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Nano formulations as curative and protective agent for fish diseases: Studies on red spot and white spot diseases of ornamental gold fish *Carassius auratus*

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Abstract
In the present study prevention and treatment of ornamental gold fishes against red spot and white spot diseases was attempted using silver nanoparticles. The uptake of silver nanoparticles and the biochemical changes were studied and their resultant toxicity was also studied. The starch stabilized silver nanoparticles could penetrate all tissues including the brain through Blood Brain Barrier. The results also demonstrated a cure from red spot and white spot diseases within 3days without showing any toxicity. The fishes showed immediate cure from infection and the infection was not observedfor more than one year till the death of the fish and hence a one time dose may give life long protection at very low concentration (10ng/g body weight) by simple bathing method. This is the first report on silver nanoparticle treatment forprotozoan and fungal infection in fishes (Patent application 2267/CHE/2012)

Keywords: Gold fish, Silver nanoparticles, White spot disease, Red spot disease, Therapeutic and protective agent

1. Introduction
The goldfish belongs to the family Cyprinidae which was one of the earliest fish to be domesticated, and is one of the most commonly kept aquarium fish. Goldfish and other Ornamental fish culture is fast emerging as a major branch of aquaculture Prevalence of fish diseases has negative economic impact on aquaculture. White spot and red spot are the very common goldfish diseases. White Spot Disease (*Ichthyophthiriasis* - Ich) is actually caused by protozoa *Ichthyophthirius multifiliis* with Salt-like specks on the body fins and excessive slime with breathing problems. Epizootic ulcerative syndrome (EUS) or 'red-spot' disease caused by pathogenic fungus, *Aphanomyces invadans.* In aquaculture, chemotherapeutic agents such as commercial antibiotics and disinfectants (chemicals) [1] are commonly employed for disease management, although this is not applicable due to high cost, environmental hazards, and the antibiotic resistance developed by many pathogens [2]. There are three phases to the life cycle of these protozoa. Ich is susceptible to treatment at only one stage of the life cycle, Vaccination of goldfish *Carassius auratus* against *Ichthyophthirius multifiliis* (Ich) was performed with live theronts, trophonts, and sonicated trophonts and with Ich antigens [3-15]. The convergence of nanotechnology with nanomedicine has added new hope for disease detection and treatment.Silver nanoparticlesare among the most commercialized nanoparticles due to their antimicrobial potential, exceptional physical and chemical properties. Silver nanoparticles based cosmetics, therapeutic agents and household products are in wide use.
In the present paper we have studied the possibility of using silver nanoparticles for the treatment of white spot and red spot disease by observing the biochemical changes to assess their resultant toxicity in the ornamental goldfish. The results demonstrated a cure within 3 days without any apparent toxicity thereby suggesting the possible application of nanoparticles in aquaculture.

2. Materials and Methods
2.1 Synthesis of silver nanoparticles
Silver nanoparticle was synthesized from silver nitrate (1mM) using green synthesis capped with starch (1%) - a modified method as reported in our earlier paper [16].

2.2 Characterization of silver nanoparticle
Silver nanoparticles synthesized were characterized by UV-Visible absorption spectroscopy, Fluorescence spectroscopy, Transmission electron microscopy and FITR.

2.2.1 Ultra violet-visible spectroscopy
The biosynthesis of silver nano particles was monitored periodically in a UV–visible spectrophotometer as a function of reaction time and biomaterial dosage at a resolution of 1 nm.

2.2.2 Transmission microscopic analysis
For transmission electron microscopy (TEM), a drop of aqueous solution containing the silver nano materials was placed on the carbon coated copper grids and then dried under an infrared lamp for 30 min. Micrographs were obtained using a Philips EM208 microscope operating at 200 kV.

2.2.3 Fluorescence spectroscopic analysis
All the fluorescence data were obtained on a Perkin-Elmer LS50B luminescence spectrometer, equipped with a 450-W xenon source and configured with double monochromators for both emission and excitation, with a 1s integration time. The excitation wavelength used was 380 nm.

2.2.4 FTIR spectroscopic analysis
FTIR spectroscopic analyses were carried out using a Jasco Fourier Transform Infrared Spectrometer 410. FTIR spectrophotometer was connected to a photoacoustic cell in the spectral range from 4000 to 400 cm^{-1}.

2.3 *In vivo* Fish study
In order to study the uptake, toxicity and effect on diseased condition, Gold fishes normal healthy and fishes with white spot and red spot diseases were collected from the market shops. Five groups (one batch of healthy and two groups for each type of infection) with 3 fishes per batchwere kept in oxygenated, dechlorinated tap water at 37 °C. The standard method recommended by the Animal welfare committee was followed. For each group, 3 fishes were maintained in 60 liters of water. Experimental (Diseased) groups were treated by bathing method in 20ml of water with silver nanoparticles at a concentration of 10ng for 20 seconds one time. Control fish (normal and diseased) were exposed to dechlorinated tap water without the addition of particles. After the treatment period, whole body X-Ray was taken. Blood samples were collected from live fish by cutting caudal peduncle for biochemical and UV visible spectroscopy, FTIR analysis.

2.4 Toxicity study
2.4.1 Biochemical assay
Blood samples were collected from the caudal vein for total protein, albumin, phosphorus, calcium and other levels in blood plasma analysis and were assayed using commercially available kits (Roche).

2.4.2 Enzyme assays
Blood biochemistry analysis such as alkaline phosphatase activity, Na^+ K^+ -ATPase activity, GSH level, Catalase assay and LPO activity were done following the methods of Ronner *et al* [17]; Beutler *et al* [18]; Beers & Sizer [19] and Ohkawa *et al* [20] respectively.

2.5 Biological distribution pattern
In order to study the pattern of uptake and tissue distribution of nanoparticles in fish, X-Ray detection was done after one month of silver nanoparticle treatment The quantity of silver nanoparticles present in all tissues were obtained from the UV-Visible absorption spectra at 420 nm specific for silver nanoparticles.

2.6 Statistical Analysis
Statistical analysis was performed using the statistical package. The data were analyzed using STDEV (Microsoft Excel, Microsoft Corporation, USA.).The data are expressed as Mean ± SD.

3. Results
Silver nanoparticles synthesized were found to be stable in solution at room temperature and showed no signs of aggregation. The UV visible spectrum of synthesized silver nanoparticles showed absorption maximum at 420nm (`Fig.1).

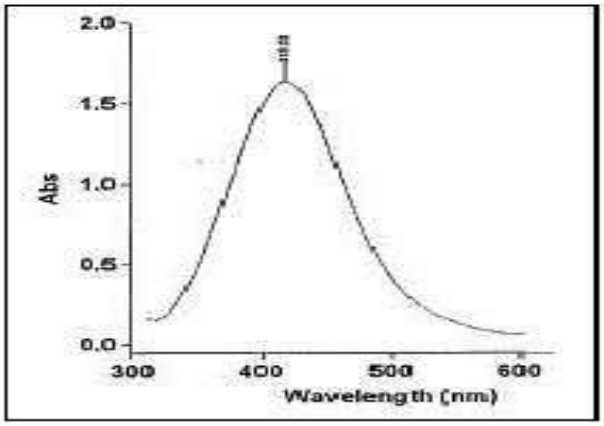

Fig 1: UV-Visible Spectrum of silver nanoparticles

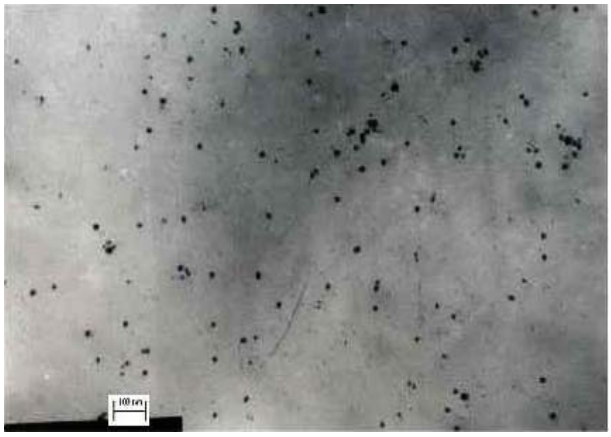

Fig 2: Transmission Electron Microscopic image of starch stabilized silver nanoparticles

Transmission Electron Micrograph of starch stabilized silver nanoparticles shows the presence of particles at an average size range of 10 nm to 20nm (Fig.2).

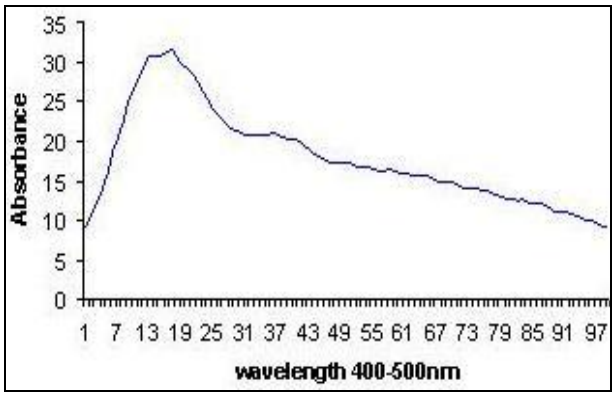

Fig 3: The fluorescence spectrum ofthe synthesized silver nanoparticles

The silver nanoparticles were also found to be mono dispersed uniformly. The fluorescence spectrum showed maximum fluorescence at 413-417nm (Fig. 3).

Fig 4: Curing property of silver nanoparticles.Red Spotdiseased fish before (1) and after (3) treatment, White Spotdiseasedfish before (2) and after (4) treatment

Bathing of diseased fish at a concentration of 10ng for 20 secondscompletely cured from the red and white spot diseases as the observed symptoms of infection disappeared immediately (Fig. 4).Approximately 3 fold increases in body weight was observed as the fishes were restored back to normalcy after total disinfection because of nanotreatment (Fig. 5) and the fish were healthy and alive for more than a year without any reinfection. Interestingly during the period of study, the fishes did not show any significant changes in behavior that might have indicated the neurotoxic effects.

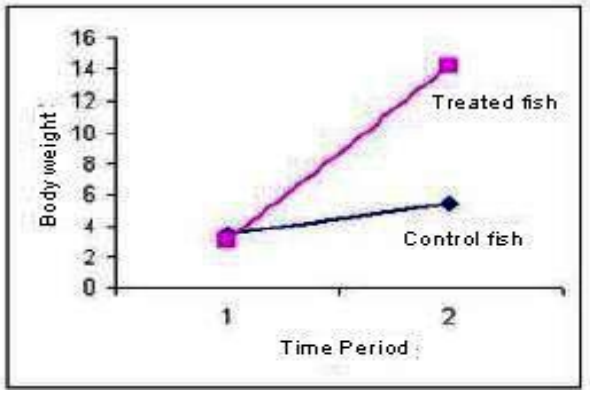

Fig 5: Body weight of silver nanoparticle treated (pink) experimental fish and Control (blue) fish

Serum biochemistry in response to metal nanoparticle exposure was studied in freshwater ornamental gold fish *Carassius auratus*. Fish were exposed to 10ng concentration for 3 days to determine changes in the levels of biochemical parameters silver nano particles in blood serum and whole body.

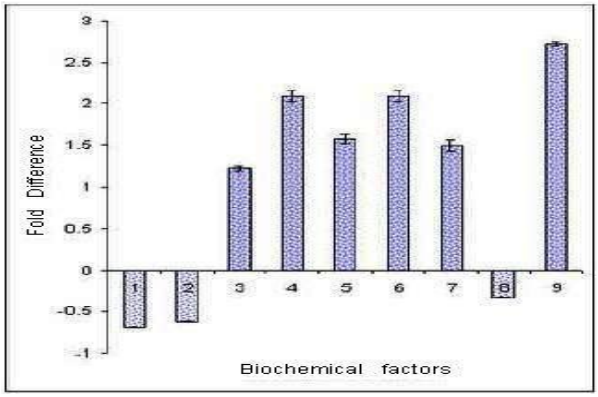

Fig 6: Fold Difference in 1. Urea 2. Creatinine 3. Sugar 4. Albumin 5. Protein 6. Cholesterol 7. Bilirubin 8. Calcium 9. Phosphorous content between control fish and Silver nanoparticle treated fish.

Various biochemical parameters analyzed in blood of control and experimental fish showed significant difference (Fig. 6). There was significant increase in sugar, albumin, protein cholesterol, bilirubin and phosphorus. All other components like creatinine, urea, and calcium decreased. The enzyme activities such as LPO, Na^+ K^+ ATPase, alkaline phosphatase, and catalase increased in contrast to GSH content which showed reduction in their activities in experimental fish than control fish (Fig. 7).

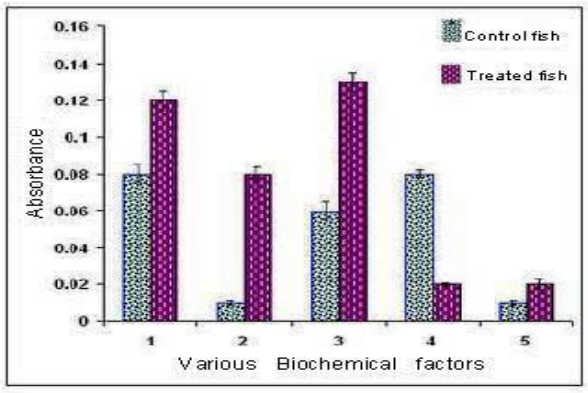

Fig 7: Comparison of various enzyme activities1.LPO 2.GSH 3. Na^+K^+-ATPase 4.Alkaline phosphatase 5.Catalase in control and silver nanoparticle treated fish. Control fish Treated fish

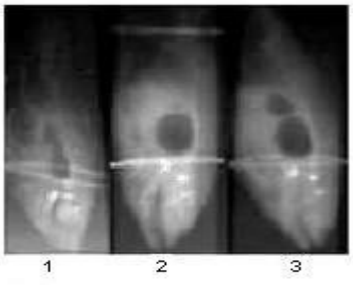

Fig 8: Whole body X-Ray image ofControl (1), Red spot disease treated (2) and White Spot disease treated fish (3).

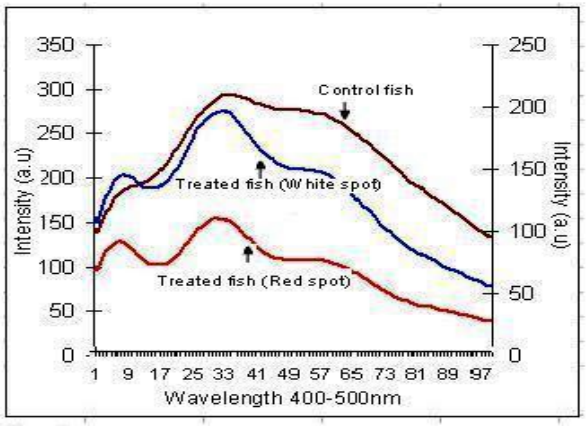

Fig 9: The fluorescence spectrum of whole body tissue of Red spot disease treated & White Spot disease treated fish compared with control fish.

In the present, study silver nanoparticles were detected in all organs including brain as observed in whole body X-Ray (Fig. 8). Silver nanoparticles were seen in blood even after two months of treatment. The fluorescence spectrum of whole body tissue (Fig. 9) showed the binding of other protein and a shift in the absorption spectrum and florescence spectrum. To determine the change in chemical bonding between the synthesized silver nanoparticles and the particles present *in vivo* in association with proteins, FTIR measurements of both samples were performed (Fig. 10). The whole body homogenate exhibited two very intense absorption bands in the 1560-1500cm^{-1} and 1350-1300cm^{-1} region of the spectrum arising from stretching vibrations of the highly polar nitrogen–oxygen bonds. For N substituted compounds, such as proline, only the- NH_2 stretching vibrations are involved and these appear at lower frequency. A relatively prominent band between 2200 and 2000cm^{-1} is found in the spectrum of most aminoacids and can be clearly seen in the spectrum of valine at 2130cm^{-1}. There is an additional band for O-H stretching vibration near 3200cm^{-1}

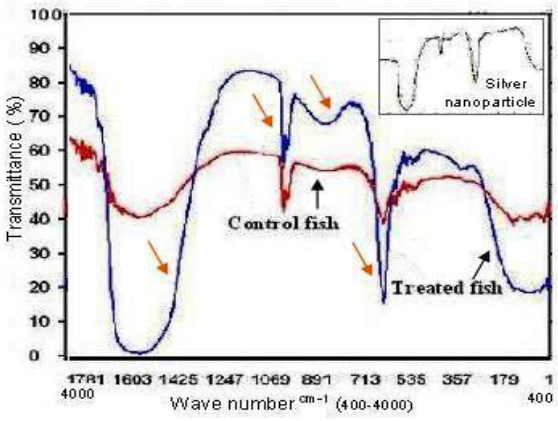

Fig 10: The FTIR pattern of control and experimental fish with silver nanoparticle pattern as an insert. Orange arrows indicate changes in FTIR pattern compared to control

4. Discussion

Drug targeting through nanoparticles may improve therapies yet a thorough understanding of the feature that regulates the effect of carrier nanoparticle is needed to translate this approach into the clinical application. Hence the present study was carried out to understand the toxicity and curing property of silver nanoparticles against White spot and Red spot Diseases of gold fish.

The UV visible spectrum of synthesized silver nanoparticles showed absorption maximum at 420nm (Fig.1) due to Mie scattering which responds only to silver metal [21].

In order to evaluate the toxic effect of silver nanoparticles, silver nanoparticles were introduced in to fishes and various biochemical parameters were analyzed in blood of healthy control and white and red spot diseased fishes. Increased concentration of GSH content and an increased capacity to maintain glutathione in the reduced state plays an important role in the detoxification of H_2O_2 and thus inthe protection against oxidative stress as observed by Inoue [22]; Jaeschke [23]; Kaplowitz & Tsukamoto [24]; Bautista *et al* [25] in human and rat liver. Maintenance of cellular GSH could be the result of elevated activity of catalase [23, 26] and to neutralize the impact of ROS, both enzymatic and nonenzymatic antioxidants which are activated [27, 28] in the cases of vertebrates and fishes. The increased catalase activity and decreased GSH content in the present study indicate the basal level oxidative stress maintenance condition of the animal because of the presence of silver nanoparticles in the body.

The Na^+ K^+ -ATPase helps in maintaining resting potential, active transport, and regulates cellular volume, ROS, as well as intracellular calcium [29]. Entry of seawater enhances intestinal and branchial Na^+ K^+ - ATPase activity in non-anadromous rainbow trout [30].

Our study showed very significant increase in alkaline phosphatase activity compared to control revealing the advantages of silver nanoparticles in maintaining the body physiology and health [31].

In Cu-exposed fishes, metal exposure increased cholesterol concentration, but decreased calcium levelin the serum. The Cl^- level decreased in Ag-exposed fishes [32].

Reduction of lipid peroxidation in the gills of fish exposed to the high concentration of N10 silver, despite a large accumulation of silver in the gills was reported by Tessa *et al* [33]. In contrast several studies, demonstrated that silver NPs generated ROS and caused oxidative stress [34-36] in mouse brain and caused increased lipid peroxidation [37].

Silver nanoparticles were distributed in all organs as observed in whole body by X-Ray (Fig. 7) similar to that observed by Raynal *et al* [38]; Briley-Saebo *et al* [39] and Quan-Yu Cai *et al* [40] in animals with iron oxide nanoparticles and gold nanoparticles [41] and with silver nanoparticles in mice, rat and Zebra fish [42-45], on medaka fish [46]. Silver nanoparticles were seen in blood even after two weeks of injection in rat. Increased half-life of ultra-small particles as seen in the present study (Fig.8) was also observed by Quan-Yu Cai *et al*. [40] with reference to gold nanoparticles. The nanoparticles were detected in the brain indicating that silver nanoparticles have the ability to penetrate blood brain barrier as observed in Zebra fish [47, 43, 44], mice and rat model [40, 16]. It was suggested that the nanoparticles could enter the cells through many routes, some of which include diffusion or endocytosis through the skin of embryos [46, 48, 49].

White Spot Disease and red-spot disease are the most common fish diseases. In aquaculture, chemotherapeutic agents are not preferred due to high cost, environmental hazards, and the antibiotic resistance developed by many pathogens [2]. Protection of goldfish against *Ichthyophthirius multifiliis* by immunization with a recombinant vaccine was attempted by He *et al* [5]. Goldfish immunized with live theronts by

immersion or injection acquired high levels of immunity and protection against infection with *Ichthyophthirius Multifiliis* [3, 4].

The therapeutic potential of silver nanoparticles against red spot and white spot diseases was demonstrated in this study. Results observed in this study showed that by20 second bathing with silver nanoparticles at 10nM concentration for three days gave complete cure from *Ichthyophthiriasis* and *Ichthyophthirius multifiliis* infection and at the same time showed long term protection from reinfection.

Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to *Danio rerio* early developmental stage was reported earlier [48-52]. Results showed that silica, zinc, and nickel nanoparticles in aqueous suspensions delayed zebra fish embryo and larva development, decreased their survival and hatching rates, and caused tissue damage [50-53]. The mortality was due to the use of very high concentrations of nanomaterials. No toxicity was observed at 0.19 nM and at 160 ng concentrations of silver nanoparticles in the Zebra fish embryos [54] and adult Zebra fish [44] respectively.

5. Conclusion

In our study young Guppies were let to swim in silver nanoparticle solution maximum for 20 seconds. The above results clearly indicated that there was no toxicity developed against silver nanoparticles and it could penetrate all tissues including the brain through BBB. Life time protection can be given to diseased fishes or as prophylactics to healthy young ones at a very low concentration by simple bathing method. This is the first report on silver nanoparticle therapy against protozoan infection in fishes.

6. Acknowledgment

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