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Shaziea Majeed
M.Phil. Research Scholar, Department of Life Science, Rabindranath Tagore University, Madhya Pradesh, India

Pragya Shrivastava
Professor, Department of Life Science, Rabindranath Tagore University, Madhya Pradesh, India

Corresponding Author:
Shaziea Majeed
M.Phil. Research Scholar, Department of Life Science, Rabindranath Tagore University, Madhya Pradesh, India

Impact of gold nanoparticles on the growth and development of *Zebrafish*

Shaziea Majeed and Pragya Shrivastava

Abstract
The present thesis encompasses a work on the effect of Gold Nanoparticles on the growth, development and behaviour of Zebrafish. In the present times, when science and technology has made progress by leaps and bounds, had at the same time its side effects on the human population as well as on other species. Nanoparticles are particles with the minimum measurement between 1 and 100 nm. Because of their peculiar size and characteristics, nanoparticles are used in very delicate missions in the biomedical field. They have been actively used for the drug distribution, cellular imaging and other diagnosis. These nanoparticles have revolutionized the medical field. Gold nanoparticles being a type of this category have also been widely used for drug distribution, detection and diagnostic methods. But along with these benefits, there are apprehensions that these nanoparticles at the same time may have an affect the growth of the organism. In this work, an attempt has been made to gauze the possible neurotoxic effects of gold nanoparticles on the growth and development of Zebrafish (*Danio rerio*), particularly its behaviour and axon development.

Keywords: *Zebrafish*, gold nanoparticles, embryo, technology

Introduction

Particles having the dimensions below the range of 100 nm are called as Nanoparticles. They are having fascinating and peculiar mechanical, electrical and optical characteristics, because of their minute dimension. Their scope of in commercial applications are very much diverse as they cover a large number of fields which includes textile cosmetics, water treatment, industry, electronics, disinfection of medical devices, plastics, and others (Lines 2008). They have revolutionized the medical sector as well, as through them the specific cells or organs are targeted and treated which till now was only a dream for the medical practitioners (Panyam and Labhasetwar 2012) [2]. It is because of their minor dimension, the nanoparticles reach out to every place of the human body and that too with greater efficiency and accuracy, when compared with the pre Nano technological devices (Kreyling *et al*. 2002) [3]. At the cellular level, nanoparticles may enter cells via different pathways. But at the same time, the physicochemical characteristics of nanoparticles are generally to known to influence the uptake pathway.

Nanoparticles are usually categorized into two broad categories, i.e. organic and inorganic nanoparticles. Carbon and carbon associated nanoparticles are categorized under the organic nanoparticles. The magnetic nanoparticles, semiconductor nanoparticles (titanium dioxide and zinc oxide) and noble metal nanoparticles (silver, gold and platinum) are grouped into inorganic nanoparticles. The applications of inorganic nanoparticles have been preferred in field of drug delivery because of their various special features e.g. good in function, controllable, target orientation, biocompatibility of nature (Xu *et al.*, 2006) [4]. These metal origin nanoparticle, naturally occurring on the earth, show extraordinary properties which they generally do not show when they are in bulk form. It is because of these characteristics and benefits; it has generally been in a great demand across the different disciplines, especially in the field of biomedicines.

Gold nanoparticles

Gold is an important constituent of almost of all culture as it is used in jewellery, coins, electronic devices as well as in medicines for being very beneficial in different types of treatments over the centuries without any harmful effects.

With the development of Gold nanoparticles the researchers use it in such places that were too small for bulk gold to reach and therefore bring it with new potential applications. They received a special attention in the field of nanotechnology and its i.e. of Gold nanoparticles use has increased tremendously over the past years. They have become a part of special interest in the field of biomedicine because of their unique optical and electrical properties (Giljohann *et al*. 2010) [5]. In biomedicine, the Goldnanoparticles are used in various aspects which includes cell uptake, drug delivery, gene transfection, cancer cell detection, antiviral activity, imaging (Javier *et al*. 2008) [6] and in many others. Besides they are used to deliver antibiotics or other antibacterial agents to different affected areas of an organism by way of conjugating the Gold nanoparticles with the antibiotic substances. Along with it, they have yielded promising results in when used as therapeutic vaccines, because in a research carried out on rats by Wang and co-workers.

However, despite of the highest biocompatibility of the Gold nanoparticles in comparison to other nanoparticles, their side-effects interms of its toxicity is still unidentified. Their small size provides them the larger surface in comparison to larger compounds, therefore provides greater chances for greater chemical and biological reaction per mass (Kreyling *et al*. 2006). These nanoparticles are causing greater effect than the larger sized particles when applied in the same concentration. Moreover, various findings togetherly has proved that particle size, coating composition and aggregation, are critical to understand cell uptake mechanism and kinetics, that is very crucial in understanding harmfulness. Therefore it became imperative that there still a dire need in conducting more and more research in overall world on the various aspects of nanoparticle toxicity, so that any possible side effects could be pin pointed and measures could be taken to address the same.

Exposure of zebrafish to *Gold Nanoparticles*

For studying the toxicity of the Gold nanoparticle, generally rats were used, but from the recent times, this trend has got changed as now other species has been used, prominent among which is Zebrafish. The Zebrafish (*Danio rerio*) is a tropical fresh water fish that originates from Southeast Asia and is the premier non mammalian vertebrate, a well-established model organism in biological studies. (Aleström *et al*. 2016) [7].

Because of the ease of accessibility, small size, robustness, reproductive capacity and short generation, easy to maintain under experimental conditions, low cost of the Zebrafish in comparison to other traditional animal models such as rat and other favorable characteristics of the Zebrafish for biological research, Zebrafish has been actively used as model for the study of various human diseases, behaviour and development. Similarly, it has become as model for the study of developmental toxicity, including neurotoxicity in humans and other species. The use of Zebrafish for study of predicting toxicity effects in humans diseases and other issues is because of its close homology with the human genome, as well as physiological and anatomical similarities including endothelial cells, presence of blood brain barrier and immunogenic responses. Besides this, Zebrafish and animals have demonstrated same biological responses to toxic substances, such as induction of metabolizing enzymes and oxidative stress. At the early larvae stage, when the Zebrafish used to be transparent, its development gets supervised through the microscope. Before five days post fertilization, Zebrafish larvae has been defined as non-animals and are therefore considered an *in vitro* model and an alternative to conventional animal testing.

Because of the apprehensions about their potential as developmental neurotoxins, my key goal of this work is to study the effects of Gold nanoparticles on Zebrafish behaviour as role of particle size in effecting the toxicity, as the preceding studies has established that the uptake of the particles is more effective at dimensions of about 40 nm in comparison to bigger and lesser particles. Besides this, it is also aimed at seeing the effect of Gold nanoparticles on the functioning of heart.

Location of the Research gap: Large number of the research studies has been conducted in the same domain. Notable research studies are; X.I. Zeng and T. P. Zhong (2018) [8], Weger M *et al*., (2020) [9], Srinath Patibandla *et al*., (2017) [10], Lisa Truong *et al*., (2019) [11], Jia, H.-R., (2019) [12]. However, there may be hardly any research study which has been explored in the same research area that has been selected for the present.

Statement of the research problem: The statement of the research problem is as under:

"Impact of Gold Nanoparticles on the growth and development of *Zebrafish"*

Purpose of the study: The present study consists of the below mentioned objectives:

- To study the effects of Gold nanoparticles on the growth and development of fish.
- To study the effects of Gold nanoparticles on the behaviour of fish.
- To study the combination and details classification of Gold nanoparticles (by green synthesis method).

Hypothesis

- Gold nanoparticles exposure will disrupt usual growth that will result in changing the behaviour of Zebrafish.
- Gold nanoparticles exposure will lead to change in cardiac functions of Zebrafish.
- Zebrafish embryos exposed to gold nanoparticles will show reduction in the locomotors movement in the dark phase while as no movement in light phase.

Methodology

The methodology of the present study has been stated under the following headings:

Gold nanoparticles of three different sizes, 20, 40 and 80 nm, of the product line Nanoxact gold nanospheres (Nano Composix) were used and the same were provided aqueous solutions, diluted in distillated water and coated with citrate. Besides this, all the three Gold nanoparticles was maintained in stock solutions at 1000 µg/mL. The stock solutions were orderly diluted to achieve concentrations of 500, 100, 50 and 5 µg/mL by adding distillated water. Transmission Electron Microscopy (TEM) and Nanoparticle Tracking Analysis (NTA) method was used for the characterization of each three gold nanoparticles stock solutions. The effect of the Gold Nanoparticles was seen on the Zebrafish and the selection of same specie material for this investigation was based on the availability, resistance and feasibility. Besides this, Fertilized Zebrafish embryos has been used in this work

Analysis of the date: The date has been analysed as under table 1-3:

Table 1: Components of the stock solution (Nano Composix MSDS). The solution was supplied in a concentration of 1 mg/ml.

Ingredients	Percentage by Mass
Gold	0.005
Sodium Citrate Dehydrate	5.9×10^5
Water	*>99*

Table 2: Time table of feeding the Model Fish at Morning 8:00 and Afternoon 2:00

Larve <20 dfp	SDS 100
Juvenile 60-100 dfp	SDS 200-300
Adult Fish >100 dfp	SDS 400

Table 3: Composure of the water used for Model Fish Unit

	Normal	Strong Range
pH	7.5- 7.8	7-8
NO_2	<0.1mg/L	<0.1mg/L
NO_3	< 30 mg/L	<60 mg/L
NH_3	<006 mg/L	<0.1 mg/L
Temperature	29 ^{0}C	25-32^0 C
Conductivity	450	300-600

Table 4 and 5 Number of larvae analysed in the behavioural test at each Gold nanoparticles size and concentration.

Table 4: Embryo Exposure

	Size 20	Size 40	Size 80
Distillated water	55	50	57
500 µg/ml	55	50	57
100 µg/ml	55	52	49
50 µg/ml	54	50	56
5 µg/ml	55	51	54
Untreated	58	52	58

Table 5: Larvae Exposure

	Size 20	Size 40	Size 80
Distillated water	51	54	54
500 µg/ml	56	56	54
100 µg/ml	60	59	57
50 µg/ml	59	53	57
5 µg/ml	57	50	56
Untreated	51	59	52

Table No. 6-11 Modelled results from Linear Mixed Effect model analysis of both the distance covered and time spent active of larvae exposed to gold nanoparticles at embryonic stage (2 hpf).

Table 6: Zebra fish Embryo exposure to Gold Nanoparticles of Size 20 nm - Distance Covered

	Estimate	DF	T	P
Distillated water	1161,41	364	19,01	0,000
Untreated	25,38	364	0,36	0,719
5 µg/mL	-146,84	364	-2,06	0,040
50 µg/mL	-133,25	364	-1,86	0,064
100 µg/mL	-144,59	364	-2,03	0,044
500 µg/mL	-188,41	364	-2,59	0,010
1000 µg/mL	-191,41	364	-2,59	0,010

Table 7: Zebra fish Embryo exposure to Gold Nanoparticles of Size 40 nm - Distance Covered

	Estimate	DF	T	P
Distillated water	1217,22	348	12,11	0,000
Untreated	-17,26	348	-0,22	0,828
5 µg/mL	-163,00	348	-2,04	0,042
50 µg/mL	-185,87	348	-2,31	0,021
100 µg/mL	-179,67	348	-2,26	0,024
500 µg/mL	-220,94	348	-2,78	0,006
1000 µg/mL	-146,26	348	-1,85	0,065

Table 8: Zebra fish Embryo exposure to Gold Nanoparticles of Size 80 nm - Distance Covered

	Estimate	DF	T	P
Distillated water	1152,60	346	16,78	0,000
Untreated	-0,71	346	-0,01	0,993
5 µg/mL	-156,36	346	-2,00	0,046
50 µg/mL	-180,49	346	-2,33	0,020
100 µg/mL	-272,07	346	-3,39	<0,001
500 µg/mL	-256,37	346	-3,12	0,002
1000 µg/mL	-328,15	346	-3,83	<0,001

Table 9: Zebra fish Embryo exposure to Gold Nanoparticles of Size 20 nm – Time Active

	Estimate	DF	T	P
Distillated water	152,65	364	17,98	0,000
Untreated	9,67	364	1,05	0,292
5 µg/mL	-12,09	364	-1,30	0,194
50 µg/mL	-15,72	364	-1,68	0,093
100 µg/mL	-13,85	364	-1,49	0,137
500 µg/mL	-23,97	364	-2,53	0,012
1000 µg/mL	-19,78	364	-2,05	0,040

Table 10: Zebra fish Embryo exposure to Gold Nanoparticles of Size 40 nm – Time Active

	Estimate	DF	T	P
Distillated water	153,56	348	12,76	0,000
Untreated	6,94	348	0,69	0,493
5 µg/mL	-12,37	348	-1,21	0,225
50 µg/mL	-16,42	348	-1,61	0,109
100 µg/mL	-15,24	348	-1,51	0,133
500 µg/mL	-22,98	348	-2,27	0,024
1000 µg/mL	-10,49	348	-1,04	0,297

Table 11: Zebra fish Embryo exposure to Gold Nanoparticles of Size 80 nm – Time Active

	Estimate	DF	T	P
Distillated water	149,85	346	17,55	0,000
Untreated	7,32	346	0,76	0,449
5 µg/mL	-11,73	346	-1,19	0,234
50 µg/mL	-16,86	346	-1,73	0,085
100 µg/mL	-29,74	346	-2,95	0,003
500 µg/mL	-22,98	348	-2,27	0,024
1000 µg/mL	-40,21	346	-3,73	<0,001

Table No. 12-17. The results from Linear Mixed Effect (LME) model analysis of both the distance covered and time spent active of larvae exposed to gold nanoparticles at larval stage (72 hpf).

Table 12: Zebra fish Larvae exposure to Gold Nanoparticles of Size 20 nm – Distance Covered

	Estimate	**DF**	**T**	**P**
Distilled water	985,43	369	11,54	0,000
Untreated	128,54	369	1,47	0,144
5 µg/mL	-23,79	369	-0,30	0,765
50 µg/mL	-53,15	369	-0,67	0,501
100 µg/mL	-11,79	369	-0,14	0,881
500 µg/mL	39,46	369	0,49	0,622
1000 µg/mL	50,23	369	0,63	0,527

Table 13: Zebra fish Larvae exposure to Gold Nanoparticles of Size 40 nm – Distance Covered

	Estimate	**DF**	**T**	**P**
Distilled water	1234,54	373	13,59	0,000
Untreated	-221,18	373	-2,59	0,010
5 µg/mL	-330,54	373	-3,71	<0,001
50 µg/mL	-111,41	373	-1,27	0,205
100 µg/mL	-185,56	373	-2,17	0,031
500 µg/mL	-366,44	373	-4,23	<0,001
1000 µg/mL	-369,82	373	-4,23	<0,001

Table 14: Zebra fish Larvae exposure to Gold Nanoparticles of Size 80 nm – Distance Covered

	Estimate	**DF**	**T**	**P**
Distilled water	1123,32	373	9,18	0,000
Untreated	-149,33	373	-1,74	0,082
5 µg/mL	-186,58	373	-2,22	0,027
50 µg/mL	-136,03	373	-1,62	0,105
100 µg/mL	-75,88	373	-0,91	0,366
500 µg/mL	-140,81	373	-1,66	0,098
1000 µg/mL	-201,22	373	-2,38	0,018

Table 15: Zebra fish Embryo exposure to Gold Nanoparticles of Size 20 nm – Time Active

	Estimate	**DF**	**T**	**P**
Distilled water	124,47	369	13,23	0,000
Untreated	15,07	369	1,44	0,151
5 µg/mL	-0,93	369	-0,10	0,923
50 µg/mL	-2,30	369	-0,24	0,807
100 µg/mL	1,42	369	0,15	0,880
500 µg/mL	4,74	369	0,50	0,619
1000 µg/mL	10,42	369	1,01	0,272

Table 16: Zebra fish Embryo exposure to Gold Nanoparticles of Size 40 nm – Time Active

	Estimate	**DF**	**T**	**P**
Distilled water	156,04	373	14,67	0,000
Untreated	-17,86	373	-1,76	0,080
5 µg/mL	39,52	373	-3,73	<0,001
50 µg/mL	-17,69	373	-1,70	0,091
100 µg/mL	-20,92	373	-2,06	0,040
500 µg/mL	-41,91	373	-4,08	<0,001
1000 µg/mL	-48,93	373	-4,71	<0,001

Table 17: Zebra fish Embryo exposure to Gold Nanoparticles of Size 80 nm – Time Active

	Estimate	**DF**	**T**	**P**
Distilled water	147,18	373	9,04	0,000
Untreated	-10,44	373	-0,94	0,346
5 µg/mL	-19,18	373	-1,76	0,078
50 µg/mL	-15,60	373	-1,44	0,150
100 µg/mL	-2,86	373	-0,26	0,792
500 µg/mL	-16,76	373	-1,53	0,127
1000 µg/mL	-25,88	373	-2,37	0,018

Interpretation and results of the data

The data has been interpreted as under the above presented data revealed that when the Zebrafish were exposed to Gold nanoparticles of size 2 hpf has generally shown the effects of reduction in motility. While as the Zebrafish that was exposed to Gold nanoparticles at 72 hpf has resulted in changes in behaviour, but this change in behaviour has not been a linear trend. In case of reduced mortality, we found that it was more dependent on the size of nanoparticle rather than that of exposure time. Besides this we have found that gold nanoparticle also affects the locomotive motor activity of the Zebrafish as it shrinks the length of axon as well as its heart beat.

Besides this, the embryos that were exposed to gold nanoparticles at 2 hpf moved less during the dark phase compared to controls irrespective of particle size, but the same general effect was not ostensible in the larvae exposed at 72 hpf. Therefore this suggest that gold nanoparticles may have an effect on CNS development, as the brain has been developed by 48 hpf, rather than being a general neurotoxic effect. Therefore, while summarising this study, we can say that the power of the effect of Gold nanoparticles is dependent on the size and tenure of exposure of the target organ/animal to gold nanoparticles. Moreover, we have also found that the gold nanoparticles coated with citrate effect the axon development through and in this method too, the size and time of the target organ/animal to gold nanoparticles exposure matters. The same gold nanoparticles coated with citrate have an influence on heart rate in larvae also and the same we have demonstrated on the Zebrafish in this study. And the same influence on the heart rate may increase or decrease the metabolic rate of the larvae which in turn affects its behavioural activity. The Zebrafish has also shown greater possibilities for being the suitable model for the study of gold nanoparticle effects the humans and other species.

Future recommendation

In order to carry on the current work, the future recommendations include:

- To study in more detail how exposure time and particle characteristics effect behavior.
- To study the effects of gold nanoparticles in motor neuron development *in vivo*
- In order to elicit the toxic mechanisms of gold nanoparticles, work should focus on gold nanoparticles uptake and biological fate, and molecular pathways, because we have found the evidence of health concern associated with the gold nanoparticles.

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