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INFLUENCE OF NANOSILVER EXPOSURE ON CHOLINESTERASE ACTIVITIES, CD41 AND CDF/LIF-LIKE EXPRESSION IN ZEBRA FISH

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Annotation

Metal nansolicoparticles are suspected to cause diseases in a number of organisms, including man. In this paper, we report the effects of nanosilver (Ag, 1-20 nm particles) on the early development of the zebrafish, a well established vertebrate model.

Embryos at the midgastrula stage were exposed to concentrations ranging from 100 and 0.001 mg/L to verify the effects on different endpoins: lethality, morphology, expression of cholinergic molecules and development of the immune system.

Key words: nanosilver toxicity in zebrafish larvae.

Кілт сөздер: балықтың данио дернәсілдеріндегі нанокүміс қышқылдары.

Ключевые слова: токсичность наносеребра в личинке данио рыб.

One of the important problems of theoretical and practical medicine and physiology is the study of the responses of the organisms to environmental cues, with the goal of enhancing prevention of the main diseases induced by environmental stress.

An emerging risk is represented by the wide diffusion of nanoparticles, such as the silver nanoparticles, that were among the first metal nanoparticles to reach the market. Manufacturers have exploited their exceptionally efficient antibacterial, anti-viral and antifungal activity [1,2] by adding them to cleaning products, toys, clothing, and coatings inside washing machines. As reported in the review of Chen and Schluesener, nowadays the products containing nanosilver are increasing, as well as their worldwide diffusion for industrial processes and treatments In daily life, consumers may have nanosilver containing room sprays, laundry detergents, water purificants and wall paint[3,4]. Their high catalytic activity is due to the particularly small size (1 to 20 nm), that highly increases the metal surfaces [1]. Nevertheless, exposure to silver nanoparticles has been associated with "inflammatory, oxidative, genotoxic, and cytotoxic

consequences" [3]. According to these authors, the particles primarily accumulate in the liver and have also been shown [4] to be toxic in other organs including the brain. Thus, the balance between the advantages and risks of nanosilver employment as a water disinfecting agent is puzzling, in part due to the scarcity of validated alternative models for testing the behavior in the aquatic environment and the effects of chronic exposures..

In this work, we have used the first developmental stages of the cyprinid zebrafish, *Brachydanio rerio (Danio rerio)* as a vertebrate model to test the effects of nanosilver in the aquatic environment. In this work, we will use the abbreviation ZF for zebrafish. During the last decades, this fish has been chosen as a reliable vertebrate model for understanding basic events in developmental biology.

Characterization of Ag NPs

Ag NPs were obtained from Polytech (Germany, type WM 1000-c), supplied as a 1000 ppm in deionized water suspension of metallic silver (Ag particles encapsulated in liposomes) with a NP size between 1 and 10 nm. This "nano-suspension" did not need further sonication, since it was very stable and it did not form any agglomerates [5]. Size range and zeta potential of Ag NPs were evaluated by Dynamic Light Scattering (DLS) analysis (Malvern, UK).

Exposure

At 12 h dead and anomalous embryos were discarded, and the healthy ones were divided into multiwell dishes containing nanosilver particles (Ag) suspended in ultrafiltered freshwater at concentrations ranging from 100 to 0.001 mg/L.

Control eggs were maintained in ultrafiltered fresh water for the time of the experiment.

All the dishes were placed in a thermostat at T= 25°C and the control and exposed specimens were allowed to develop for further 24 and 48 h, up to hatching.

After those times, the survived larvae were counted and measured. Developmental anomalies were registered and classified.

Fixation for immunochemistry reactions

After incubation, the living larvae were fixed in paraformaldehyde (PFA) 3% in phosphate buffer saline (PBS) + 70% cold methanol for 20 min, and then rinsed and re-hydrated in 0.1%BSA/PBS for 10 min before processing for immunohistochemical reactions.

Localization of molecules immunologically related to CDF/LIF and to CD41

Samples were incubated overnight at T= 5°C in the primary antibodies diluted 1:200 in PBS containing 0,5% BSA, 0,1%NGS. The primary antibodies were: anti-Leukemia Inhibitory Factor (CDF/LIF), raised in goat (Sigma, IT), or anti-CD41 (ABCAM, IT, l1024) raised in mouse. After rinsing in PBS/BSA, the samples were incubated overnight at 5°C in the secondary antibodies (chick anti-goat Alexafluor 488 and rabbit anti-mouse igG, respectively), 1: 300 in PBS/BSA. Nuclei were counterstained with 1μM RNAase followed by 2μM propidium iodide (PI). The preparations were mounted on a slide with the anti-fading Gelvatol [6]. Images (1024x1024x8 bit) were acquired on a Leica TCS SP5 AOBS confocal laser scanning microscope (Leica Microsystems Mannheim,Germany), using a one Airy disk unit pinhole diameter and an HCX PL APO 20x/0.70 objective; magnifications were obtained by scan field selection. Alexa Fluor 488 was excited with the 488 nm line of the Ar laser, and its fluorescence was collected in a

spectral window of 500-530 nm. Propidium iodide was excited with the 543 line of the He-Ne laser, and its fluorescence was collected in a spectral window of 600-640 nm. Laser scanning transmitted light images were obtained using the 488 nm laser line.

Homogeneous measures

For homogeneous measures (body length, enzyme activity), a one-way ANOVA was performed to test for differences among the effects of different nanosilver concentrations. Prior to running analyses, homogeneity of variances was tested by Levene's test; whenever necessary, data were transformed and re-tested. When transformation did not produce homogeneous variances, we set a=0.01, in order to make up for the increased likelihood of type 1 error [7]. Post hoc multiple comparisons after ANOVA were made by Tukey's test of honestly significant differences. Descriptive statistics are reported as mean ± standard deviation. All analyses were performed using the free PAST software package version 2.17c [8].

The occurrence of dead embryos for each exposure was different among the embryos exposed to the different Ag concentrations, with a trend to increase from control to the more concentrated exposures, but no homogeneity among the different groups of embryos was present. The RR elaboration of the data showed an exponential risk probability of about 1.2 folds respect to the control for the exposure to 0.001 mg/L; average risk of 1.7 folds for the embryos exposed to 0.01 mg/L, 0.1 mg/L; 1 mg/L, and a risk of 2.56 folds for the exposures to 10 mg/L Ag concentration (Fig. 1). The exposure to 100 mg/L caused 100% death in almost all the experiments.

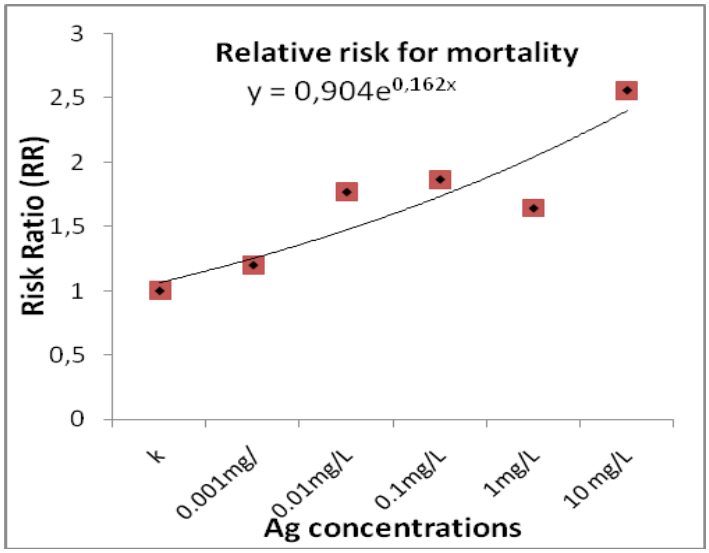

Fig. 1 - Death risk probability (RR) according to the different Ag concentrations (0, 0.001, 0.01, 0.1, 1, 10 mg/L).

X axis= Ag concentrations;

Y axis = risk ratio respect to unexposed samples, assumed as 1

Localization of molecules immunologically related to CDF/LIF (Fig. 2).

In Control samples, the CDF/LIF-like fluorescent immune reaction marked a complex net of vessels in the head, in the thymus (not shown) and the main vessels above described. In control larvae, CDF/LIF IR defined the particular structure of the vessels and of the varicosities, and the thin intersegmental vessels (ISLV), joining the DLLV. CDF/LIF-like IR also stained the walls of the vessels and molecules released among the muscle fibres (Fig. 2 A). The samples exposed to 0.001 mg/L nanosilver showed an aspect very similar to the controls, IR-positive cells were present inside the PCLV varicosities and ISLVs departing from them (Fig. 2 B). The samples exposed to higher concentrations of Ag showed decreasing distribution of positive sites (Fig. 2 C,D,E). The ISLV were not decored in the samples exposed to 0.01 and 0.1 mg/L (Fig. 2 C,D);

in the PCLV the LIF IR appeared irregularly distributed, and weaker in the samples exposed to 1 mg Ag/L (Fig. 2 E). Traces of released LIF–like molecules were seen inside the ISLVs in samples exposed to 1mg/L, while the samples exposed to 10 mg/L only showed IR traces, scattered inside the DLLV, The SILV and the PCLV (Fig. 2 F).

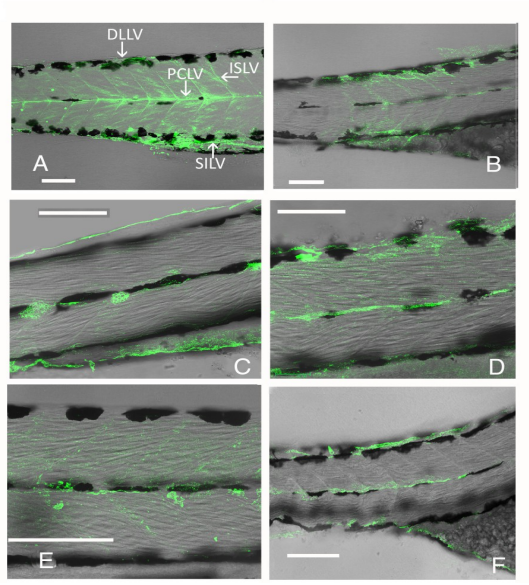

Fig. 6 – CDF/LIF IR, confocal sections of 72 h larvae, stained *in toto* by immunofluorescence. A= control larva; B= larva obtained from embryo exposed to 0.001 mg/L nanosilver; C,D= larvae obtained from embryos exposed to 0.01 and 0.1 mg/L Ag; E, F= larvae obtained from embryos exposed to 1 and 10 mg/L Ag, respectively

The relationship between AChE activity and blood [9] and in thymocytes [10] is known since more than 20 years and recently it was associated to stress events in several aquatic organisms, such as prawn exposed to ChE-inhibiting pesticides [11]. In general, the cholinergic anti-inflammatory system and α7 nicotinic receptors in macrophages have been proposed to play a role in neuroimmunomodulation of resistence and relief in mammalian inflammatory processes [12]. In the high vertebrates interrelationship between the lymphatic system cell mobilization and neurotransmitter systems was also demonsrated [13,14,15].

The effects of exposure to NPs show a trend to impairment of immune responses, possibly related to the degree of inhibition of the AChE and PChE activities. This hypothesis paves the way to further studies on the presence of molecules related to the cholinergic system in the immune cells of different organisms and the competitive effects possibly exerted by the Ag NPs.

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НАНОКҮМІСТІҢ ХОЛИНЭСТЕРАЗН ҚЫЗМЕТІНЕ ӘСЕР ЕТУІ CD41 ЖӘНЕ CDF/LIF-ТЕКТЕС БАЛЫҚТА ҚАЛЫПТАСУЫ (ДАНИО РЕРИО) ДЕРНӘСІЛДЕРІ

Резюме

Металдық нанобөлшектер, зерттеумен қарастырылғандай, кейбір организдерде, сонымен қатар адамдарда да ауыру тудыруы мүмкін. Бұл жұмыста біз хабарлағандай нанокүміс (Ag, 1-20 нм бөлшектері) омыртқалы модельдердің жақсы үйлесілімділігінің және балықтарда данионың басты кезеңдерінде пайда болуы анықталған. Эмбрион орта кезеңдерде 100-ден 0,001 мг/л диапазонына дейін гаструлы концентрацияға тап болады яғни әртүрлі нүктенің соңына дейін тексеру әрекеттері үшін: тез арада, морфология, холинерголиялық молекулалар экспрессиясы мен иммундық жүйенің дамуына.

Кілт сөздер: балықтың данио дернәсілдеріндегі нанокүміс қышқылдары.

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ВЛИЯНИЕ НАНОСЕРЕБРА И ВОЗДЕЙСТВИЯ НА ХОЛИНЭСТЕРАЗНУЮ ДЕЯТЕЛЬНОСТЬ CD41 И CDF/LIF-ПОДОБНЫЕ ВЫРАЖЕНИЯ ЗЕБРА РЫБ (ДАНИО РЕРИО) ЛИЧИНОК

Резюме

Металлические наночастицы, как предполагается, вызывают заболевания в ряде организмов, включая человека. В данной работе мы сообщаем о последствиях наносеребра (Ag, 1-20 нм частиц) на ранних стадиях развития у рыбок данио, хорошо организованной позвоночных модели. Эмбрионы на стадии середины гаструлы подвергались концентрации в диапазоне от 100 до 0,001 мг/л для проверки воздействия на различные точечный конец: летальность, морфологии, экспрессии холинергических молекул и развития иммунной системы.

Ключевые слова: токсичность наносеребра в личинке данио рыб.

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