Immunity

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Abbreviations: Nano-Se: Nano-Se; NZW: New Zealand White; IL-1 β : Interleukin 1beta; TNF-A α : Tumor Necrosis Factor; Se NPs: Selenium Nanoparticles; SEM: Scanning Electron Microscope; BW: Body weight; HK: House Keeping; WG: Weight Gain; NO: Nitric Oxide; APPs: Acute Phase Proteins; OECD: Organization for Economic Co-Operation and Development; SCGE: Single Cell Gel Electrophoresis

Abstract

This work was conducted to explore the potential for dietary administration of Nano-selenium (Nano-Se) versus bulk selenium (sodium selenite, SSE) in New Zealand White (NZW) rabbit and its effects on growth efficiency, hematological parameters and immune responses. Green synthesis of Se NPs was achieved using the reducing power of iron present in the fenugreek (*Trigonella foenum-graecum L*) seed extract. Sixty female rabbits (8 wk old) were grouped randomly into 5 groups; 6 rabbits each in two replicates, Se was added to the ration at two concentrations (0.1, 0.3 mg / kg). Feeding was continued for two months, then growth parameters were investigated. Rabbits were experimentally infected with *S. aureus* bacteria to investigate the effect of Se in both forms on immune system.

Results from SEM image of Nano-Se has shown that the size of the particles is >50 nm in diameter so that they are converted into nano se. Growth rates were increased in rabbits with Nano-Se feed additive compared to SSE and control. Hematological parameters showed that nano se increased WBC counts, haemoglobin levels, RBCs and neutrophils but decreased lymphocyte counts relative to bulk se.. Lysozymes displayed no clear pattern with nano / bulk se, the detected nitric oxide was higher in nano se-fed rabbits compared to control and bulk se groups. The cytokines genes expression of the rabbits innate immune response to infection (IL 6, IL 1 β and TNF- α) revealed that nano se (0.3mg/kg) increased these genes expression than all other groups. It was clear from the comet assay that there was no definite trend can be obtained to say that nano se has a risk effect on the DNA of the rabbit. It can be concluded that Nano-se supplementation has induced positive changes in blood constituents which have improved the growth performance and the immune function of growing NZW rabbits more than SSE.

Introduction

Since rabbit production makes up only a very small percentage of the total animal production in the world, there is little information available or research performed on the nutrition of the rabbit. Rabbit meat may play a great role in man nutrition. Rabbit efficiency in producing meat compares favorably with most other domestic animals and may play a significant role in solving a part of meat shorting in Egypt especially during poultry crisis as bird flu. Animals feed supplementation of minerals, some elements and vitamins to ration promotes more rapid growth (e.g. growth promoters), maintain health or stimulate an immune response and disease resistance. Selenium is an essential trace element important

as animals feed additive for many physiological processes, especially for immune and defense functions, as well as metabolism of thyroid hormones. The most important action of selenium biological functions comes from several specific seleno-proteins, some of which are involved in thyroid hormone metabolism, while others play an antioxidant defense role. Selenium is also necessary for immune function regulation and has a vital role in non-specific immune response [1,2]. The weakened immune system is a result of Se deficiency [3].

Application of Nanotechnology in the agri-food sector potentially offers huge benefits for both consumers and manufacturers in a

wide range of applications. Many of these benefits result from the ultrafine dimensions of Nano-particles, which enable them to reach new locations in the body, and thus can perform their biological functions more efficiently [4]. According to many literatures, consumers are more concerned about food produced by the application of Nanotechnology [5]. Nano-Se has attracted more attention because of its high bioavailability, high catalytic efficiency, strong adsorbing ability and low toxicity compared with selenite in rats (Jia, et al.), mice [6] and goats [7]. The immune system provides body defense against invading microorganisms as well as being importantly involved in tissue repair after injury. Immune responses can be divided into nonspecific (or innate) and specific (or acquired) immunity [8]. Non-specific immunity exists from birth and operates in a generalized fashion without regard to the exact identity of the pathogen. It serves as the body's first line of defense against invasion.

There are a wide variety of innate immune defenses, ranging from anatomic (e.g., the integrity of the skin; tears that wash out bacteria), to physiological (e.g., lysozymes in mucus cleave bacterial cell walls; acid in the stomach kills pathogens), to phagocytic (e.g., macrophages engulf and destroy bacteria) [9]. Both non-specific and specific immune responses are regulated by mediators such as cytokines. Cytokines are a diverse group of non- antibody proteins that act as mediators between cells. They were initially identified as products of immune cells that act as mediators and regulators of immune processes, but many cytokines are now known to be produced by cells other than immune cells and they can have effects on non-immune cells as well. Cytokines are currently being used clinically as biological response modifiers for the treatment of various disorders. Molecular understanding of signaling mechanisms orchestrating the immune responses is required to define new targets for future treatments of bacterial infections [10]. *Staphylococcus aureus* (*S. aureus*) is an adaptive opportunistic pathogen, capable to persist and replicate under various conditions. This microbial species causes a wide range of diseases in both men and animals [11].

In rabbits, problems of staphylococcosis arise when *S. aureus* bacteria infect small dermal lesions and invade subcutaneous tissue [12], provoking a number of lesions including pododermatitis, visceral abscesses and mastitis [13]. Occasionally, abscesses in internal organs, most commonly lungs, liver and uterus, are observed [14]. This research work studies the evaluation of Nano selenium as a feed additive in enhancing the processes of growth and immunity in rabbit (*Oryctolagus cuniculus*). Growth will be evaluated through growth parameters and measurements. Evaluation of immunity will be carried out based on molecular studies by examining some immune genes expression (interleukin 6 (IL6), interleukin 1beta (IL-1 β) and tumor necrosis factor (TNF-A α)) as a proinflammatory cytokines after *Staphylococcus aureus* experimental infection (Challenge) using the high technology of real-time PCR (qPCR).

Immunity will be assessed also on a biochemical bases exemplified by nitric oxide and lysozymes activity measurements . Finally, comet assay will be carried out to assess if there is a hazardous effects of using nano selenium in comparison to bulk selenium on rabbit tissues DNA?

Materials and Methods

Ethical Approval and Place of Experimental Work

Rabbits were handled according to the EU Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. The experimental work of the present study was carried out at the Animal Reproduction Research Institute, Giza, Egypt.

Experimental Animals, Batteries , Feed and Management

Sixty female White New Zealand rabbits were purchased from El-fayoum farm, 8 weeks old, with average weight of 1 to 1.5 kgs and housed in batteries in a special lab. The rabbit room was naturally ventilated by wired windows and provided with sided fans. Each cage had its feeder and automatic nipple drinker, feed and water were offered. All animals were kept under the same managerial, hygienic and environmental conditions and were maintained and treated according to the accepted standards of animals treatment (Figure 1). Rabbits were vaccinated with viral immunization (SER-VAC RHDV Vaccine) which was given by Pathology department, Animal Reproduction Research Institute, Giza, Egypt.

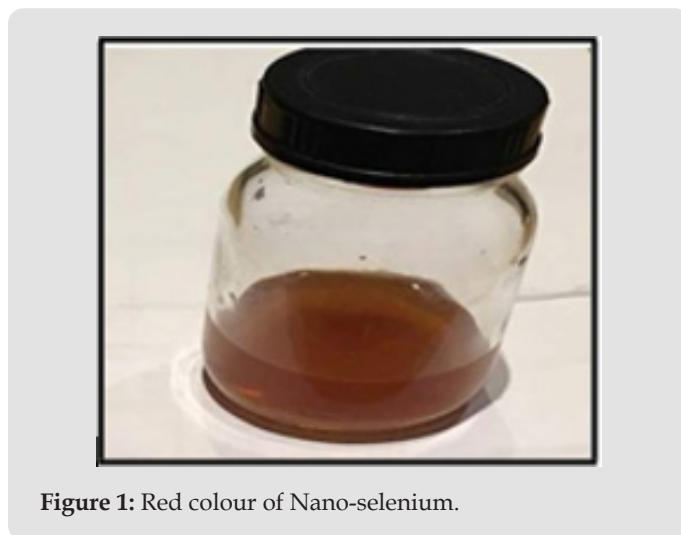


Figure 1: Red colour of Nano-selenium.

Feed and Feed Supplements

The experiment was conducted, and ration was purchased with factorial arrangement of treatments, being selenium additive-free rabbit ration. Ingredients and chemical analyses of the basal diet are presented in Table 1 following NRC (1977) recommendations.

Selenium Additive

In the present study, selenium bulk (Sodium Selenite) was supplied by the Egyptian National Research Center. The use of Green

synthesis to generate selenium Nanoparticles was demonstrated so, the procedure is free from the drawbacks of using any chemicals. Green synthesis of selenium Nanoparticles (Se NPs) was achieved according to Ramamurthy, et al. [15], by a simple biological procedure using the reducing power of iron present in the fenugreek (*Trigonella foenum-graecum L*, Fabaceae family) seed extract. The plant is cultivated worldwide as a semi-arid crop (Table 1).

Table 1: Ingredients and chemical composition of the basal diets.

Feed Ingredients	Percent %
Yellow corn	14
Barley	8.0
Soybean meal	7.0
Wheat bran	26.2
Alfalfa hay	40.0
Molasses	2.0
Dicalcium phosphate	1.2
Limestone	1.0
Sodium chloride	0.2
Vit. Min. Premix	0.4
Total	100
Digestable energy (Kcal/kg)	2687
Crude Protein (%)	16.20
Crude Fiber (%)	13.20
Ether extract (%)	3.00
Calcium (%)	1.20

Fenugreek seeds were purchased from a super market then washed well and left overnight to dry, dried seeds were crushed well and turned into powder, five grams of the powder were added to 500 ml distilled water (DW) and left for 24 hrs to obtain extract. Filtration of the solution was carried out by using filter paper to obtain a pure extract. A mixture of 0.02 gm of selenium + 2ml ethanol + 1ml ascorbic acid was prepared and two ml of the fenugreek extract were added to the mixture prepared. The solution was shaken well then placed in a dark bottle at room temp for 24hrs. After 24hrs, the extract was turned from white into red color (Figure 1) and selenium was expected to be converted into Nano selenium. Chitosane added to Nanoselenium as stabilizer (Figure 1).

Scanning Electron Microscope Analysis

Scanning analysis using scanning electron microscope (SEM); JEM-2100) was carried out in the National Research Centre, images were taken for the analysis of size and shape of SeNPs. The size of selenium Nanoparticles was <50 nm.

Experimental Design

Selenium was added to the ration in the designed rates, Rabbits (no. 60) were housed in grow-out batteries with access to water and commercial feed, they were randomly divided into 5 groups (Control and 4 treatments). Se was added to the ration in two concentrations (0.1 and 3 mg/kg). Feeding was continued for two months.

Growth Parameters Estimation

Body weight (BW) was measured at the beginning of the experiment (8th week of age). Growth performance was calculated as weight gain through an equation:

$$\text{Weight gain (WG \%)} = \frac{(\text{final weight} - \text{initial weight}) \times 100}{\text{initial weight}}$$

Challenge Test

To evaluate the immunity function in rabbits after feeding for 2 months on bulk and Nano-forms of selenium supplemented feed, a challenge test was carried out through *Staphylococcus aureus* experimental bacterial infection in rabbits and examination of some immune genes expression.

Staphylococcus aureus

Staphylococcus aureus was supplied by Microbiology department of animal reproduction institute. The bacteria cultured and the bacterial density was determined by McFarland's tube. The strain of *S. aureus* was grown in 250 ml of brain heart infusion broth. Heat shock stress conditions were imposed as the culture broth was incubated at 25, 37 and 50 °C respectively. At each temperature, samples were taken at intervals after heat shock, and crude cell extracts were prepared.

Experimental Infection

Rabbit groups were immunized by experimental infection with sub lethal pathogenic bacteria *Staphylococcus aureus* (8×10^8 CFU).

Clinical Investigations

Rabbits were clinically investigated for disease symptoms and investigated for the internal organs and tissue lesions.

Samples Collection

Blood Sampling and Analysis of Some Immune Response:

Three days after the experimental infection of different rabbit groups under investigation, blood samples were collected from the marginal ear vein of the immunized rabbits into plain sterile glass tubes; EDTA supplemented for blood count. and free tubes for serum analysis. Blood samples for serum analysis were left to clot, then centrifuged (4000 rpm) for 10 min; the clear serum was collected and stored at -20°C until the subsequent assessment of cellular (nitric oxide and lysozymes). The animals were then euthanized and liver and spleen samples were collected and kept in RNA lysis buffer and stored at -80°C for further RNA extraction and Real-Time PCR analysis and immune genes expression quantification.

Methods of Analysis

Estimation of Serum Nitric Oxide and Lysozymes Levels:

Measurement of nitric oxide was assessed according to the assay described by Ramadan, et al. [16].

Molecular studies (IL6, IL1-β and TNF-A Genes expression):

Gene expression was assessed using Real-Time PCR, RNA Extraction was carried out using Gene-Jet RNA Purification Kit following the manufacture’s recommended protocol, cDNA Synthesis: using Maxime RT PreMix Kit and Polymerase Chain Reaction (Real-time PCR) was carried out using TaqMan Universal Master. NCBI website (<http://www.ncbi.nlm.nih.gov/>) was used for searching for the target genes; interleukin 6 (IL 6), interleukin 1-β (IL1-β) and tumor necrosis factor alpha (TNF-α) sequences and the house keeping (HK) gene (rabbit GAPDH). Primers and probe were designed using NCBI Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and GenScript (<http://www.genscript.com/>) (Table 2). Rotor-Gene Q - QIAGEN instrument was used with 36 wells using Qiagen PCR Tubes for quantification of gene expression in both target and housekeeping genes (Table 2).

Table 2: Illustrates the sequences of both the target and housekeeping genes.

Rabbit Genes	Sequence(5'→3')	Strand
Interleukin 6 (IL 6)	AGGCCAAGTTCAGGAGTGAC	F
	CTTGAGGGTGGCTTCTTCAT	R
	TCAGCACCATGCTGACCCTGG	P
Interleukin 1β IL 1- β	CCTGCAGCTGGAGAGTGATAG	F
	GTGCTGATGTACCAGTTGGG	R
	CAAGCTGGAATTTGAGTCTGCCCA	P
Tumor necrosis factor (TNF-A)	TGTGCCTCCCTTCACTTATG	F
	AGTAGGGCGGTACCAACAC	R
	TGACCCGTCGCTACATCACCG	P
GAPDH	TTCCAGTATGATTCCACCCA	F
	GATCTCGCTCCTGGAAGATG	R
	TCTCAGCCTTGACCGTGCCG	P

F denotes to a forward, R denotes to reverse. L denotes to forward primer, R denotes to reverse primer, P denotes to Probe. Probes are modified with 5’Fam - 3’Tamra as (reporter- quencher).

Assessment of Genotoxicity in Rabbit Tissues by the Single Cell Gel Electrophoresis (SCGE)/ Comet Assay: The comet assay was carried out according to the technique described by Singh, et al. [17], a protocol that can be used to detect DNA damage.

Statistical Analysis

The obtained data in the present study were statistically analyzed using the computer program (SPSS version 15 for Windows), and comparison were made using one-way ANOVA.

Results

The scanning electron microscope (SEM) image of Nano-Se is shown in Figure 2. It can be seen that the particle sizes is > 50 nm in diameter; they were spherical in shape and there were some agglomerations among the NPs.

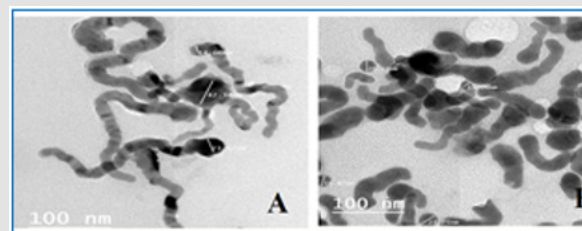


Figure 2: TEM image of Selenium Nanoparticles with diameters < 50 nm; A: 14.6, 42.34 and 23.62 nm, B: 11.06, 20.23 and 18.87nm.

Rabbit Mortality Rates

No mortalities were recorded along the course of the experiment in all the control and the challenged groups.

Clinical Investigation after the Experimental Infection

Experimentally infected rabbits showed some illness exhaustion, and lack of appetite for food, examination of the internal organs revealed cases of lung hematoma and some hemorrhagic patches in 40% of the cases. Macroscopic examination revealed deeply red consolidated area in the lungs of the infected rabbits (Figure 3).



Figure 3:
A. Lung hematoma,
B. Haemorrhagic pneumonia and
C. red consolidated areas.

Growth Performance in Rabbits

The effects of feeding diets supplemented with selenium and Nano-Selenium for two months on weight gain in rabbits are presented in Figure 4. The effects of dietary Se supplementation on the growth performance of NZW rabbits showed that, among the tested groups of conventional and Nano- Se supplementation, it was noticed that; the weight gain of rabbits increased than the control group which was fed on ration without any supplementation. There was a positive relationship between the increase in the

concentration of Se and the weight gain in groups fed on Se supplemented feed (Either bulk selenium or Nano selenium) with observation that Nano-Se supplementation enhanced the growth rates more than bulk selenium in respect to the two concentrations

(0.1 and 0.3mg/kg.) with observation that 0.3 mg/kg Nano-Se enhanced the growth rates better than the concentration of 0.1 mg/kg (Figure 4 & Table 3).

Table 3: Blood hematological parameters of New Zealand (NWZ) rabbit groups fed with test diets for 8 weeks.

Item	Test Diet				
	Control	Bulk Se (0.1mg/kg)	Bulk Se (0.3mg/kg)	Nano Se (0.1mg/kg)	Nano Se (0.3mg/kg)
Hb (g/100 mL)	14.7 ± 0.37	14.9 ± 0.36	9.63 ± 0.38	15.8 ± 1.60	17.6 ± 1.3
RBCs (× 10 ⁶ /μL)	4.2 ± 0.24	4.32 ± 0.13	4.39 ± 0.17	4.72 ± 0.63	6.2 ± 3.2
PCV (%)	43.33 ± 2.40	43.56 ± 1.73	44.00 ± 1.73	47.33 ± 5.93	51.23 ± 4.23
MCV	96.34 ± 0.41	97.17 ± 1.19	97.66 ± 0.13	97.74 ± 1.11	98.11 ± 5.51
MCH	32.03 ± 1.53	30.91 ± 0.10	30.26 ± 0.45	30.69 ± 1.26	32.43 ± 1.7
MCHC (%)	32.88 ± 3.33	31.80 ± 0.49	31.15 ± 0.51	31.45 ± 1.18	32.11 ± 82
WBCs (× 10 ³ /μL)	9.600 ± 2.31	7.3 ± 2.51	9.99 ± 0.39	10.5 ± 5.31	12.8 ± 2.71
Neutrophils (%)	10.00 ± 0.58	9.00 ± 1.03	11.00 ± 0.58	14.33 ± 2.85	14.77 ± 6.41
Lymphocytes (%)	82.00 ± 1.00	82.00 ± 1.19	82.00 ± 1.11	76.33 ± 2.19	81.93 ± 1.13
Monocytes (%)	7.33 ± 0.88	8.00 ± 0.00	6.50 ± 0.21	8.00 ± 0.58	9.1 ± 1.42
Eosinophils	0.33 ± 0.33	0.50 ± 0.29	0.50 ± 0.52	1.00 ± 0.00	0.35 ± 6.11
Basophils	0.43 ± 0.22	0.50 ± 0.29	0.00 ± 0.00	0.33 ± 0.33	0.41 ± 0.22

Values expressed as means ± SE (n = 3). Different superscript letters indicate significant differences for each pair comparison.

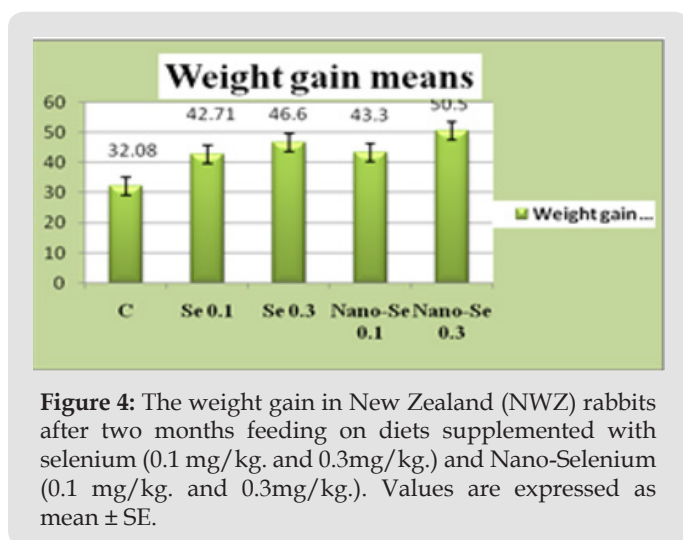


Figure 4: The weight gain in New Zealand (NWZ) rabbits after two months feeding on diets supplemented with selenium (0.1 mg/kg. and 0.3mg/kg.) and Nano-Selenium (0.1 mg/kg. and 0.3mg/kg.). Values are expressed as mean ± SE.

Blood Hematological Parameters of Tested New Zealand (NWZ) Rabbit Groups

Table 3 illustrates the effect of utilizing different Se forms (Nano and bulk) as a feed additive in the New Zealand (NWZ) rabbit. In the present study, blood hematological parameters was found to be affected by Se additive where nano se increased the WBC counts, hemoglobin levels, RBCs and neutrophils values and some decrease in the Lymphocytes count when compared to bulk se.

Lysozymes Analysis and Nitric oxide Production Analysis

Data of Lysozymes and nitric oxide production in the present study is illustrated in Figure 5. It was noticed that no specific or regular form of immune response was observed in the form of

lysozyme using the feed additives of selenium, in the two forms of nano and bulk Se and different concentrations, where it was clear that immunized rabbit groups that were fed on Nano selenium produced higher increase in the mean values of serum nitric oxide production three days post vaccination (72.53 & 70.05 μg/ml) for Nano selenium additives concentrations (0.1 mg/kg and 0.3 mg/kg, respectively) in comparison with vaccinated non treated control group (35.17 μg/ml) and immunized groups that were fed on bulk selenium (44.98 and 46.4 μg/ml for bulk selenium additives concentrations (0.1 mg/kg and 0.3 mg/kg, respectively) (Figure 5).

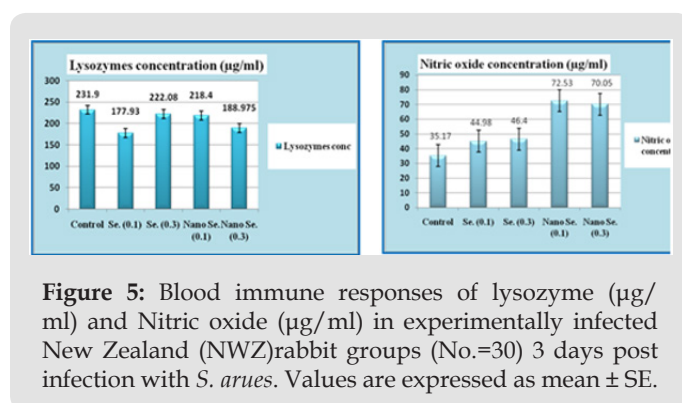


Figure 5: Blood immune responses of lysozyme (μg/ml) and Nitric oxide (μg/ml) in experimentally infected New Zealand (NWZ) rabbit groups (No.=30) 3 days post infection with *S. arues*. Values are expressed as mean ± SE.

Expression of IL6, IL1-β and TNF-A Genes Response to the Bacterial Infection

Real time PCR results were analyzed, for all samples, dissociation curves were acquired for quality control purposes. In addition, amplification products were visualized by agarose gel electrophoresis. For gene expression quantification, we used the comparative Ct method. First, gene expression levels

for each sample were normalized to the expression level of the housekeeping gene encoding GAPDH within a given sample (ΔCt); the difference between the treated groups (Fed on selenium bulk and Nano) compared to the control (feeding without any selenium supplementation) was used to determine the $\Delta\Delta Ct$. The $\log_2 \Delta\Delta Ct$ gave the relative fold change in the gene expression of the test versus the control condition. The relative changes in mRNA transcript levels for IL6, IL 1 β and TNF- α were presented in Figure 6. The results clearly demonstrated that the mRNA expression of

the three genes under investigation was increased in tissues of rabbits fed on ration supplemented with Nano Se in comparison to rabbits fed on feed supplemented with bulk selenium within the same concentration of selenium used either 0.1 or 0.3 mg/kg, but the values were comparable between the 0.1 mg/kg Nano Se group and the 0.3 mg/kg bulk se group. All Se treated groups revealed better expression of studied genes when compared to the control group with observation that groups fed on Nano Se gave higher expressions over the other groups (Figure 6).

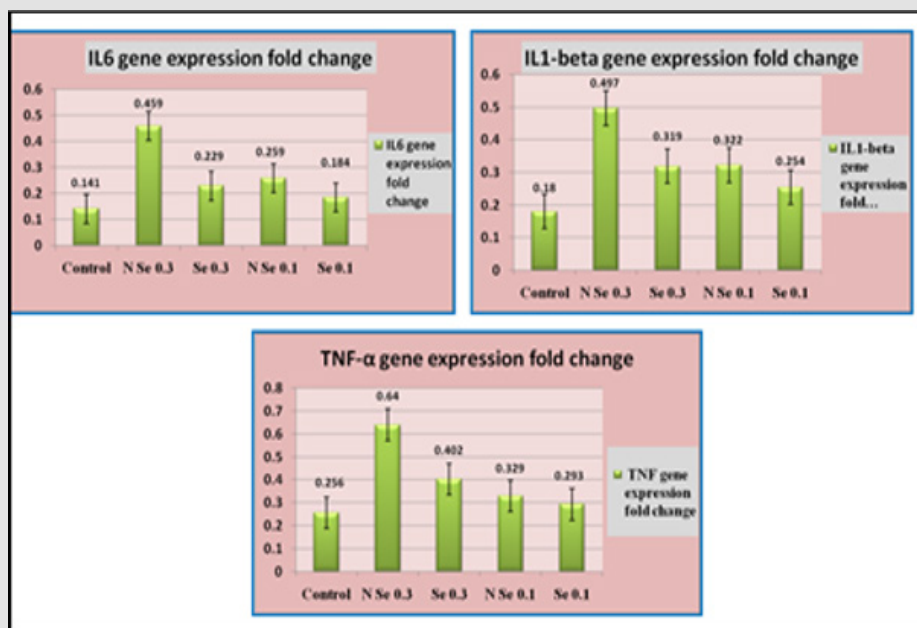


Figure 6: IL 6, IL 1 β and TNF- α genes expression in experimentally infected New Zealand (NWZ) rabbit groups (No.=30) 3 days post infection with *S. arues*. Values are expressed as mean \pm SE.

Comet Assay Data Analysis

Comet assay or single cell gel electrophoresis has been shown to be a simple and sensitive genotoxic approach for evaluating DNA damage (Frenzilli, et al.). In this study, comet assay was carried out to assess the hazard effects on DNA after using Nano selenium in comparison to bulk selenium. Results indicated that there are a rate of comet cells among different rabbit groups (Figure 7) ranged between 13.15% and 17.08% with a comparable values (Figure 8). There is an observation that the percent of comet cells was higher

in rabbits group which were fed on ration supplemented with Nano selenium (Concentration = 0.1 mg/kg feed) but still near the rate in other groups even the control (Figure 8 & Table 4). Concerning the tail length and DNA percent in comet tails, Table 4 illustrates that the tail length was lower in comet cells of rabbits fed on feed supplemented with se than the control which had a feed without se. Moreover, the shortest tail length appeared in cells of animals fed on nano se concentration (0.3mg/kg). The DNA percent in the tail showed irregular image and had no specific trend.

Table 4: The effects of selenium feed additive on rabbits Genotoxicity using comet assay (mean \pm S.E): Comet data analysis.

Group conc (mg/kg)	Comet %	Tail Length (px) Mean \pm SE	%DNA in Tail	Tail Moment	Olive Moment
Control	13.37 \pm 1.16a	7.34 \pm 0.94a	9.8 \pm 2.11	0.65 \pm 5.03	1.2 \pm 3.26
Se 0.1	15.075 \pm 2.43	6.3 \pm 0.77 a	9.57 \pm 36.22	0.78 \pm 0.97a	1.18 \pm 0.84
Se 0.3	13.15 \pm 2.35a	6.78 \pm 1.27	9.82 \pm 0.79	0.82 \pm 2.11a	1.33 \pm 17.3
N Se 0.1	17.08 \pm 0.86	6.59 \pm 1.15	9.85 \pm 5.13	0.91 \pm 32.1	1.32 \pm 19.64
N Se 0.3	15.03 \pm 4.17a	5.68 \pm 2.7	9.34 \pm 9.06	0.69 \pm 0.99	1.19 \pm 8.66

Data are shown as mean \pm SD. 1px is equal to 0.24 μ m for unit conversion.

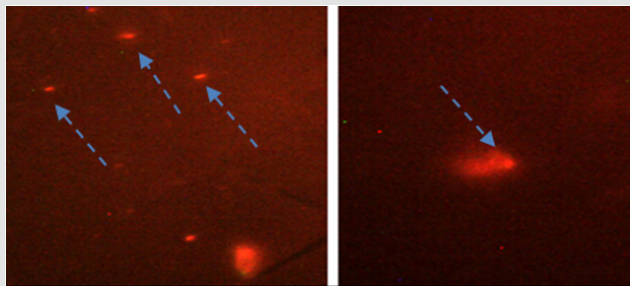


Figure 7: Comet cells present in different rabbit groups.

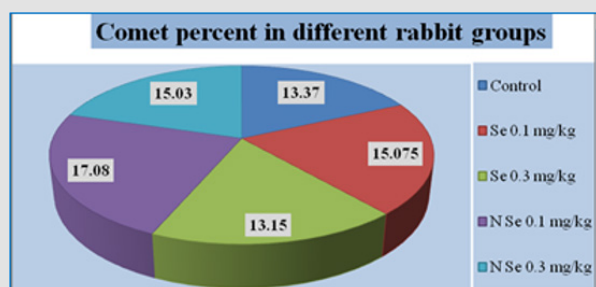


Figure 8: Comet cells percent in different New Zealand (NZW) rabbit groups.

Discussion

Se NPs were synthesized from sodium selenite by a simple and eco-friendly approach using fenugreek plant extract as an effective reducing and stabilizing agent. On addition of fenugreek extract, the sodium selenite solution changed to yellow within a minute and attained to red colour within few minutes. This indicates synthesis of Se NPs [18], also SEM indicated the nano particles of se. Concerning the mortality rate, no deaths of rabbits occurred during the duration of this study. The results indicated positive effects of using nano-selenium supplementation to enhance growth performance of growing NZW rabbits more than those of sodium selenite or bulk selenium, this result is consistent with a previous results of Emara, et al. [19], where they found that dietary supplementation of nSe revealed heaviest live body weight and highest WG and the lowest mortality rate when compared to the control and bulk Se diets, suggesting a better absorption and higher bioavailability of nano-Se. However, previous studies reported that dietary supplementation of Se at 0.1 ppm produced an improvement in fetal and birth weight of offspring in rabbit does, but the level of 0.3 ppm was not effective [20].

Ebeid, et al. [21] reported that feeding male California rabbits on diets supplemented with organic Se (0.15 or 0.30 ppm) resulted in an increase in live body weight and daily weight gain compared with their control counterparts and these results are consistent with the results of this work. The different physiological effects of Nano-Se and sodium selenite may be attributed to the different absorption process and metabolic pathways. It has been reported that the superior performance of nanoparticles may be attributed

to their smaller particle size and larger surface area, increased mucosal permeability, improved intestinal absorption and tissue depositions, while nanoparticles show new transport and uptake characteristics and exhibit higher absorption efficiencies [22]. Mohapatra, et al. [23] found that feeding diets supplemented with nano Se up to 0.30 mg/kg from 9 to 20 weeks of age positively affected feed conversion ratio of pullets but when the level of added nano Se reached 0.60 mg/kg FCR was negatively affected, this in part is matching our results where, nano Se raised the growth rates but was positively consistent with the increase of concentration.

The improvement in the performance of rabbits due to Se supplementation may be due to that decreases the oxidative stress and helps in the scavenging of ROS from the tissues, because Se is a cofactor for many antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase [24]. However, data of this work in investigating growth rates resulted from feeding on nano Se feed additive was inconsistent with the study of Dokoupilova, et al. [25] who noted that added Se (Se-enriched yeast) did not affect FCR of growing rabbits. Also, Dorra, et al. [26] found that addition of nano-Se had no significant effect on body weight gain (WG) of rabbits at all age intervals and the whole experimental period. In general, these differences in results and opinions could be due to the species-specific differences or the differences in diet contents and the nano material types and levels [27]. The contradictory findings of different scientific literature studies on the responsiveness of growing animals to dietary supplementation with nano selenium may also be attributed to a variety of factors such as species, genotype, age, dietary composition (amino acid profile), length of analysis, nano selenium processing, and dose used.

Three days after the experimental infection with *S. aureus*, external clinical investigations showed have showed some illness symptoms such as lethargy, fatigue, and lack of appetite for food in infected animals that are susceptible to *S. aureus* Bacterial disease. Examination of the internal organs revealed cases of lung hematoma and some hemorrhagic patches, macroscopic examination revealed deeply red consolidated areas in the lungs of the infected rabbits. Rabbits are important natural hosts of *S. aureus*, . The strains in rabbits can be divided into low virulence and high virulence. Infection of low-virulence strains is limited to a few birds, while infection of high-virulence strains indicates an outbreak spread through the entire flock causing major economic losses [28]. *S. aureus* poses a major threat to rabbit farming. Manifestations of infected rabbits include subcutaneous abscesses, mastitis and pododermatitis [29]. At the end of August 2017, a serious respiratory disease broke out in a rabbit farm with about 1,000 adult female rabbits in the Fujian Province of south-eastern China, killing approximately 1,000 rabbits in a four-week timeframe.

Infected rabbits had clinical symptoms of weight loss, coughing and purulent nasal discharge, haemorrhage in trachea and purulent pneumonia was found in dead rabbits [30], These results are

consistent with our findings in the concern. Blood hematological parameters was found to be affected by Se addition where nano se increased the WBC counts, hemoglobin levels, RBCs and neutrophils values if compared to bulk se. When comparing the effect of SeNPs (red selenium) (orally 1 mg•kg⁻¹ BW) and inorganic selenium (orally 1 mg Se•kg⁻¹ BW) on the antioxidant function of neutrophils and haematological parameters in sheep for 30 days, it was found that thiobarbituric acid-reactive levels were significantly higher in both groups than in control animals on the 30th day, in contrast to the predicted decline. There were no major variations between the amount of packed cells and the number of red blood cells in the experimental and control groups. The white blood cell count in the Nano-Se group showed a substantial increase on the 20th and 30th days and in the Na₂SeO₃ group on the 20th day compared to the control group. There was also a substantial rise in neutrophil count and a large decrease in lymphocyte count on day 10 in the Nano-Se group compared to the second experimental group and control group and on days 20 and 30 in all experimental groups compared to the control group [31].

Differences between studies can be explained as the fact that combined synergistic effects of additives might be different from individual effects. Selenium has been shown to be commonly concentrated in organs of immune response such as liver, lymph nodes, and spleen [32]. To rise immunogens; treatment with Se may increase the production of antibodies and broaden complementary responses via various mechanisms that include different selenoproteins. Nitric oxide (NO) is an important molecule which is a general characteristic of activated macrophages [33]. Nitric oxide (NO) initially described as a physiological mediator of endothelial cell relaxation. It is an intercellular messenger and has been recognized as one of the most versatile players in the immune system. Immediately after engulfment of the bacteria, inducible nitric oxide synthase enzyme is expressed in the phagocytic cells (Antoine, et al.) and the levels of NO sharply increase. In the current study dietary administration of nano Se to immunized rabbits resulted in a substantial increase in mean values of serum nitric oxide production, approximately two-fold and approximately 1.6-fold, compared to rabbits fed on feed supplemented with bulk selenium two days post-immunization.

The current NO results are in opposition to those of Abd-Allah, et al. [34], who recorded no differences in NO level due to Nano-Se, although ours were significantly high (72.53 µg/ml and 70.05 µg/ml for the concentrations 0.1 mg/kg & 0.3mg/kg respectively) and about twice the effect of the bulk Se (44.98 µg/ml & 46.4 µg/ml for the concentrations 0.1 mg/kg & 0.3 mg/kg respectively) and to the control (35.17 µg/ml). Lysozymes is an active molecule in the defense system of the organisms which is secreted by neutrophils and phagocytes. Lysozyme is responsible for lysing pathogenic bacteria with the key feature of pathogen restriction by targeting and disrupting the glycoside bonding of the peptidoglycan substrate [35]. Lysozyme activity is known to be part of a non-specific immune re-

sponse and is found in leukocytes. It is a natural enzyme that acts as an antimicrobial and has had an immune modulating effect. It serves as a non-specific defensive mechanism, representing the behaviour of macrophages [36]. The activation of phagocytic activity would therefore be reflected in the activity of the serum lysozyme.

The present study has revealed that, dietary administration of Se additive for 60 successive days, either nano or bulk showed revealed no specific trend of elevating of the values of lysozymes, moreover the control had higher values in all groups. Carroll, et al. [37] in their study reported that, Although normal rabbit serum and plasma serum also contain identical (though reduced) levels of lysozyme, they differ significantly in their bactericidal ability. These results clearly indicate that the predominant antibacterial element in rabbit serum is not lysozymes and that the exact source of serum bactericide in these animals is not the same. They continued to indicate this explanation as Further evidence that lysozyme is not the primary bactericidal agent in rabbit serum comes from an examination of the purified enzyme in the standard killing assay. When tested at concentrations equivalent to those in 2 to 5% serum or plasma, rabbit lysozyme exhibits little bactericidal activity. In another study of Weksler, et al. [38] they said that, differences observed for rabbit serum and plasma serum indicate that activity is “released” from cellular elements during coagulation or immune injury, and several investigators have presented evidence suggesting that platelets are the source of rabbit serum bactericides.

The role of lysozyme in host defense against infection remains relatively elusive although elevated circulating lysozyme concentrations were evidenced after antigen stimulation [39]. In the same way, Pawlikowska, et al. [40] reported that lysozymes concentrations increased before the efficient production of specific antibodies. Georgieva, et al. [41] in their study to evaluate the changes in the concentrations of major blood proteins associated with experimental E. coli infection in New Zealand rabbits revealed that E. coli infected rabbits exhibited considerable increases of the serum lysozymes concentrations on days 11 and 18, confirming the role of this protein in the innate immunity and that lysozyme can be considered as a late major positive acute phase proteins (APPs) with its low values, data from our work is consistent with those studies and it could be proved that the low values of lysozymes in this study may be due to that lysozymes is a late major positive acute phase proteins (APPs) and there are other immune elements that had the main role as a bactericidal agent.

The relative changes in mRNA transcript levels for some innate immune genes exemplified by IL6, IL1β and TNF-α were analyzed. Results clearly demonstrated that the mRNA expression of these genes was unregulated or increased in spleen and liver of the rabbits fed on nano Se more than bulk organic Se when compared with the control group. These results revealed feeding on nano Se supplemented diet, at 0.3mg/kg showed the highest expression status compared with the other protocols. Such amelioration of

proinflammatory cytokines expression as an immune response to bacterial infection can be ascribed to the activation effect of Se on signaling pathways that react with the signal transduction of Toll-like receptors in monocytes and thereby stimulate the secretion of pro-inflammatory cytokines [42]. In parallel with our result, Schnupf, et al. [43] reported that, during the natural infection, *Shigella* infection of rabbit ileal loops led to a large increase in mRNA abundance for the pro-inflammatory cytokines IL-1b, TNF- α , IL-6, IL-4, IFN- γ and IL-8.

Using nano ZnO as a feed additive in rabbits, they said that the administration of zinc nanoparticles to growing rabbits as an alternative to conventional zinc sources could elicit favorable influences on growth performance, serum parameters and splenic expression of IL6. Also, a study conducted by Sundaresan, et al. [44] reported that the expression of IL6 in the ovary and oviduct of hens was up regulated significantly during zinc-induced molting. Experimental infection with a sub-lethal dose of *Staphylococcus aureus* bacteria caused increased expression of IL1- β after two days of the experimental infection. Raw real-time threshold cycle (Ct) data showed an improvement in IL1- β expression in rabbit groups supplemented with nano Se in their feed more than in the bulk selenium and the control groups. Also, the nano selenium concentration 0.3 mg/kg revealed higher value more than 0.1 mg/kg. In line with the result obtained from this study concerning the higher rate of NO in case of dietary supplementation of nano Se, iNOS is involved in immune response and is regulated by cytokines and determined primarily by the novo synthesis and stability of iNOS mRNA, the expression of interleukins was found to be higher also in case of utilizing the nano Se, this may indicate the same trend of the proinflammatory cytokines and the NO production [45].

Genotoxicity endpoints are crucial in assessing the safety of chemicals. The Organization for Economic Co-operation and Development (OECD) has published guidelines for several validated and standardized *in vitro* and *in vivo* methods including genotoxicity assays covering different endpoints. It is clear that the methods and standard procedures used to identify the toxicity of chemicals may not be entirely sufficient for the safety evaluation of NMs. NMs can interfere with test components or detection systems for standard toxicity tests [46]. In 2006, the OECD reviewed the Manufactured Nanomaterials (WPMN) with the goal of updating the OECD guidelines on genotoxicity and evaluating their suitability for NMs. Magdolenova, et al. [47] analyzed the genotoxicity methods used in 112 papers published between 2000 and 2012 on the possible genotoxicity of NMs. Similarly, Azqueta, et al. [48] reviewed 102 papers evaluating the genotoxicity of NMs with possible use in medicinal products. Based on the findings of both reviews, where the authors of this paper were personally involved, the comet assay and the micronucleus assay are the most commonly used *in vitro* and *in vivo* techniques, the comet assay being the most commonly used *in vitro* and the micronucleus assay *in vivo* studies.

In this work, assessment of genotoxicity by the single cell gel electrophoresis (SCGE)/ comet assay was carried out to assess if there is any hazard effect in using nano se supplement. Dietary supplementation of nano se versus bulk organic se for rabbits produced comparable rates of comet cells like the control after 60 days post immunization. With regard to tail length and DNA percent in comet tails, Table 4 shows that the tail length in comet cells of rabbits fed on feed complemented with se was lower than the control which had se-free feed. In addition, the shortest tail length was observed in cells of animal that fed on nano-se (concentration 0.3 mg / kg). The DNA percentage in the tail displayed an unusual picture and had no clear pattern. As SeNPs have been shown to be a promising agent to regulate chronic toxicity caused by exposure to heavy metals. Prasad, et l. [49] studied the effects of T-synthesizing SeNPs. Arjuna leaf extract on human lymphocytes treated with arsenite (As+III) [50-55].

Studies of cell viability using MTT assay and DNA damage using comet assay showed the protective effect of SeNPs against As+III-induced cell death and DNA damage. This approach may be used in the future to mitigate ROS-induced arsenic-mediated toxic hazards, especially in areas with arsenic-contaminated groundwater and the prevalence of arsenicosis. However, if nano-selenium has been used as a protective agent against induced cell death and DNA damaging materials, it is a fact that it does not have the character of causing cell death or DNA damage. Finally, We may infer that Nano-se supplementation is capable of causing positive changes in the blood composition, making it possible to boost the growth efficiency of growing NZW rabbits and improve its immunity more than supplementation with bulk Se [56-60].

Conclusion

From this study it could be mentioned that administration of Se nanoparticles to growing rabbits as an alternative to conventional Se sources could improve growth performance, immune system function utilized by some hematological parameters, serum No and expression of some proinflammatory cytokines like IL 1- β , IL6 and TNF- α . From the economic point of view 0.3 mg/kg of Se nanoparticles is the optimal concentration for use. Comet assay revealed that using nano selenium showed no hazardous effect on liver and spleen cells, but still a great efforts in research is needed to improve our knowledge of SeNPs involvement in the functionality of organs and the potential role of SeNPs in unwanted disorders.

Conflict of Interest

Authors hereby that there is no conflict of interest concerning this work.

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