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SCOLICIDAL EFFICACY OF SELENIUM NANOPARTICLES AGAINST PROTOSCOLECES OF HYDATID CYST

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Abstract

The hydatid disease known as hydatidosis or echinococcosis is a cyclozoonotic disease widely distributed in the viscera and other organs of humans and animals. The aim of the present study was to evaluate the efficacy of different concentrations of selenium nanoparticles (Se NPs) (100, 250 and 500µg/ml), albendazole sulfoxide (ABZ sulfoxide) (50µg/ml) and NaCl 0.9% as control with different exposure times (10, 20, 30, 60 min and 3 days) on protoscoleces of *Echinococcus granulosus* (*E. granulosus*). They were cultured in test tubes containing RPMI 1640 medium. Viability was examined by use of 0.1% eosin solution and scanning electron microscopy (SEM) was performed for different samples. Exposure to Se NPs at 100µg/ml had a scolicidal effect 100% after 60 min and 3 days only while they killed 93.7%, 95.3%, 98.7% at 10, 20 & 30 min application respectively. Also, on exposure to a concentration of 250 µg/ml, the scolicidal activity of Se NPs was 100% after 20, 30, 60 min and 3 days while it was 99.7% after 10 min. On exposure to a concentration of 500µg/ml of Se NPs, all protoscoleces (100%) were killed at all times. Furthermore, exposure to albendazole sulfoxide exhibited partial scolicidal efficacy. SEM of different samples showed obvious alterations with Se NPs 500 µg after 3 days of exposure included invaginated scolex, degenerated rostellum and more severe affection of internal tissue with loss of integrity of the tegument and appearance of blebs.

Key words: Selenium, nanoparticles, hydatid, albendazole

Introduction

Echinococcosis or hydatid disease is a parasitic infection caused by the larval stage of dog tapeworm *E. granulosus*. It is a major community health economic problem worldwide (Rahimi *et al*, 2015). Systemic chemotherapy, puncture of the cyst with aspiration and surgical removal are the three main dealings for hydatid cysts (Lv *et al*, 2013), with surgery being one of the best selections for considering echinococcosis (Topcu *et al*, 2009). The probability of cyst rupture with leakage of contents and spreading of a large number of protoscoleces during surgical operation can cause secondary echinococcosis (Moro and Schantz, 2009).

The frequently known scolicidal agents; hypertonic saline, silver nitrate, cetrimide and ethanol were used for inactivation of cyst contents. Those scolicidal agents present different dangerous side effects such as methemoglobinemia, sclerosing colangititis and liver necrosis (Mahmoudvand *et al*, 2014). The classically used drugs against *E. granulosus* are the benzimidazoles. Mebendazole was the first drug used for hydatidosis treatment then it was replaced by albendazole due to its better bioavailability (Alvela-Suarez *et al*, 2014). However, long time use of albendazole and mebendazole showed different adverse effects such as severe leucopenia, thrombocytopenia, hepatotoxicity and alopecia (Junghanss *et al*, 2008; Mahmoudvand *et al*, 2014). Praziquantel and nitazoxanide are other anthelmintic chemotherapeutic agents used against echinococcosis, but effectiveness was inferior to benzimidazoles (Alvela-Suarez *et al*, 2014). Thus, progress of new scolicidal agents with few side effects and more efficacies was a serious requirement for the hydatidosis treatment (Adas *et al*, 2009).

Selenium (Se) is a micronutrient metalloid commonly exists in the form of sodium selenite, selenomethionine and methyl selenocysteine. It is fused in the structure of enzymes such as glutathione peroxidases, iodothyronine deiodinases and thioredoxin reductase

used in antioxidant defense, detoxification and metabolism respectively (Messarah *et al*, 2012; Forootanfar *et al*, 2014).

Se NPs own antibacterial, antiviral and antioxidant properties proposing they could be suitable as beneficial applicants against infectious diseases. Moreover, Se NPs were identified to be more efficient than sodium selenite and selenomethionine in growing glutathione S-transferase activity (Wang *et al*, 2007)**.** It was evidenced that nanoparticles (NPs) due to their large surface-volume ratio presented several unique properties and they were also able to go in cells more commonly than other particles (Tran and Webster, 2011).

Nanostructured nanoparticles can be synthesized using bacterial and fungal cells as biological catalytic agents, providing a non-toxic and environmentally appreciated approach for the making of nanoparticles, including Se NPs (Xiangqian *et al*, 2011). Frequent microbial strains can reduce the toxic selenite oxyanion to the less toxic elemental selenium through the progress of either intracellular or extracellular Se NPs with a typical spherical shape and a diameter of 50-400nm (Lampis *et al*, 2014).The mechanism of effectiveness of selenium against microorganisms remains indistinct but there are some studies offered that the inorganic forms of selenium could react with membrane peroxidases to generate oxygen free radicals, such as superoxide anion (O2) (Mézes and Balogh, 2009; Tran and Webster, 2011). Se NPs are itemized as the most promising nanosystem with high anticancer action and better biocompatibility (Huang *et al*, 2013; Nie *et al*, 2016). The capability of biogenic Se NPs to induce apoptosis in another form of eukaryotic cell, the *Leishmania major* promastigotes was reported (Beheshti *et al*, 2013).

The present study aimed to test the scolicidal effect of Se NPs against the protoscoleces of *Echinococcus granulosus*.

Materials and Methods

Biosynthesis and characterization of Se NPs: Se NPs were synthesized according to the method designated elsewhere (Shakibaie *et al*, 2010). Briefly, a sterile nutrient broth (NB) medium was supplemented with the Seþ4 ions (100mg/L; equal to 1.26mM SeO2 solution) and 100 ml of this medium was relocating to a 500 ml Erlenmeyer flask. Medium was inoculated with 1ml of fresh inoculums (OD600, 0.1) of *Bacillus* sp. MSh-1 and was kept aerobically at 30^{o}C in a shaker incubator (150rpm). After 14h, bacterial cells and Se NPs were detached from culture medium by centrifugation at 4000g (10min). Pellets were washed with 0.9% normal saline solution using centrifugation, transported to a mortar and frozen by adding liquid nitrogen and were then disrupted by a pestle. The resulting slurry was ultrasonicated at 100W for 5min and washed three times by sequential centrifugation (10,000g, 5 min), with a 1.5M TriseHCl buffer (pH 8.3) comprising 1%SDS and deionized water. Next step involved extracting and purifying the Se NPs by an organic-aqueous partitioning system (n-octyl alcohol-water). In the current study, particle size of Se NPs was < 10nms, sample volume was 10 ml and sample code was NS0009, Nano streams Co.

Collection of protoscoleces: Protoscoleces of *E. granulosus* were obtained from the livers of naturally infected sheep slaughtered at Shebin El Kom Slaughter House, and carried to Laboratory of Parasitology Department, Faculty of Medicine. Hydatid fluid was aspirated by a 20 ml syringe and transported into a container and left to set for 30 minutes for protoscoleces to settle down into the bottom. Then, they were centrifuged at 800 rpm for 5 min. Supernatant was discarded and protoscoleces were washed two times with PBS solution. Protoscoleces measured approximately 0.3-0.4 mm. Their number per ml was adjusted as 2×10^{3} protoscoleces in 0.9% NaCl solution with at least 90% viability rate. Protoscoleces viability was confirmed by flame cell motility and impermeability to

0.1% eosin stain under a light microscope. Live protoscoleces were stored at 4°C for investigations (Mahmoud *et al*, 2016; Barabadi *et al*, 2017).

Scolicidal assay: To investigate the scolicidal effects of Se NPs against protoscoleces of hydatid cysts, three concentrations of the Se NPs (100, 250 & 500µg/ml), albendazole sulfoxide (ABZ sulfoxide) (50µg/ml) and 0.9% NaCl solution as a control were used with different exposure times (10, 20, 30, 60 min & 3 days). At first, 0.5ml of the protoscoleces (2×10^3/ml) solution was placed in test tubes containing RPMI 1640 medium. Then, 0.5ml of various concentrations of Se NPs was added to each tube. Tubes were gently mixed and incubated at 37°C for 10, 20, 30, 60min & 3 days. At the end of each incubation the upper phase was carefully removed to determine viability of protoscoleces.

Determination of viability of protoscoleces: In order to evaluate the viability of protoscoleces, eosin solution with a concentration of 0.1% was mixed with protoscoleces in a ratio 1:1 and incubated for 15 min. Solution upper portion was discarded. The remaining pellet of protoscoleces was smeared on a glass slide, covered with a cover glass and examined under a light microscope. Dead protoscoleces percentages were determined by counting 100 protoscoleces per microscopic field (three fields for each specimen). Dead protoscoleces exposed to biogenic Se NPs absorbed eosin and colored red, but live protoscoleces remained colorless with characteristic muscular movements and flame cell activity (Rahimi *et al*, 2015).

Scanning electron microscopy (SEM): Parasites were processed for scanning electron microscopy at different time points after the initiation of treatment with different concentrations of Se NPs at Electron Microscopy Unit of Tanta University. Fixed specimens were then washed in distilled water, treated with 1% uranyl acetate for 30 min, subsequently washed extensively in distilled water and dehydrated by incubation in sequentially increasing concentrations (50%, 70%, 80% and 90%) of ethanol. Samples were then washed in PBS (pH 7.2) and treated with 1% uranyl acetate for 30 min. They were then coated, inspected and examined (Wang *et al*. 2015).

Statistical analysis: Statistical package of the social signs SPSS version 20 software (SPSS Inc. Chicago, ILL Company) was adopted, epicalc version 1.02 software and excel sheet to perform the analysis. All data were presented as number and percentage. Chi square test was used to compare groups of categorical data.

Results

Scolicidal effects of selenium nanoparticles: The scolicidal efficacy of different concentrations of Se NPs (100, 250 & 500µg/ml), albendazole sulfoxide and 0.9% NaCl solution for 10, 20, 30, 60 min and 3 days against protoscoleces of *E. granulosus* was studied. Se NPs in all concentrations exhibited significant scolicidal effects as compared with control group ($P < 0.001$). On exposure to 500µg/ml of Se NPs, all protoscoleces (100%) were killed after 10, 20, 30, 60 min & 3 days. On exposure to 250 µg/ml, the scolicidal activity of Se NPs was 100% after 20, 30, 60 min and 3 days while it was 99.7% after 10 min. Exposure to Se NPs at 100µg/ml, it had a scolicidal effect 100% after 60min and 3 days only, But, they killed 93.7%, 95.3%, 98.7% at 10, 20 & 30min of application respectively. Exposure to albendazole sulfoxide exhibited partial scolicidal efficacy at any exposure time, they killed 37.3%, 43.7%, 51.7%, 66.3% & 71.3% after 10, 20, 30, 60min & 3 days application respectively. Exposing protoscoleces to NaCl 0.9% solution killed only 1% after 30, 60 min & 3 days while it had no efficacy at 10 or 20min. So, by increasing the exposure time with Se NPs in all concentrations, mortality rate significantly increased (Tab.1). The results showed potent *in vitro* scolicidal efficacy for biogenic Se NPs 2.5cm at various concentrations for various times.

Table 1: Scolicidal effects of Se NPs against protoscoleces of hydatid cyst at various concentrations after various exposure times.

Group			time					Total
			10 min	20 min	30 min	60 min	3 days	
Se NPs 100µg	dead	Count	281	286	296	300	300	1463
		% within time	93.7%	95.3%	98.7%	100%	100%	97.5%
	living	Count	19	14	4	0	0	37
		% within time	6.3%	4.7%	1.3%	0%	0%	2.5%
	Total	Count	300	300	300	300	300	1500
		% within time	100%	100%	100%	100%	100%	100%
Se NPs 250µg	dead	Count	299	300	300	300	300	1499
		% within time	99.7%	100%	100%	100%	100%	99.9%
	living	Count	1	0	0	0	0	1
		% within time	0.3%	0%	0%	0%	0%	0.1%
	Total	Count	300	300	300	300	300	1500
		% within time	100%	100%	100%	100%	100%	100%
Se NPs 500µg	dead	Count	300	300	300	300	300	1500
		% within time	100%	100%	100%	100%	100%	100%
	Total	Count	300	300	300	300	300	1500
		% within time	100%	100%	100%	100%	100%	100%
ALB. sulfoxide	dead	Count	112	131	155	199	214	811
		% within time	37.3%	43.7%	51.7%	66.3%	71.3%	54.1%
	living	Count	188	169	145	101	86	689
		% within time	62.7%	56.3%	48.3%	33.7%	28.7%	45.9%
	Total	Count	300	300	300	300	300	1500
		% within time	100%	100%	100%	100%	100%	100%
Nacl 0.9%	dead	Count	0	0	3	3	3	9
		% within time	0%	0%	1%	1%	1%	0.6%
	living	Count	300	300	297	297	297	1491
		% within time	100.0%	100.0%	99.0%	99.0%	99.0%	99.4%
	Total	Count	300	300	300	300	300	1500
		% within time	100%	100%	100%	100%	100%	100%

By use of pearson Chi-Square test, p value was < 0.001 in groups I and III

Discussion

Hydatidosis or cystic echinococcosis (CE) is a major public health disease especially in developing countries (Khademvatan *et al*, 2018). In Egypt, zoonotic hydatidosis was reported in man (Hassanain *et al*, 2016) as a silent health problem mainly among children (Haridy *et al*, 2008) and in the farm animals (Amer *et al*, 2015). Moreover, echinococcosis was reported in street dogs in urban and rural areas (Elshazly *et al*, 2007). It is identified as a parasitic infection of dog tapeworm *E. granulosus* (Fasihi *et al*, 2012). Inactivation of the scoleces by using a scolicidal agent earlier to removal of hydatid cyst is forcefully suggested during surgery of CE to reduce the risk of intraoperative spillage of the cyst contents and reappearance of hydatidosis (Beheshti *et al*, 2013).

Till now, the usage of scolicidal agents as hypertonic saline, ethanol (95%), H_2O_2, silver nitrate, chlorhexidine gluconate, cetrimide, povidone iodine, mannitol, honey, albendazole and some plant extracts in various studies was confirmed (Mahmoudvand *et al*, 2014). However, most of these scolicidal agents may lead to undesirable complications that diminish their usage in treatment of CE (Hosseini *et al*, 2006). For these reasons, studies for finding of a fast scolicidal agent with no unwanted side effects during surgery are obligatory (Adas *et al*, 2009). In the current study, scolicidal efficacy of different concentrations of the Se NPs (100, 250 and 500µg/ml), albendazole sulfoxide and NaCl 0.9% for 10, 20, 30, 60min and 3 days against protoscoleces of *E. granulosus* was determined.

In accordance to the present study, four concentrations of the Se NPs (50, 125, 250 and 500µg/ml) were used with different exposure times (10, 20, 30 and 60 min) in the study of Mahmoudvand *et al*. (2014) and discovered that all protoscoleces were killed after 10min of exposure to concentration of 500µg/ml of Se NPs. Besides, after 20min exposure of a concentration of 250µg/ml, the scolicidal activity was 100%. Se NPs at concentration125µg/ml killed 41.4%, 73.4%, 86.6% & 100% of the protoscoleces and at concentration 50µg/ml destroyed 16.2%, 27.8%, 41.6% & 56.5% of them after 10, 20, 30 & 60min application, respectively.

Scolicidal effects of Se NPs at concentration 500µg/ml were compared with scolicidal effects of albendazole sulfoxide and NaCl 0.9% as previously reported (Kayaalp *et al*, 2001; Caglar *et al*, 2008; Adas *et al*, 2009). Also, albendazole was effective in treating cystic hydatidosis than mebendazole (Sadati *et al*, 2016).

Moreover, albendazole-encapsulated nanosize liposomes as albendazole-encapsulated conventional and albendazole loaded polyethylene glycol (PEG) liposomes were investigated *in vitro* to detect their efficiency in treatment of cystic hydatidosis; they were 81% and 72%, respectively (Panwar *et al*, 2010). Also, the use of 0.9% NaCl (saline) in the study of Caglar *et al*. (2008) as a control agent had no scolicidal effect corresponding to the current study.

Based on *in vitro* and *in vivo* studies of Beheshti *et al*. (2013) biogenic Se NPs could be considered as novel therapeutic agents for treatment of the localized lesions of cutaneous leishmaniasis caused by *L. major*. Also, *Trypanosoma* and other higher microrganisms needed trace amounts of selenium ions (Lobanov *et al*, 2006).

Moreover, Shakibaie *et al*. (2010) reported no biochemical changes from the orally administration of 2.5, 5 & 10mg/kg of Se NPs to male mice for two weeks, but a dose of 20mg/kg of Se NPs gave signs of toxicity including lower body weight and changes in clinical chemistry and hematological parameters.

Barabadi *et al*. (2017) found that scolicidal activity of green synthesized gold nanoparticles (AuNPs) utilizing mycelia-free culture filtrate of *Penicillium aculeatum* against hydatid cyst protoscoleces of *E. granulosus* was potential. High scolicidal activity of various concentrations of biosynthesized silver nanoparticles (Ag-NPs) from aqueous aerial *Penicillium aculeatum* extract against *E. granulosus* protoscoleces *in vitro* at different exposure times proved to be potential, safer and non-toxic compared to other chemical materials (Rahimi *et al*, 2015). *In-vitro* efficacy of arsenic trioxide (ATO) against *E. granulosus* protoscoleces incubated with 2, 4, 6, & 8mol/liter, showed that ATO had a potent ability to kill protoscoleces and that ATO represented a new strategy in treating hydatidosis (Wang *et al*, 2015).

In the current study, SEM of protoscoleces of *E. granulosus* of GI treated with Se NPs 100/µg/kg revealed minimum ultrastructural changes included contracted soma and loss of some hooks. In GII treated with Se NPs 250µg/kg, ultrastrucrural changes were more obvious and included contracted soma, loss of some hooks, collapsed scolex and appearance of blebs in the tegument. In GIII treated with Se NPs 500µg/kg, SEM showed more aggravated altered structures with loss of hooks, contracted soma to very small size, degenerated scolex and rostellum. After 3 days, there were more detectable alterations included invaginated scolex, degenerated rostellum and more severe affection of internal tissue with loss of integrity of the tegument and appearance of blebs. In group IV treated with albendazole sulphoxide, less ultrastructural changes revealed than groups treated with Se NPs, included collapse of sucker region and inavaginated scolex. Regarding group V treated with NaCl 0.9%, no ultrastructural changes were determined in the scoleces of *E. granulosus* by SEM.

Similar to the present study, SEM using gold nanoparticles (AuNPs) against protosc-

oleces of *E. granulosus* revealed that the live protoscoleces had turgid soma and scolex regions (Barabadi *et al*, 2017). Hooks arranged microtriches and uniform tegum ranged microtriches and uniform tegumental layer were observed. After treatment with AuNPs, loss of turgidity particularly with the soma region and damage of tegument were seen among protoscoleces.

In line with this study, protoscoleces cultured with 8µmol/liter arsenic trioxide (ATO) had more obvious damage than those cultured with 2, 4, & 6µmol/liter ATO. At 3 days of 8µmol/liter ATO treatment, SEM showed hooks loss, shedding of microtriches and reduced volume (Wang *et al*, 2015).

In Loos and Cumino (2015) SEM of protoscoleces and meta-cestodes incubated with 10mM of metformin and its combination with albendazole sulfoxide for 4 days showed that control protoscolex was with normal sucker and microtriches; treated one was with soma region contracted and scolex region showed loss of hooks and shedding of microtriches.

Conclusion

No doubt, echinococcosis/hydatidosis is an Egyptian public health zoonotic problem.

Se NPs had potent scolicidal effects on protoscoleces of *E. granulosus* with increasing dose and time of exposure proved by ordinary examination by usage of 0.1% eosin and electron microscopic study.

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Explanation of figures

Fig. 1: Dead protoscoleces after exposure to 0.1% eosin stain.

Fig. 2: Live protoscoleces after exposure to 0.1% eosin stain.

Fig. 3: SEM of protoscoleces of *E. granulosus* showed no ultrastructural alterations during whole incubation period with NaCl 0.9% treated group. Scolex is everted with hooks. Hooks inserted into rostellum with one row overlapping each other. Each hooklet about 20 to 40um

long, rounded basally and sharpens distally towards point of insertion into rostellum. Protoscoleces contain intact germinal layer with different cell types.

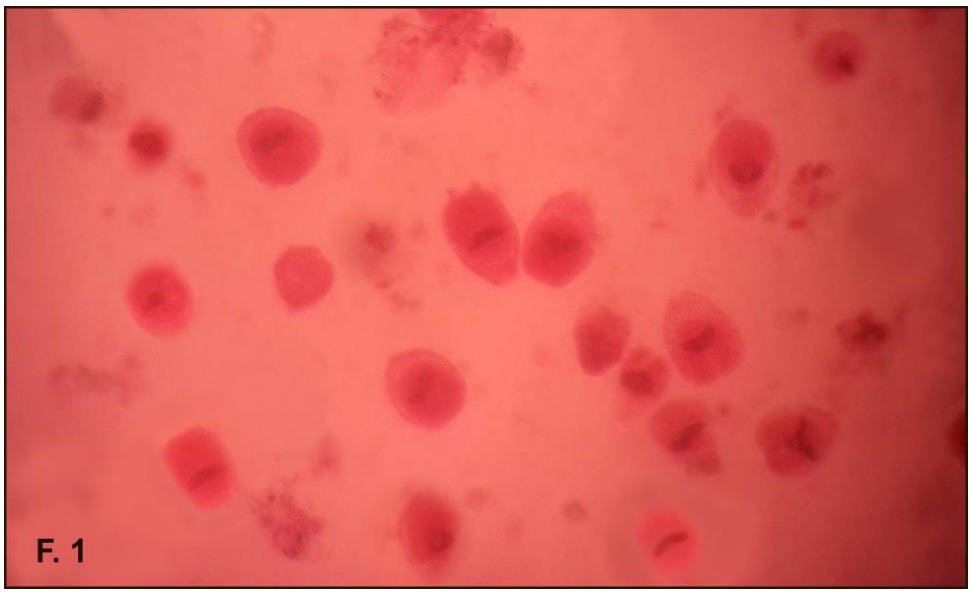
Fig. 4: SEM of protoscoleces of *E. granulosus* cultured *in vitro* in a medium containing RPMI 1640 for 30 min after treatment with Se NPs 500 µg/ml showing: altered structures, loss of hooks, contracted soma to very small size, degenerated scolex and rostellum.

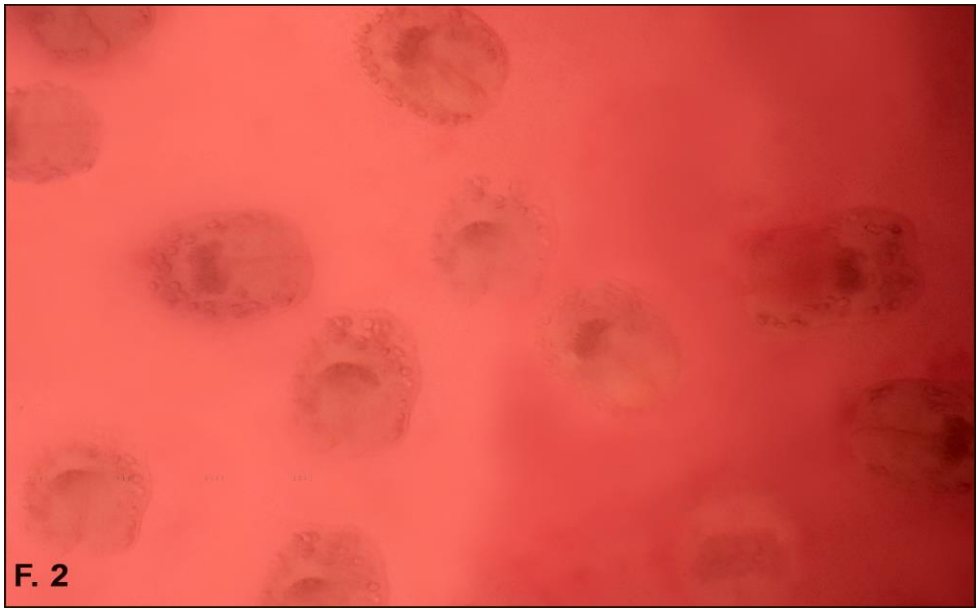
Fig. 5: SEM of protoscoleces cultured *in vitro* in a medium containing RPMI 1640 for one hour after treatment with Se Nps 500µg/ml showed altered structures, collapse of sucker region, loss of hooks, invaginated scolex, degenerated rostellum, internal tissue affected with loss of integrity of tegument and appearance of blebs.

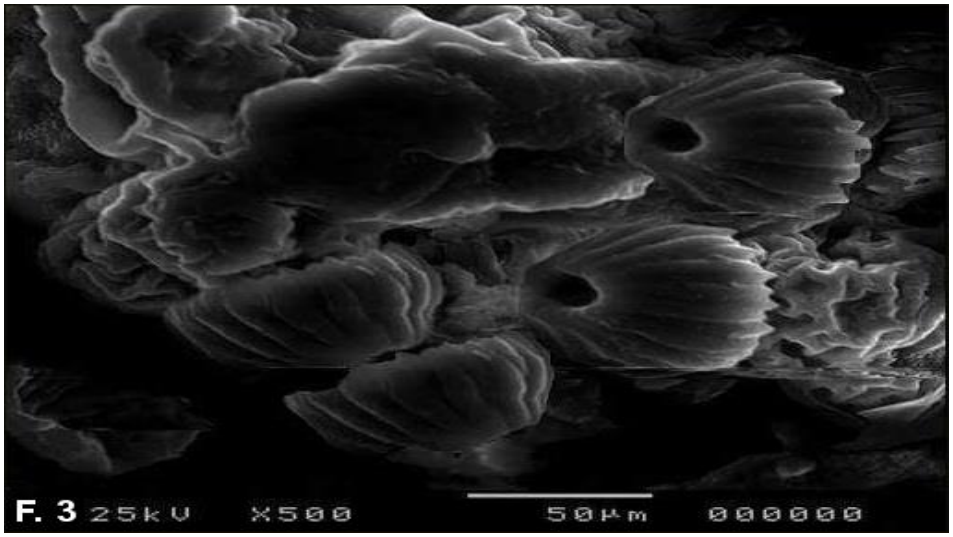
Fig. 6: SEM of protoscoleces cultured *in vitro* in a medium containing RPMI 1640 for 3 days after treatment with Se Nps 500µg/ml showed altered structures, loss of hooks, invaginated scolex with shedding of microtriches, degenerated rostellum, tegumental alterations with loss of integrity of the tegument with appearance of blebs and severe alterations of the internal tissue.

Fig. 7: SEM showed variable sizes of Se Nps sited on protoscoleces of hydatid cyst.

SEM of protoscoleces of GI (treated with Se NPs 100 µg/kg) showed minimum changes even after 3 days of treatment: Contracted soma and loss of some hooks. In GII (treated with Se NPs 250 µg/kg), changes increased with contracted soma, loss of some hooks, collapsed scolex and appearance of blebs in tegument. In GIII (treated with Se NPs 500 µg/kg), showed more aggravated altered structures with loss of hooks, contracted soma to very small size, degenerated scolex and rostellum (fig. 4 & 5). In the same group, after 3 days, more obvious alterations with invaginated scolex, degenerated rostellum and more severe affection of internal tissue with loss of integrity of the tegument and appearance of blebs (fig. 6). In GIV (treated with albendazole sulphoxide), less ultrastructural changes than groups treated with Se NPs, included collapse of sucker region and inavaginated scolex. Regarding GV (treated with NaCl 0.9%), no ultrastructural changes observed in scoleces of *E. granulosus* by SEM (fig. 3).



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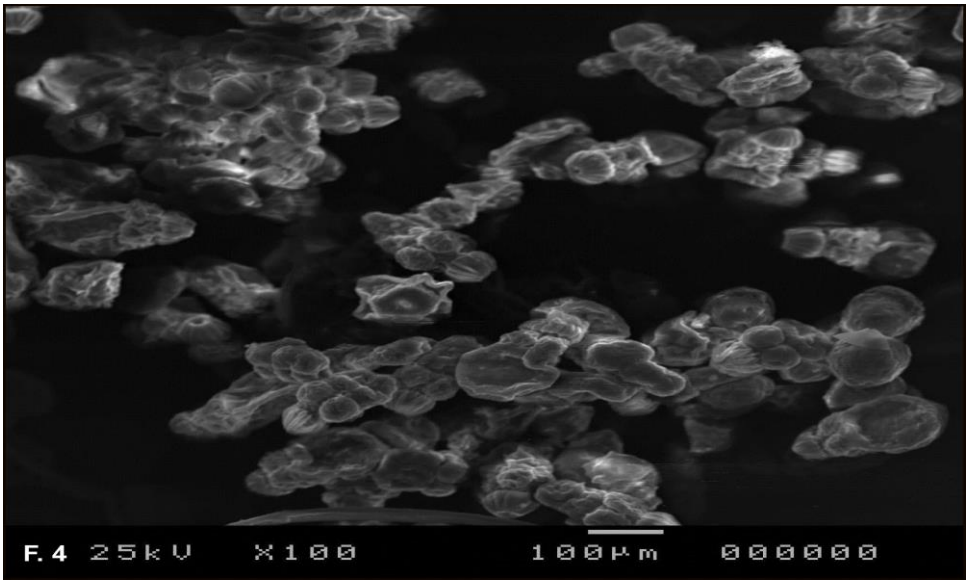
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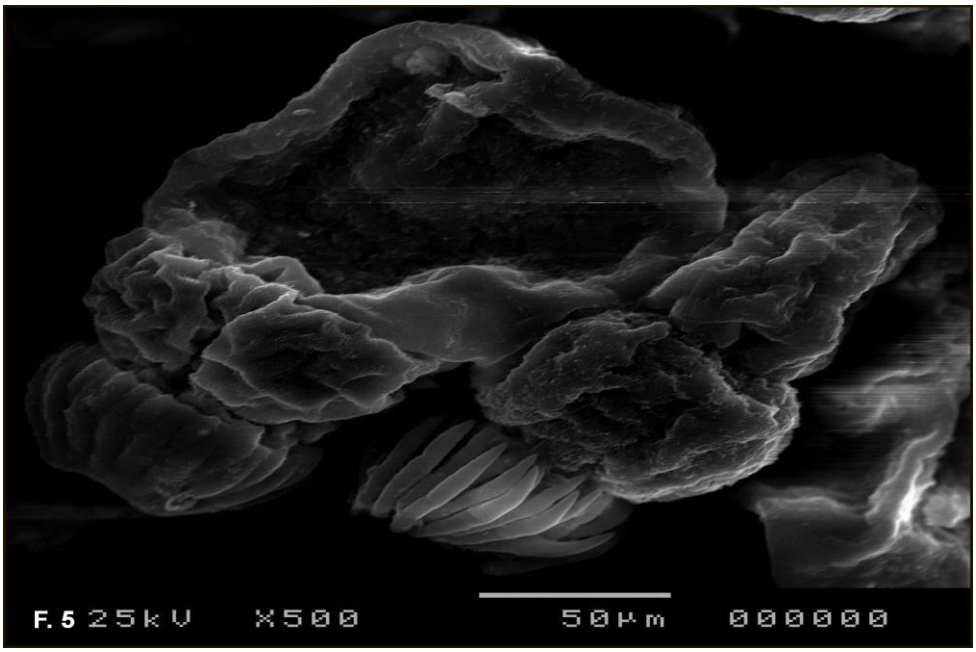
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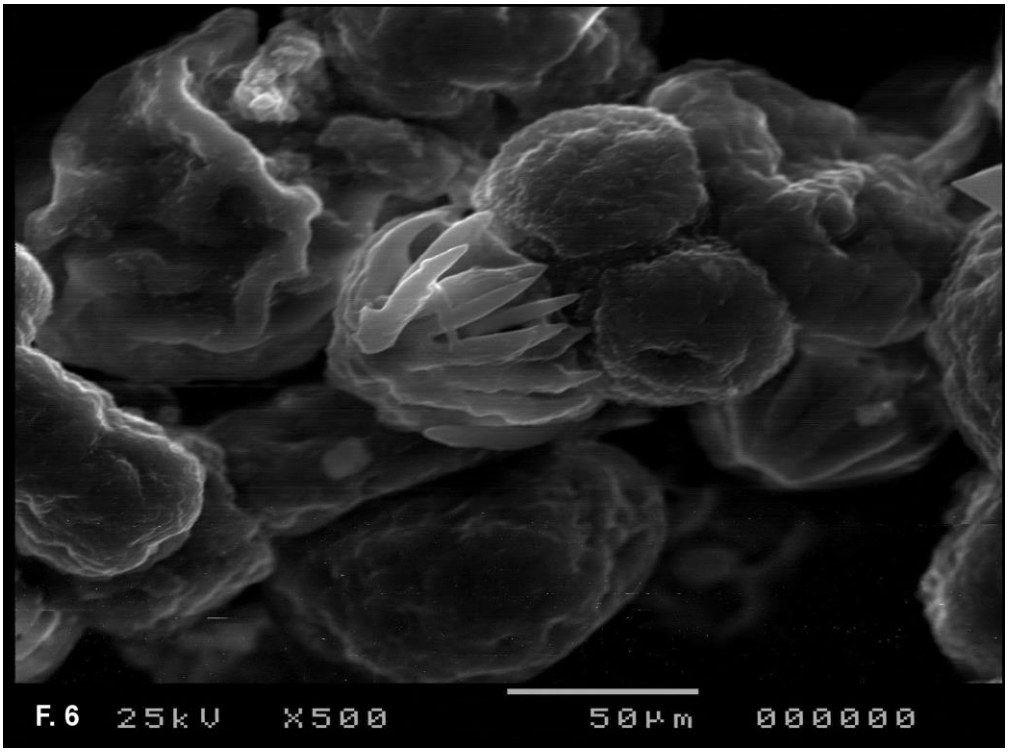
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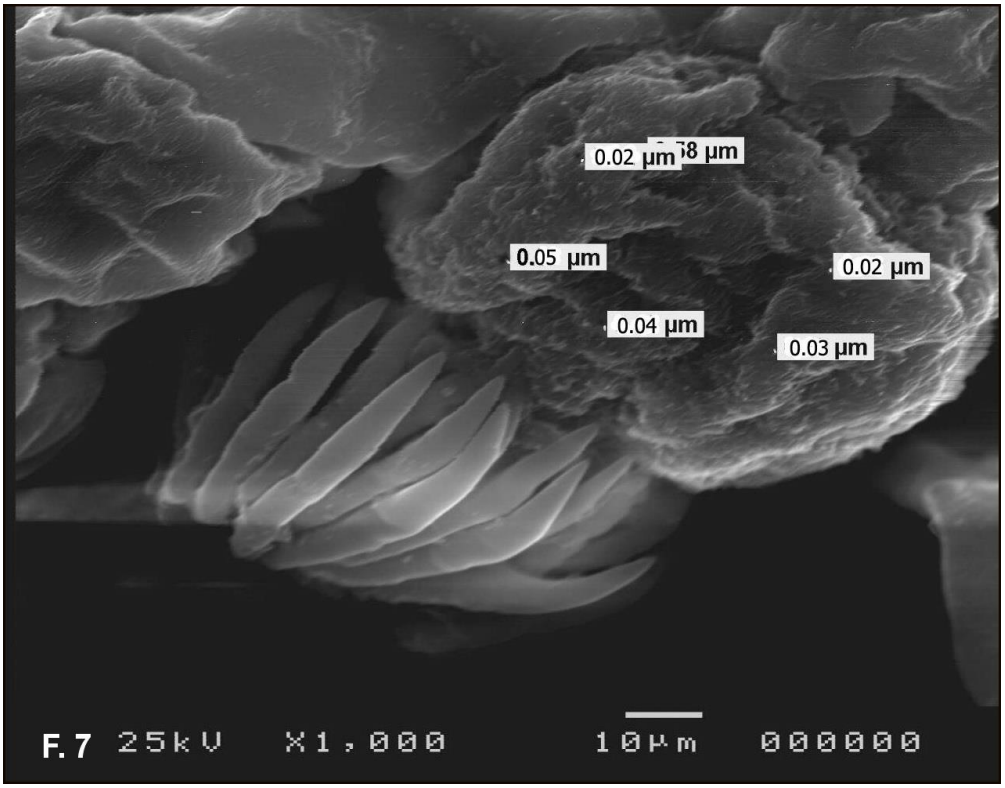
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0.05 µm
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