Efficacy of Filter Water with Nanosilver-coated Natural Zeolite in Controlling Water Molds Infection on Bunnei (Barbus Sharpeyi)Eggs

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ABSTRACT

The effect of indirect use of nanosilver particles (AgNPs) was studied for control water molds infection on Bunne eggs during incubation period in the hatchery. This effect was studded with different concentrations of nanosilver-coated natural zeolite (0.5,1and 2 % AgNPs) and these concentrations were compared with unmodified natural zeolite as water filter in semi-circulatory incubation system. All incubators were inoculated with water molds-infected Bunne eggs for testing the effect of AgNPs on inhibition of fungal infection. Dead and infected eggs were removed periodically ,the efficacy of the filters was assessed by estimation the survival rates from fertilization to accomplishment of the yolk-sac absorption stage. Survival rate was increased about 5.3% (filters with 0.5% AgNPs) from fertilization to larvae compared to control ($P<0.05$).The additional option of active carbon (absorbent media) along with AgNP-coated zeolite filters caused an increase of about 13.5% in the survival rate for the larval stage ($P<0.05$).No infection with water mold were observed in the incubators during the incubation period in the incubators with water supply from filters with AgNP-coated zeolite in contrast to the control group with about 12% water mold infection. The end results enhanced that the indirect usage of AgNPs in the aforementioned filters were significantly effective for control water mold infections on semi-circulation system for incubation of Bunne's eggs, making them a candidate for exchange the chemical reagents currently used in eggs incubation in hatchery system. The indirect use of nanosilver materials for the disinfection of water for eggs incubation was applied for the first time in fish hatchery in Iraq.

Keywords: Silver nanoparticles ,coated natural zeolite, Bunnei, water mold, water filter, fish hatchery.

INTRODUCTION

Nanotechnology is the science of production and use of nano-sized materials and its many applications are starting to appear in the agriculture sciences, particularly aquaculture, where the antimicrobial properties of certain nanomaterials are of especial interest [1]. Al-Wahda fish hatchery is the main fish hatchery which located at Swaira, south of Baghdad in Iraq. It propagate different fish species belonging to Cyprinidae [2]. Most of the pathogenic microorganisms transferred to fish aquaculture by water, thus many diseases controlling methods are based on water disinfection. Saprolegniasis is a major economic problem with many fish hatcheries[3,4],it caused by water molds, particularly *Saprolegnia* , *Achlya* and *Aphanomyces* [5] .Saprolegniasis controlled by regular fungicide treatments [6]. However, usage of disinfectant chemicals may have unfavorable effects on the cultured fish and surrounding environment, malachite green is highly effective agent to control water mold infections in fish and fish eggs, yet it is known to be carcinogenic, teratogenic and mutagenic [7,8], thus its application is limited to the

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treatment of eaten fish. Formalin is one of aquatic fungicides [9] , but it is not completely effective for controlling water mold infections in fish and fish eggs[10] and about its effect on both human handling and the environment [11]. The use of other fungicides, as ozone, hydrogen peroxide and copper is not widespread [12,13,14]. Thus, new methods need to be developed for controlling water mold infections in aquaculture, from these methods using inorganic materials containing silver

which some of them had already applied in commercial [15,16]. Silver is well known have relatively safety and a wide antimicrobial spectrum [17,18]. The direct use of colloidal nanosilver particles in the fish eggs incubation

system [19].The direct use of colloidal nanosilver in water for disinfection might causes disastrous impact on the organisms and the environment [20,21].notwithstanding ,silver nanoprticles have a special property

that they can easily use to coat or mix with other substances , making them a good for the indirect treatment of water via coated filter media if the process of coating is performed with exact manner [22,23].

Accordingly, this study applied the indirect use of silver nanomaterials as antifungal treatment in aquaculture by coating natural zeolite with (AgNPs) to the water filter of Bunne eggs incubators to control water mold infection.

Materials and Methods

Characterization of filter media

Determination of crystalline phase of the nanosilver-coated zeolite achieved by X-ray diffraction (XRD) by using a Philips X'Pert-MPD X-ray Diffraction System (Netherlands).Scanning Electron Microscope (SEM) photographs of the coated and uncoated zeolite were performed using a Philips XL30 SEM (Netherlands).

Preparation and modification of filters

Filters used in this experiment are commercially available in local markets (housing filter JEWIN CFR) this filter contain three layers, first layer is natural zeolite this layer modified by coating it with silver nanomaterials concentrations 0.5%, 1% and 2% (W/W) by sol-gel dip coating technique [24,25].Second layer uncoated natural zeolite and last layer is active carbon. Water was dechlorinated by adding 1 $mg.l^{-}$ sodium thiosulfate then this water was left at least 72 h in 750 liter reservoir tanks, total water volume in this experiment was 1000 liters, water was pumped by centrifugal pump (6-7 $l.min^{-1}$), after passing through the filter ,water was returned to reservoir and approximately 50% of water was exchanged with oxygenated fresh water every day. Chemical parameters like sulfide, ammonium, potassium, magnesium, total hardness, calcium hardness and chloride were all measured with Palintest photometer Modle: 8000, UK, Table 1 show these parameters as compared with the world slandered values in [26]

In this study semi-circulatory system (picture 1) was used in eggs incubation, the schematic diagram shown in Figure1 each system consisted of standard cylindrical glass Hungarian type jars (with capacity 9-10 liters). 500g of fertilized eggs were transferred to each incubator (2500 eggs per jar), water temperature (18±1C°) water temperature and chemical parameters were routinely monitored during the incubation period and eggs jar were covered to be protected from direct light, any dead eggs(with dark color) and embryos were removed periodically by sucking it manually with silicon tube , the survival rate estimated as the percentage of the initial number of eggs 24 hours after fertilization.

Isolation and Inoculation of *Saprolegnia* spp. into the incubators

As the method mentioned by [27] with few modifications the isolates of *Saprolegnia* spp. obtained during incubation period infected cultured eggs collected from hatchery incubators. During the artificial propagation season randomly, eggs coated with fungal mycelium were collected and transferred to the laboratory, then water molds isolates were examined microscopically for the morphology and colonies to determine if one or more types of fungi grew concurrently. A portion of the mycelium was cultured into sterile glass petri dishes contain Corn Meal Agar with Raper's ring to limit yeast [28], with chloramphenicol (100 µg / ml) to limit bacterial growth . Cultures were examined periodically for two months this to monitor the development of sexual structure depending on the classification key [29].

Filters

Depending on the method used by [30] with few modifications, Seven filter types were evaluated in this study, six of them were treated with silver nanomaterials and one as control which consisted of 500 g uncoated natural zeolite. The first group of filters included treated filter media, consisting of 500 g natural zeolite coated with 0.5, 1and 2 % of silver nanoparticles. while, the second group of filters included a nanosilver-coated media layer plus (AFM) an absorbent filter media layer. AFM consisting of a layer of 250g natural zeolite along with a layer of 250g granular activated carbon. The details of all the filters are shown in Table 2, and all were tested in triplicate. To measure the release of silver ions or silver nanomaterials from the modified filters into the water, samples were taken from each trough daily and after egg fertilization. The water samples were acidified with HNO3 to reduce the pH to less than 2 and then placed in dark glass vessels at 4 C^0. Prior to the measurements, the water samples were digested using 69 % HNO3 and the total silver concentrations were measured using a Perkin Elmer Atomic Absorption Spectrophotometers (Model 370) – AAS [31].

Statistical analysis

The standard errors of the means (SE) and standard deviations (SD) and were calculated. All the statistical analyses were performed using SPSS Statistics 17.0 software. The data were analyzed using a one-way analysis of variance (ANOVA). Significant means were compared using Duncan's test. ($P<0.05$) was considered statistically significant.

Results and Discussion

Characterization of filter media

The XRF results showed the chemical composition of the zeolite coated with different percentages of silver nanoparticles. As seen in Table 3, the presence of silver was evident in the AgNPs-coated zeolite. For the nominal 0, 0.5, 1, and 2 % AgNPs-coated zeolite, the actual percentages of silver were 0, 0.322, 0.723, and 1.729, respectively; according to this, the actual percentages of nanoparticles incorporated in the natural zeolite were less than the nominal percentages, this revealed that the method of coating zeolite was performed correctly. All other oxides were with acceptable values.

The XRD results indicated that there were no peaks related to crystalline silver identified in the AgNPs-coated natural zeolite and no distinct differences in the XRD patterns between the AgNPs-coated and uncoated zeolite samples as showed in Figure 2, this indicating that silver nanoparticles which coating zeolite surfaces were amorphous, this agreed with [30] who suggest that the crystals undetectable by XRD are very small crystals and seemed structureless.

Photographs from Scanning Electron Microscope (SEM) of the AgNPs-coated and uncoated natural zeolite Plate 1 showing circular-shape silver nanoparticles uniformly coated the surface of the natural zeolite. Data from the analysis of the SEM photographs show that the mean size of the silver particles on the surface of the natural zeolite was 55.61 nm.

Incubation and efficiency of larval production

During the incubation period, parameters of water quality were kept within the acceptable tolerance ranges for Bunnei eggs [31]. The means and standard deviations for the temperature

,dissolved oxygen, and pH during the experiment were 18 ± 0.2 C^0 ,7.1 ± 0.13 mg/l, and 7.8 ± 0.3 respectively.

Water mold infection during the incubation was only observed in the control groups (in about 6 % of the initial number of eggs) just before the eyed-egg stage. The fungal colonies as examined with microscope an septate hyphae with white to gray cotton-like growths on the eggs and mad nearby eggs to aggregate together, that cause the death of these eggs. After the eyed-egg stage and in order to continued inoculation with water mold, no observation of fungal infection was in any of the AgNPs-treated groups, including the dead eggs, it seemed that water filters with natural zeolite coated with silver nanoparticles have the ability to inhibit and control fungal infections during the incubation period of Bunne eggs . The ability to sterilize drinking water using filters containing silver nanoparticles has already been proven [22,34], this application of this filters is the first in aquaculture. The mechanisms of silver nanoparticles against microorganisms had already been reported by [35,36,37] and include: (1) damaging and changing the membrane structure of the microorganisms, (2) penetration of a microorganism and interaction with sulfur phosphorus-containing compounds, such as proteins and DNA, (3) loss the ability of the DNA to replicate , (4) inactivation of some enzymes, (5) generating free radicals and hydrogen peroxide, (6) the release of the silver ions from the nanoparticles, from the above mechanisms, two antifungal mechanisms are suggested by passing through the AgNP-coated zeolite : (1) fungicidal effect: fungi are directly killed by the silver ions released from the filters and (2) fungistatic effect: fungi are contaminated with silver ions, yet still survive however, they cannot grow into colonies on the surface of the eggs, as the silver ions affect their growth ability and replication, notwithstanding the lack of fungal infection with the AgNP treatments, all the eggs treated with 1Ag–O and 2Ag–O died during the early stages of embryonic development. Moreover according to this , increasing the percentage of nanosilver coating on the filter media significantly increased the release of silver compounds into the water. The high concentration of silver ions in the water appeared to be the cause for the egg mortality with the 1 and 2 % AgNP treatments. Yet, it is well known that silver compounds are toxic and lethal for different life stages of different types of fish [38,39,40,41,42].

Although the lack of water mold infection with the AgNPs treatments, all the eggs treated with the 1 and 2 % AgNPs-coated zeolite (1Ag–O and 2 Ag–O) died during the early embryonic development stages. The total silver concentrations measured in the water for these two groups showed a high release of silver compounds from the filters into the water. Moreover, when using the AFM layer along with a nanosilver-coated media layer in these two groups, a negligible portion of eggs reached the eyed-egg stage (1.9 % with 1Ag-AFM and 0.7 % with 2Ag-AFM), while none of them reached the hatching stage. In contrast, the 0.5 % AgNPs-coated zeolite (0.5Ag–O) treatment resulted in higher eyeing and hatching rates compare to those for the uncoated zeolite (0Ag–O or control) treatment (Figure 3, $p< 0.05$). Furthermore, the 0.5Ag-AFM, which used both AFM and 0.5 % AgNPs-coated zeolite, resulted in the highest eyed-egg stage and hatching rates when comparable to those with the 0.5Ag–O and 0Ag–O filters (Figure3, $p< 0.05$). The survival from 24 h post-fertilization to the completion of yolk–sac resorption (YSR survival rate) as well as survival from hatching to yolk–sac resorption was also significantly higher for the 0.5Ag-AFM group when compared to control groups0.5Ag–O and 0Ag–O the egg's chorion is a protective barrier against environmental stressors and many toxic chemicals [43]. In the chronic exposure to silver, it has been shown that about (80 %) of the total silver content can be absorbed by the chorion, while the rest was absorbed by the embryo [44]. With high silver concentrations, even the protective properties of the chorion are unable to prevent the toxic effects of silver. Therefore, it is believed that the antimicrobial action of nanosilver-coated filters is due to the gradual release of silver ions from the filters [23,34], this study showed that, if the released amount exceeds a certain level, it can have toxic effects on aquatic organisms, this technology is a double-edged sword, and it should be carefully select a special dose of silver nanoparticles in filter media, which it's not toxic to fish, but kill the pathogenic microorganisms. Consequently, since the quality and quantity of the silver coating showed substantial impact on the filter performance during the incubation period, more studies are needed to find better techniques for the stable coating of silver nanoparticles on the surfaces of different filter media to decrease the release of silver compounds from filters.

Silver concentration released from filters

SEM photograph (Plate 1) show the coating of natural zeolite with nanosilver particles was done successfully.

As shown in table 4, the total silver concentrations released from the different filters into the water on days 1, 3, and 5after fertilization. No silver concentration was found in the water of the control that did not use AgNPs-coated media (0Ag– O). In 0.5Ag–O group, silver was detected at concentrations of less than about 0.05 mg/l, yet there was no significant difference between the sampling days ($p<0.05$). For the 0.5Ag-AFM group, despite the application of the AFM, silver was detected on the first day after fertilization, although the concentration was significantly lower than the silver released with the 0.5Ag–O (0.005, $p<0.05$). In addition, the concentration of silver detected in the 0.5Ag-AFM group was significantly lower on the first day than after 3 days and after 5 days ($p<0.05$).

With the 1 and 2 % AgNPs-coated media treatments, high concentration of silver was detected in the water on the 3day, and even the AFM were unable to decrease the silver concentrations in these groups Table 4 . It should also be noted that, due to the high mortality rate of eggs in these groups during the first three days.

From the results, the 1 and 2 % silver nanoparticle-coated filters that were lethal for Bunee eggs, the filter media coated with 0.5 % AgNPs increased the propagation efficiency during the incubation of the fish eggs and larva; this increase according to the prevention effects of the silver nanoparticles against water mold infections in the incubation system. In addition to the use of the absorbent filters along with the filters containing silver nanoparticles significantly increased the propagation efficiency. the aim of using the absorbent filters was absorption of the silver compounds released from the AgNPs-coated media. Also , the zeolite and activated carbon filters had some effect in this regard, and the increased propagation efficiency with the 0.5Ag-AFM could be according to absorption of the silver compounds by the AFM. Moreover, some of the increase may have been due to the absorption of other harmful compounds by the AFM, as natural zeolite and activated carbon are both known as good absorbents of chemical compounds, such as drugs and malachite green, in aquaculture [45,46,47]. More studies are needed to develop better AFM with a longer and higher ability for silver absorption.

CONCLUSION

The application of 0.5 % nanosilver filters was effective in preventing water mold infections and resulted in a higher propagation output. This new type of antifungal water filters may also have the potential for widespread usage in water treatment applications in the aquaculture industry for other aquatic animals and against other microorganisms. More studies should be done to examine possible undesirable effects on the cultured fish, such as the changes in expression of genes related to silver toxicity, bioaccumulation and food consumption.

Table(1) Chemical parameters of dechlorenated water used in semi-cicerculatory system these values with the slandered limits as compared with [26].

Parameter / Unites	Cl+	NH4+	Mg+	S2-	K+	Calcium hardness	Total hardness
mg/L	2.1	0.2	31	0.0	2.9	22	167

Table(2) types of filters used in this study

Filter type (abbreviations)	Main Filter Media (MFM)	Concentrations of nanosilver-coated on MFM (%)	Measured nanosilver in MFM by XRF (%)	Absorbent Filter Media (AFM)
0 Ag-O	uncoated natural zeolite(500 g)	0	0	None
0.5 Ag-O	Nanosilver coated natural zeolite(500 g)	0.5	0.321	Natural zeolite (250 g) and granular activated carbon(250 g).
1 Ag-O		1	0.723	
2 Ag-O		2	1.729	
0.5 Ag- AFM		0.5	0.321	
1 Ag- AFM		1	0.723	
2Ag- AFM		2	1.729	

Table (3) concentrations of Chemical components of natural zeolite coated with different concentrations of silver nanoparticles specified by XRF analysis.

concentrations of silver %	Concentrations of chemical components								
	Na_2O	MgO	P_2O_5	SO_3	K_2O	CaO	TiO_2	Fe_2O_3	Ag
0	1.53	1.02	0.01	0.03	1.52	1.92	0.10	0.98	0
0.5	1.32	1.11	0.01	0.03	1.87	1.88	0.18	0.79	0.322
1	1.22	1.21	0.01	0.02	1.76	1.69	0.15	0.86	0.723
2	1.39	1.02	0.01	0.01	1.55	1.68	0.13	0.91	1.729

Table(4) Silver concentrations in the water of incubators after fertilization.

Filter type	Ag concentration mg/l (mean ± SD)		
	1^{st} daf	3^{rd} daf	5^{th} daf
0 Ag-O	0	0	0
0.5 Ag-O	$0.42\pm0.01^{a,A}$	$0.43\pm0.01^{a,A}$	$0.40\pm0.01^{a,A}$
0.5 Ag- AFM	$0.005\pm0.01^{a,B}$	$0.012\pm0.01^{b,B}$	$0.010\pm0.01^{b,B}$
1 Ag-O	$0.179\pm0.01^{c,C}$	$0.001\pm0.01^{b,C}$	-
1 Ag- AFM	$0.174\pm0.1^{c,C}$	$0.003\pm0.01^{b,C}$	-
2 Ag-O	0.97 ± 0.01^{D}	-	-
2Ag- AFM	0.88 ± 0.01^{D}	-	-

daf: day after fertilization.

^{a,b,c} means within a row with different superscripts are significantly different (P<0.05).

^{A,B,C,D} means within a column with different superscripts are significantly different (P<0.05).

Picture (1) Semi-circulatory system in Alwahda fish hatchery which used in this study .

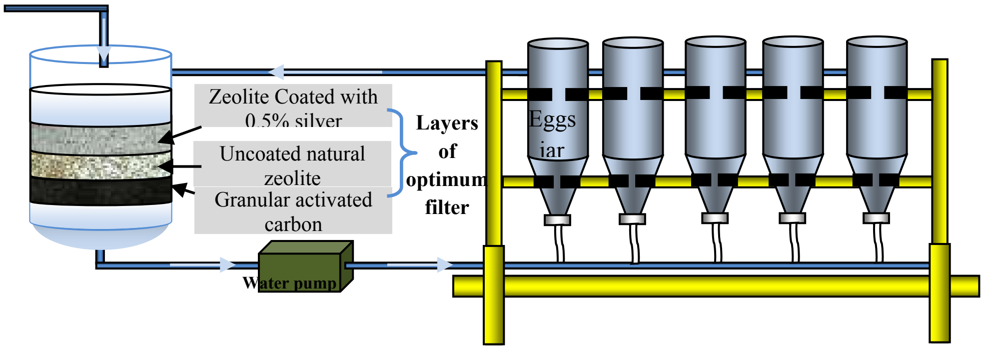

Figure (1) Schematic diagram of the semi-circulatory system used in this study.

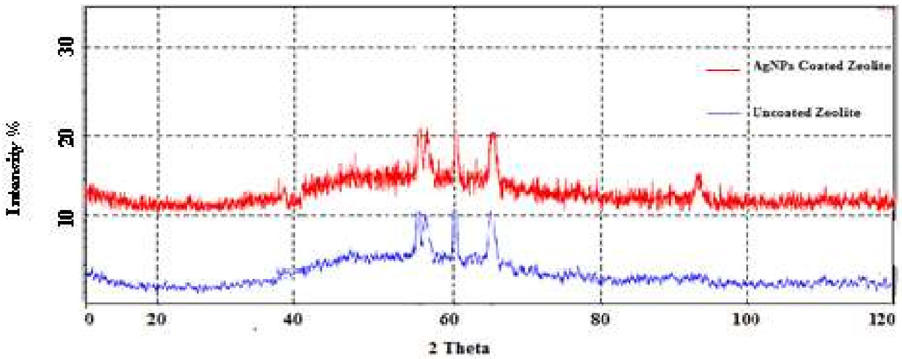

Figure (2) X-ray diffraction patterns of coated and uncoated natural zeolite with silver nanoparticles.

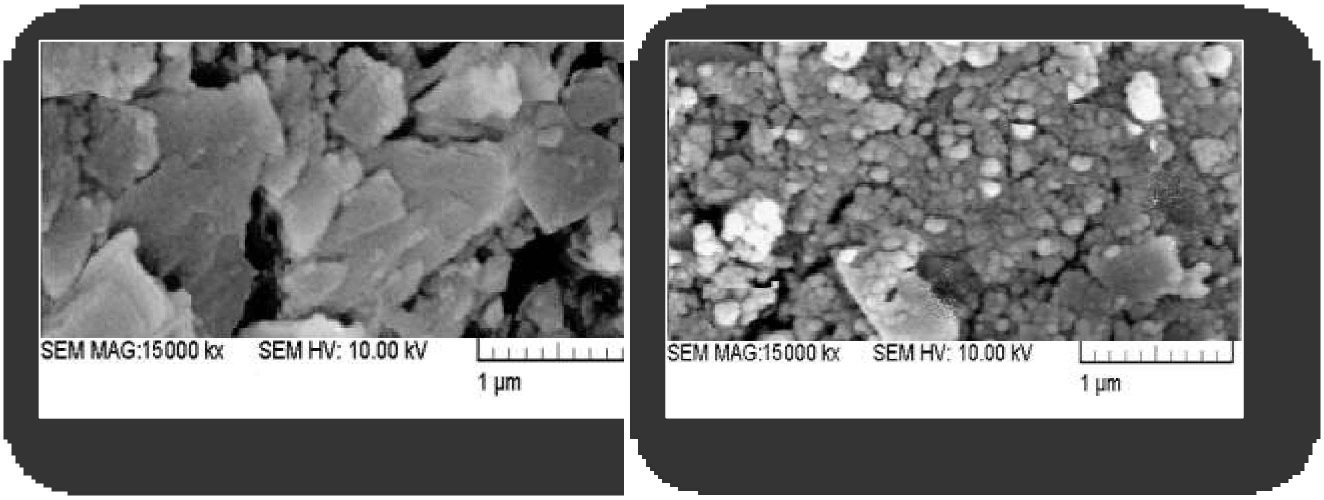

Plate (1) SEM photographs (left)uncoated natural zeolite, (right) coated natural zeolite with silvernanoparticles.

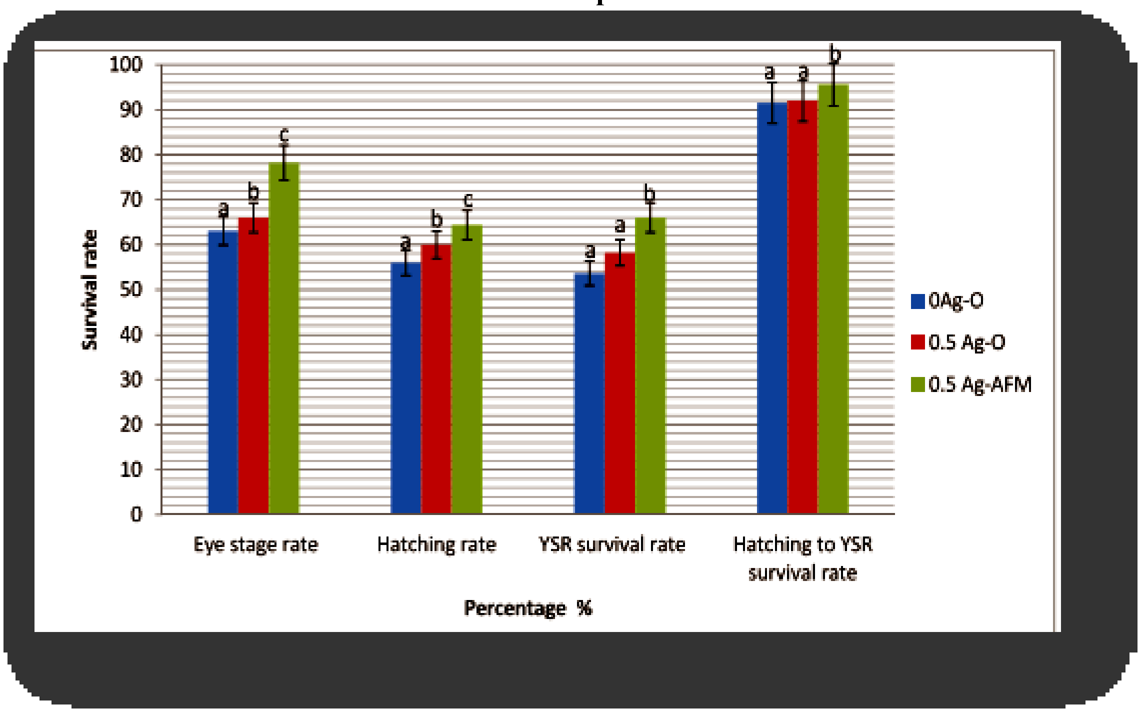

Figure (3) Survival rates from 24 h post-fertilization to eyeing (eyeing rate), from 24 h post-fertilization to hatching (hatching rate), from 24 h post-fertilization to completion of yolk–sac resorption (YSR survival rate), and from hatching to completion of yolk–sac resorption with 0Ag-O, 0.5Ag-O, and 0.5Ag-AFM (mean ± SD). Values with different superscripts (a, b, c) are significantly different (ANOVA, P< 0.05).

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