RESEARCH ARTICLE

Determination of Chronic Median Lethal Concentration of Sodium Fluoride in *Drosophila melanogaster* and Exploring Effect of Sub-lethal Concentrations on Differential Hemocyte Count

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Abstract The prerequisite for toxicity assessment of any chemical is the determination of its median lethal concentrations and thus, this study initiates with determination of chronic median lethal concentration of sodium fluoride (NaF) in a non-target organism *Drosophila melanogaster*, which was found to be 125–130 μg/mL. Further, chronic exposure to NaF at sub-lethal concentrations (10–100 μg/mL) resulted in significant reduction in total hemocyte number in general and plasmatocytes in particular, whereas, crystal cells were significantly increased in number. Since hemocytes are an integral part of innate immune system in *Drosophila*, their numerical fluctuation confirms fluoride-induced compromise in innate immunity status of *Drosophila*.

Keywords Fluoride · Fruit fly · LC_{50} · Trypan blue

Introduction

Any chemical substance, biological agent or antimicrobial disinfectant used against pest (like insects, plant pathogen, birds, mammals, fish, and nematodes) is known as pesticide. Use of pesticides is indispensable because physical and biological pest control methods are far less cost effective, thus, chemical control is an obvious choice. But most of these chemical pesticides are persistent in the environment and cause significant problem to non-target organisms. Among various pesticides used in agriculture, fluoride-containing ones form a small group. Sodium fluoride (NaF) being one such chemical, is widely used as fungicide, wood preservative for utility poles and for water fluoridation. Interestingly, it is also an active ingredient of toothpastes and mouth rinses. *Drosophila melanogaster* (fruit fly) which is genetically best known of all eukaryotic organisms is equally popular as a model organism in evaluating the toxic potential of any chemical (Mishra et al. 2013). Various studies report the effect of chemicals on the life cycle, morphology, fecundity and hatchability of *Drosophila* (Podder and Roy 2013; Rajak et al. 2013). To evaluate the toxicity of any chemical it is very important to determine its concentrations which can kill 50% of the organisms. The acute and chronic LC_{50} of another fluoride-containing pesticide, cryolite (sodium aluminum fluoride) have been worked out (Podder and Roy 2013) and the values are seen to have huge differences (acute LC_{50} is in the range of 150–158 μg/mL and chronic LC_{50} is 67,000 μg/mL cryolite concentrations). The acute LC_{50} of NaF in adult *D. melanogaster* has been reported to be 48,000 μg/mL (Mitchell and Gerdes 1973). Interestingly no report on chronic LC_{50} of the chemical in *Drosophila* is yet available. Due to this reason, the authors aim to determine the chronic LC_{50} of NaF in the larvae and pupae of *Drosophila*. This finding would essentially help in the process of dose selection for study on sub-lethal toxicity of the chemical.

Like other living organisms, *D. melanogaster* is known to combat any infection through immune system machineries, particularly the innate immune system, which comprises of humoral and cell mediated immune responses. Hemocytes are responsible for cell-mediated immune

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response and three different types of hemocytes, namely plasmatocyte, lamellocyte and crystal cells (PC, LC and CC), are reported from the fruit fly's hemolymph (Shrestha and Gateff 1982) and their structures were reported earlier by three authors (Rajak et al. 2014). The total hemocyte count in *D. melanogaster* varied to a great extent after acute exposure to acephate, an organophosphate insecticide (Rajak et al. 2014). In another study, Das et al. (2006) noted sodium fluoride (NaF) induced oxidative stress and immunotoxicity following NaF exposure in rats. Further it was shown that NaF treatment lowered cellular immunity in the rats resulting from significant diminution in peripheral blood lymphocyte, monocyte and neutrophil counts in conjunction with a reduction in splenocyte counts. Similarly, chronic fluoride (F) intoxication causes biochemical changes as well as chromosome aberrations in mice brain and bone marrow cells (Bhatnagar et al. 2006; Podder et al. 2008a, b). Thus, in this study, chronic LC_{50} of NaF in larvae and pupae of *D. melanogaster* has been determined followed by selection of sub-lethal concentrations of NaF for further experiments. The experimental findings on differential hemocyte abundance in treated insects would prove helpful to confirm the immunotoxic potential of NaF.

Materials and Methods

Model Organism

Oregon-R strain of *D. melanogaster* cultured in Standard *Drosophila* Medium (SDM) was used as the model organism. SDM contain agar–agar (0.83%), sucrose (4.16%), corn meal (4.72%), yeast extract (1.66%), nipagin (0.27%), and propionic acid (0.27%). The flies were maintained in environmental test chamber at 22 ± 1 °C temperature, 50–60% humidity and 12L:12D conditions.

Test Chemical Preparation

NaF powder (MERCK, India) was dissolved in distilled water to prepare the stock solution (1000 µg/mL). This stock was further diluted to prepare different concentrations of working NaF solutions.

LC_{50} Determination

To determine the chronic lethal concentration 50 (LC_{50}) of NaF in 3rd instar larvae of *D. melanogaster*, five graded concentrations (120,130,140,150 and 160 µg/mL) of NaF were prepared in SDM from stock solution of 1000 µg/mL. Since previous study in our laboratory recorded the chronic median lethal concentration of another fluoridated chemical cryolite (Na_3AlF_6), which was found to be within the

range 100–160 µg/mL (Podder and Roy 2013), the present study was designed to the range 120–160 µg/mL. Thirty early 1st instar larvae (freshly hatched, within 6 h) in triplicate sets were maintained for each grade along with the control set and data on LC_{50} were collected and recorded following the methodologies described by Ashburner et al. (2005).

Hemolymph Smear Preparation

Following determination of median lethal concentration newly hatched 1st instar larvae were exposed to sub-lethal concentrations of NaF (10, 20, 40, 80, and 100 µg/mL) and maintained up to 3rd instar stage. Hemolymph of late 3rd instar larvae was collected and hemocytes were counted and compared according to the method of Rajak et al. (2014). Here 10 larvae (per treatment category) were bled for hemolymph on a slide having 20 µl PBS and mixed properly using micropipette. Thereafter, hemolymph smear was prepared on the slide and was air dried, followed by fixation in methanol and stained using Giemsa stain to visualize the hemocytes. For each treatment category, three replicates of slides were prepared for differential count of hemocytes.

Trypan Blue Dye Exclusion Assay

For differentiating the living from the dead hemocytes, hemolymph collected from treated 3rd instar larvae was diluted in 20 µl of phosphate buffered saline (PBS) (pH 7.4). Then the cells were stained with 20 µl of 0.2% trypan blue staining solution for 10 min. The extra stain was removed by washing with 20 µl of PBS and observed under microscope at $(10 \times 40) \times$ magnification. Similar type of experiment was carried out for untreated (control) larval hemolymph. Total number of cells (hemocytes) were counted using hemocytometer and the number of dead and live cells were recorded from which the difference between control and treatment categories could easily be suggested. To obtain the total number of cells the following formula was applied:

$$[(\text{Total hemocyte number}/\text{Number of squares counted}) * \text{dilution factor} * 10^4] \text{ cells/mL of hemolymph.}$$

Statistical Analysis

All data are expressed as mean $\pm$ Standard Error (SE). Mean, standard error, linear regression equation and correlation coefficient of data were calculated using MS Excel 2007. Student's t-test was carried out to determine significant differences between control and treatment groups, where $p < 0.05$ was considered statistically significant.

Table 1 Mean percentages of pupae formation and adult emergence in *Drosophila melanogaster* after exposure to different concentrations of NaF

Concentrations of NaF in SDM (µg/mL)	Percentage (%) of pupae formation	Percentage (%) of adult emergence
Control	95 ± 2.88	88.33 ± 1.66
120	61.66 ± 1.66	38.33 ± 1.66
130	48.33 ± 1.66	22.33 ± 3.33
140	41.66 ± 3.33	13.33 ± 1.66
150	28.33 ± 4.4	8.33 ± 1.66
160	6.66 ± 1.66	–

Concentrations of NaF are represented in µg/mL. Data for pupae and adult formation from 30 larvae in each category is represented as mean ± standard error (SE)

Chronic median lethal concentration (LC_{50}) of NaF in the third instar larvae and pupae of *Drosophila melanogaster* was determined by the log probit transformation method as described by Finney (1971). The results of probit calculations as represented in the graphs were obtained with the help of Microsoft Excel 2007.

Results

The result shows that increasing concentrations of NaF significantly ($p < 0.05$) reduces the percentage of pupae formation (Table 1) when compared to the control data.

The percentage of pupae formation declined up to 61.66% at 120 µg/mL of NaF when compared to the control group (in which 95% pupae formation occurred). The percentage of pupae formation was least (6.66%) at 160 µg/mL. Fifty percent of larval mortality was observed between 120–130 µg/mL as represented in the Table 1. Probit analysis of the data reveals that larval LC_{50} of NaF is 125.314 µg/mL (Fig. 1). Thus 50% of the larvae failed to become pupae at this concentration.

Percentage of emergence that represents pupal mortality determined the LC_{50} of NaF in case of the pupae. Chronic LC_{50} of NaF in the pupae has been presented in Fig. 2. As stated above, the results show that pupal mortality was significantly increased ($p < 0.05$) with increasing concentrations of NaF (Table 1), that is greater number of pupae fail to emerge as adults. Maximum pupal mortality was observed at 160 µg/mL where no pupa successfully emerged as adult. From the probit analysis of the data, it was observed that 50% pupal mortality occurred between 125 and 130 µg/mL of NaF.

Hematological studies reveal mean percentage of PC for control group to be 94.147 ± 1.85% whereas for treatment groups (10, 20, 40, 80 and 100 µg/mL), the mean PC count was found to be 90.382 ± 0.728, 81.759 ± 0.798, 84.966 ± 2.178, 89.160 ± 0.777 and 80.436 ± 1.37% respectively (Fig. 3). Data of plasmatocyte number in treatment categories (20, 40 and 100 µg/mL) are significantly ($p < 0.05$) reduced when compared to the control set.
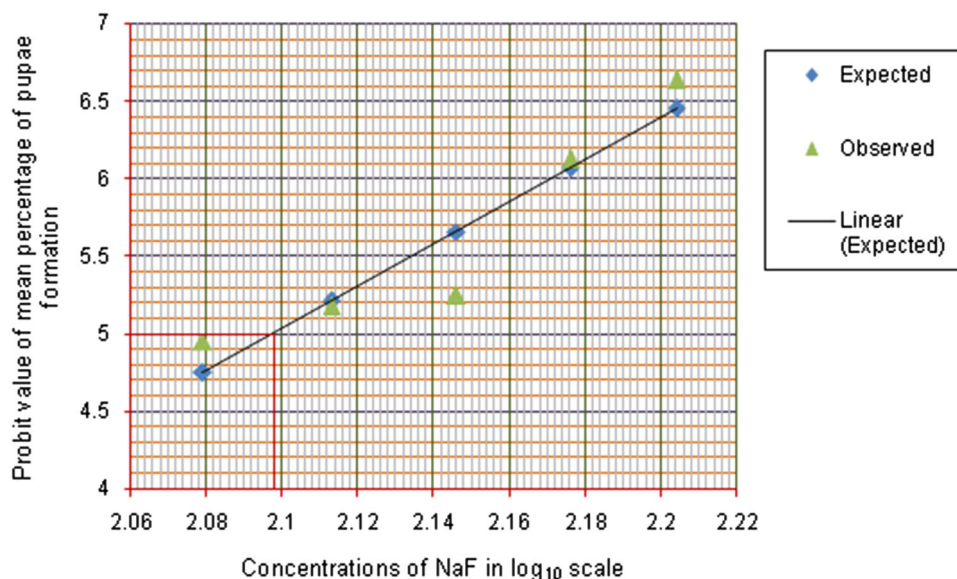

Fig. 1 The graph represents probit analysis for evaluation of chronic LC_{50} concentration of NaF in third instar larvae of *Drosophila melanogaster*. Five concentrations of NaF (120, 130, 140, 150 and 160 µg/mL) were selected and were used to observe mortality percentage in third instar larvae of *Drosophila*. The observed mortality percentages were converted into probit values (represented by '*y*' axis in graph), whereas graded concentrations of NaF were transformed into values in log10 scale (represented by '*x*' axis in graph). Probit and log values then used to plot the graph by utilizing MS Excel 2007. According to the above graph, LC_{50} falls in a log value of 2.097 which in antilog form, represents 125.025 µg/mL concentration of NaF
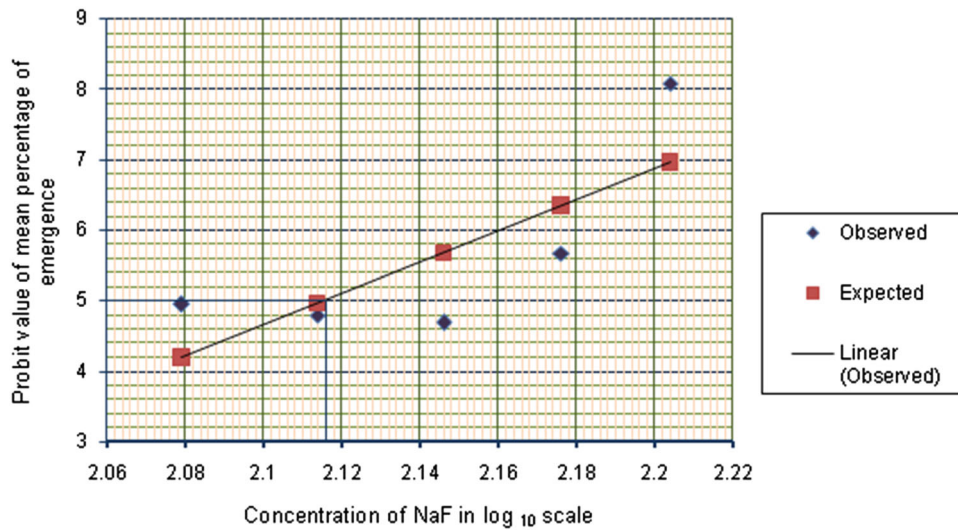
Fig. 2 Probit analysis for chronic LC_{50} determination in adult emergence of *Drosophila melanogaster*. The graph shows the probit values of observed and expected percentage of adult formation. Each treatment group comprised of twenty 1st instar larvae. Triplicate sets of such treatment groups along with control group were observed for the experimental duration of 25 days. The scattered dots represent the expected percentage of adult formation which are joined to form a trend line that help to determine the log concentration of the chemical corresponding to 50% pupal death (or 50% emergence). According to the graph the LC_{50} value of NaF in $\log_{10}$ scale is 2.116 whose antilog value represents 130.617 µg/mL NaF concentration
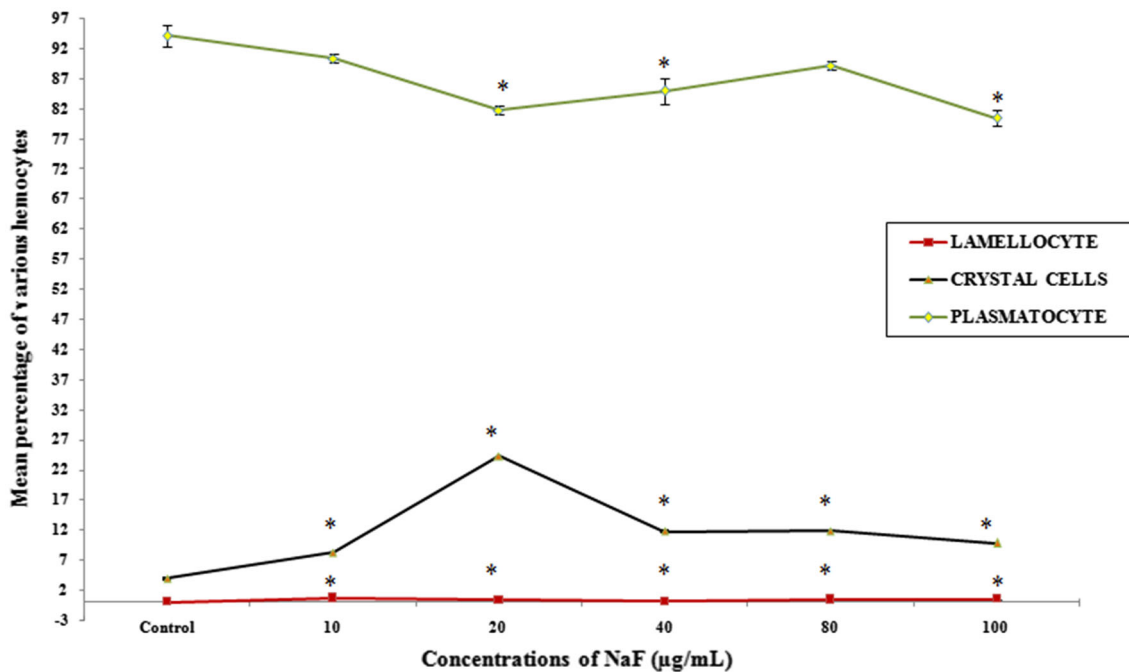

Fig. 3 Graphical representation of numerical variation of different type of hemocytes from 3rd instars larval hemolymph in *Drosophila melanogaster* in response to exposure to different concentrations of NaF (10, 20, 40, and 80,100) µg/mL along with food. Data represents Mean ± SE of particular hemocyte count at variable treatment concentrations from hemolymph collected from triplicate set and pooled from 10 larvae. The symbol '*' represent statistically significant ($p < 0.05$) when compared to control label the photo as control and treated

On the other hand, the mean percentage of LC for control group was recorded as 0 where as for treatment groups such as 10, 20, 40, 80 and 100 µg/mL, the mean LC count was found to be 0.672 ± 0.001, 0.326 ± 0.002, 0.067 ± 0.028, 0.376 ± 0.011 and $0.483 \pm 0.001\%$ respectively (Fig. 3). The number of lamellocyte in
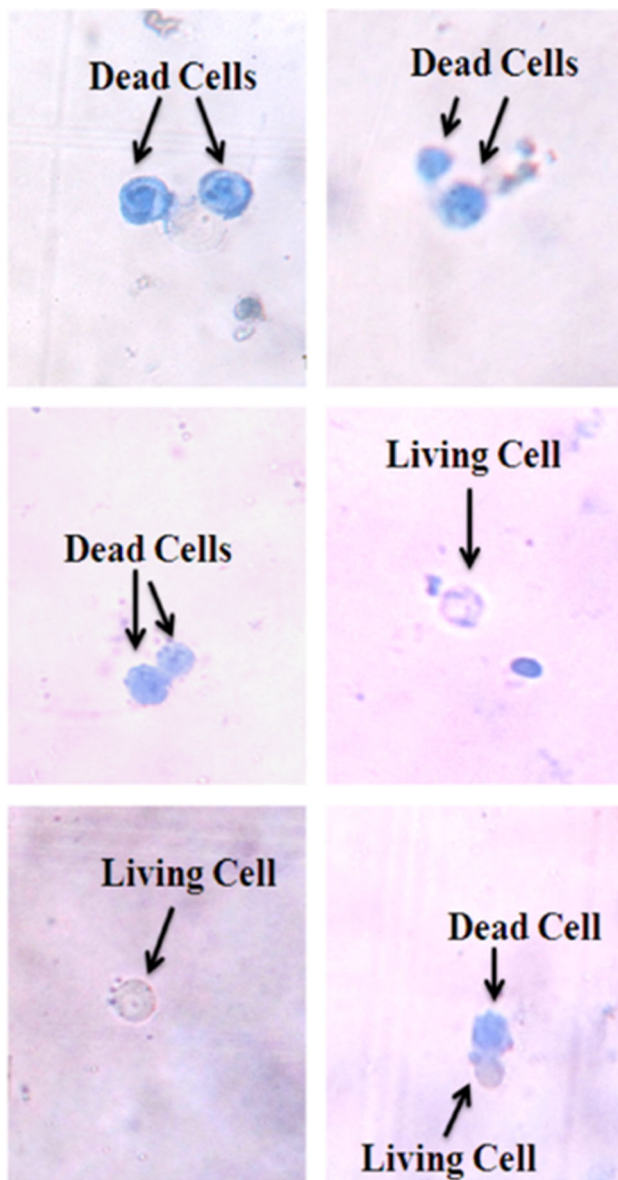
Fig. 4 Photograph showing results of Trypan blue assay. Hemocytes appearing *blue* in colour are confirmed to be dead while the ones that appear to respond negatively to the dye are living. Hemocytes are observed at $(10 \times 40)\times$ magnification (colour figure online)

different treatment categories of NaF increased significantly ($p < 0.05$) when compared to the control. Interestingly, the mean percentage of CC for different treatment groups such as 10, 20, 40, 80 and 100 μg/mL was 8.18 ± 0.017, 24.25 ± 0.020, 11.72 ± 0.024, 11.82 ± 0.014, and 9.71 ± 0.020, respectively whereas the control group showed crystal cells percentage as 3.85 ± 0.017 (Fig. 3). The number of crystal cells in different concentrations of NaF increased significantly ($p < 0.05$) when compared to the control. Since the number of LC is maintained at negligible low number, the focus

was confined to variation in the count of PC and CC in the present study.

Through trypan blue dye exclusion assay it was observed that in control larval hemolymph the number of dead cells was $12.6 \times 10^4 \pm 1.8$ /mL of hemolymph. Interestingly, the number of dead cells increased significantly with the increasing concentrations of NaF i.e., $15.942 \times 10^4 \pm 1.56$, $15.0 \times 10^4 \pm 1.50$, $18.0 \times 10^4 \pm 1.8$, $15.9 \times 10^4 \pm 0.3$, $25.8 \times 10^4 \pm 2.83$ cells/mL of hemolymph respectively (Fig. 5).

Discussion

In the context of the above LC_{50} determination, death caused by slow and delayed poisonous effect of NaF has only been considered. Hence the chronic LC_{50} is expected to be lower in comparison to the acute LC_{50}. Another fluoride-containing chemical cryolite has been shown to cause 50% of larval mortality in *Drosophila* between 150–158 μg/mL concentrations (Podder and Roy 2013). The toxicity caused by NaF depends on its easy and free dissociation into elemental fluoride ion (F^-). Further, F^- of NaF has been suggested to inhibit a variety of vital enzymes causing death (Bhatnagar et al. 2006). Hence, lower concentration might be more effective at prolonged exposure. Since larva of *D. melanogaster* is a voracious eater, hence, the selection of the route of exposure through food seemed to be quite justified. Thus the relevance of determination of LC_{50} is unquestionable.

Plasmatocyte cells (PC) are known to be involved in phagocytosis (Tan et al. 2014) and lamellocyte (LC), a variant form of PC (Rizki and Rizki 1980), is involved in encapsulation of large foreign particle which may be too large to be phagocytosed (Crossley 1964; Ware and Whitacre 2004; Tan et al. 2014). In the present study, a significant ($p < 0.05$) reduction in the number of PC in the hemolymph of *D. melanogaster* after exposure to sublethal concentrations of NaF has been observed. Interestingly, similar type of reduction in number of PC and LC is seen after treatment with the organophosphorous chemical acephate (Rajak et al. 2014), indicating compromise in immunity due to chemical insult. NaF is known to influence actin polymerization and thus is likely to interfere with the process of phagocytosis as has been reported in *Venerupis philippinarum* (Ballarin et al. 2014). Hence the present observation where significant ($p < 0.05$) reduction in the number of PC was seen can be correlated with probable compromise in phagocytic machinery.

Due to rise in the level of free radicals resulting from F^- exposure, lipid peroxidation is reported to increase in sheep (Guven and Kaya 2005) and *Drosophila melanogaster* (Dutta et al. 2017). Fluoride induces oxidative stress and

Fig. 5 Graphical representation of total number of hemocytes, living hemocytes and dead hemocytes. The line shows the variation in percentage of death after treatment with different concentrations of NaF. Data represents mean ± standard error (SE)
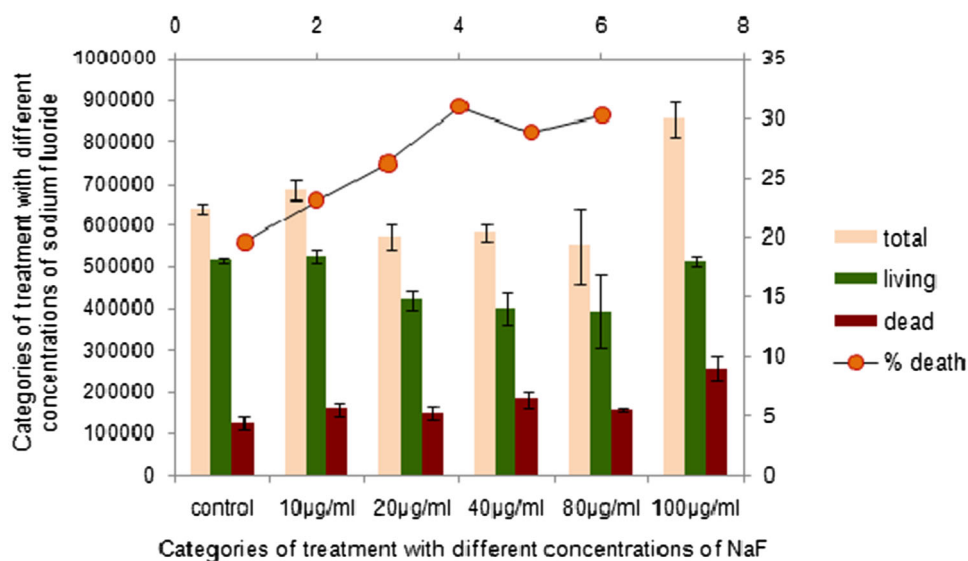
apoptosis resulting in elevation of lipid peroxidation (Karube et al. 2009; Anuradha et al. 2001). So from results of the present studies it seems that, there exists a possibility that, NaF might cause death of PC and thereby their number in circulating hemolymph would be low. NaF is also known to reduce mitosis rate which might result in reducing proliferation of hemocyte precursor cells during hematopoiesis thereby decreasing the cell count as suggested by Podder et al. (2008a, b) in mice. Since PCs are involved in phagocytosis and encapsulation of larger pathogen, their reduction might adversely affect the immune response of the insect. Another kind of hemocyte reported in *Drosophila* hemolymph are crystal cells (CC), which possess dark crystals of prophenoloxidase (proPO) inside their cytoplasm (Meister and Lagueux 2003).Various stress inducing factors like temperature, pathogen attack, exposure to toxic chemicals etc. might activate a cascade of serine protease activity that ultimately cleaves prophenoloxidase into active phenoloxidase to produce melanin. Melanin is reported to help insects to combat different stress conditions (Hamilton and Gomez 2002). In the present study, increased CC after chronic exposure to NaF seems to be associated with detoxification mechanism in the insect body as this might be helpful to protect the organism from excessive F induced damage.

As trypan blue is a vital dye, dead cells with compromised cell membrane are unable to exclude the dye; as a result, those cells appear blue in colour when observed under microscope (Fig. 4). Hence, this assay successfully confirms the decrease in number of PC and it may be

assumed that NaF being an inducer of apoptosis might cause the death of the immune cells (Fig. 5).

Conclusion

The study, for the first time reported about larval and pupal chronic LC_{50} of NaF in the range of 125–130 μg/mL in *D. melanogaster*. The sub-lethal concentrations of NaF, were found to cause significant variations in differential hemocyte count of the third instar larvae of *Drosophila*. This variation, in turn, could compromise the immune system function of the insect. Similarly, chronic exposure to NaF through pesticides, tooth paste and water might also adversely affect the immune system of genetically similar higher order organisms including human beings and result in reduced immune response against pathogenic infections and diseases.

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