3 EFFECTS OF LOW DOSES OF NONYLPHENOL ON LIVER OF ADULT MALE RATS – A SHORT-TERM STUDY

3.1 Introduction

Nonylphenol (NP) is the final degradation product of alkylphenol polyethoxylates, which is widely used in the preparation of lubricating oil additives, resins, plasticizers, surface-active agents, detergents, paints, cosmetics and other formulated products. NP is also found in polyvinyl chloride (PVC) used in the food processing and packaging industry. Concern about NP has been increased as it can mimic the natural hormone estradiol and target various organs including liver where estrogen receptors are expressed. Humans are constantly exposed to NP by contaminated water and food products. Lipophilic nature of NP leads to its accumulation in animal tissues (Guenther et al., 2002). NP has been associated with various abnormal functions of vital system such as endocrine, reproductive and immune systems in wild life and humans (Colborn et al., 1993). Previous studies have reported that endocrine disruptors like bisphenol A and methoxychlor exhibit adverse effects at “very low doses” (below NOAEL) (vom Saal and Hughes, 2005; Welshons et al., 1999). However a few studies have reported low dose effects of NP (doses below NOAEL (15 mg/ kg body weight, WHO 2004)). Administration of NP at dose level of 50 µg/ kg body weight/ day for 30 day has been shown to induce oxidative stress in blood of adult male rats (Yasemin and Recap, 2010). Previous studies from our laboratory have reported that administration of low doses of NP induces oxidative stress and decreased sperm count in rat (Chitra and Mathur, 2004). Doses selected in the present study could resemble the natural exposure to human from the environment. According to WHO report on NP, the maximum intake of NP by human through drinking water and food is 2 mg/ kg/ day (paints), 0.35 µg/ kg/ day (use of pesticide product), 0.1 µg/ kg/ day (use of cosmetics) and 0.2 µg/ kg/ day (food packing materials) (WHO, Integrated risk assessment: NP case study., 2004).
Recently a few studies have shown that NP induces insulin secretion in pancreatic β-cells via intracellular estrogen receptors, which suggests a possible role of NP in inducing a state of hyperinsulinemia and insulin resistance (Adachi et al., 2005; Alonso-Magdalena et al., 2006). On the other hand, oxidative stress has been reported to induce insulin resistance and affect glucose homeostasis in the body (Archuleta et al., 2009; Ohta et al., 2011). Therefore, it would be interesting to investigate oxidative damage in pancreas and liver. Since liver is the major organ involved in regulating glucose homeostasis in the body, the effect of NP on glucose metabolism enzymes in liver was investigated. In the present study we sought to investigate the effects of low doses of NP on glucose homeostasis in the body and to see if oxidative stress has any role to play in mediating the effects. The objectives of the present study were (i) to evaluate the low-dose effects of NP on plasma glucose, insulin and estradiol (ii) to evaluate the effects of NP on antioxidant status of pancreas and liver (iii) to evaluate the effects of NP on glucose metabolism enzymes in the liver of adult rats.

### 3.2 Materials and Methods

#### 3.2.1 Animals, body weight and growth rate

Adult male albino rats of Wistar strain (120-140g) were housed in polypropylene cages and maintained at 22-25 °C under a well-regulated light and dark (12 hour:12 hour) schedule in the Animal House Facility of the Pondicherry University. The animals were fed on standard rat chow (Sai Durga Feeds and Foods, Bangalore, India) and water ad libitum. The weights of the rats were recorded on alternate days and the animals showing poor growth rate were eliminated from the study. For seven days prior to the start of experiments, rats were handled daily for 5 min to acclimatize them to human contact and minimize their physiological response to handling.

#### 3.2.2 Chemicals

NP (99% purity), glucose-6-phosphate dehydrogenase, fructose-6-phosphate, aldolase, glycerophosphate dehydrogenase and triose-P-isomerase were obtained from
Sigma Chemical Company (St. Louis, MO, USA). NADPH and glutathione (oxidized) were obtained from SISCO Research laboratories, Mumbai, India. Bovine serum albumin, horseradish peroxidase, thiobarbituric acid and pyrogallol were obtained from Himedia Laboratories, Mumbai, India. Malondialdehyde was obtained from Merck-Schuchardt, Germany. All other chemicals used for various assays were of analytical grade and were obtained from local commercial sources.

3.2.3 Treatments, necropsy and collection of tissues

Experiments were carried out in adult male rats (80-90 days). The animals were divided into four groups and six animals were maintained in each group. NP was dissolved in olive oil (see Appendix 1.1) and was administered by oral gavage daily for 7 days. Corresponding group of control rats were administered with equal volumes of vehicle (olive oil) alone. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003).

- Group 1: Administration of NP at a dose of 15 µg/ kg body weight/ day
- Group 2: Administration of NP at a dose of 150 µg/ kg body weight/ day
- Group 3: Administration of NP at a dose of 1500 µg/ kg body weight/ day

The weights of the animals were monitored regularly until the end of the treatment. After twenty four hours of the last dosing and overnight fasting, the rats were weighed and sacrificed by cervical dislocation. Blood samples were collected from control and treated groups by cardiac puncture method, in clean centrifuge tubes containing anticoagulant, heparin (see Appendix 1.2). Blood was centrifuged at 3000 rpm for 30 min and the plasma was collected in clean centrifuge tubes. The levels of glucose, insulin and estradiol were assayed as given in the respective sections.

Liver and pancreas were collected, cleared of the adhering tissues, weighed and stored at -80 °C until analysis.
3.2.4 Preparation of tissue homogenates of pancreas and liver

A 10% homogenate of pancreas and liver were prepared in normal saline using glass-teflon homogenizer (Remi RQ-127A, Remi Motors, Mumbai, India). The homogenate was centrifuged at 800x g for 30 min at 4°C and the supernatants were used for biochemical assays. All biochemical estimations were carried out in duplicate.

3.2.5 Determination of protein

Protein contents were determined according to the method of Lowry (Lowry et al., 1951). An aliquot of 0.1 ml of the test sample was mixed with 5.0 ml of alkaline copper reagent and vortexed. They were allowed to stand for 10 min at room temperature. Folin-Ciocalteau reagent 1 N (0.5 ml) was added to each of the tubes, vortexed and allowed to stand for 20 min at room temperature. The optical density was read at 610 nm in a Systronics Spectrophotometer. A calibration curve was prepared using different concentrations of bovine serum albumin (see Appendix 1.3).

3.2.6 Determination of antioxidant status in pancreas and liver

Activities of antioxidant enzymes such as SOD, catalase, glutathione peroxidase, glutathione reductase and the levels of $\text{H}_2\text{O}_2$ generation and lipid peroxidation were assayed by the methods as described below.

3.2.6.1 Assay of superoxide dismutase

Superoxide dismutase (EC.1.15.1.1) activity was assayed as described by Marklund and Marklund (1974). The assay mixture contained 2.4 ml of 50 mM Tris HCl buffer containing 1 mM EDTA (pH 7.6), 300 µl of 0.2 mM pyrogallol and 100 µl enzyme source (see Appendix 1.4). Increase in absorbance was measured at 10 sec intervals for 3 min at 420 nm against blank containing all the components except enzyme and pyrogallol in a Systronics Spectrophotometer. Specific activity of the enzyme was expressed as nmol pyrogallol oxidized per min per mg protein.
3.2.6.2 Assay of catalase

Catalase (EC.1.11.1.6) activity was assayed as described by Claiborne (1985). The assay mixture contained 2.4 ml of phosphate buffer (0.05 M, pH 7.0), 10 µl of 19 mM hydrogen peroxide and 50 µl enzyme source (see Appendix 1.5). Decrease in absorbance was measured at 10 sec interval for 3 min at 240 nm against blank containing all components expect enzyme in a Systronics Spectrophotometer. Specific activity of the enzyme was expressed as µmol of H$_2$O$_2$ consumed per min per mg protein.

3.2.6.3 Assay of glutathione peroxidase

Glutathione peroxidase (EC.1.11.1.9) activity was assayed as described by Mohandas et al. (1984). The assay mixture contained 1.59 ml of phosphate buffer (100 mM, pH 7.6), 100 µl of 10 mM EDTA, 100 µl of sodium azide, 50 µl of glutathione reductase, 100 µl of reduced glutathione, 100 µl of 200 mM NADPH, 10 µl of hydrogen peroxide and 10 µl enzyme source (see Appendix 1.6). Disappearance of NADPH was measured at 10 sec interval for 3 min at 340 nm against blank containing all components expect enzyme in a Systronics Spectrophotometer. Specific activity of the enzyme was expressed as nmol of NADPH oxidized per min per mg protein.

3.2.6.4 Assay of glutathione reductase

Glutathione reductase (EC.1.6.4.2) activity was assayed as described by Carlberg and Mannervik et al. (1975). The assay mixture contained 1.75 ml of phosphate buffer (100 mM, pH 7.6), 100 µl of 200 mM NADPH, 100 µl of 10 mM EDTA, 50 µl of 20 mM glutathione oxidized and 50 µl enzyme source (see Appendix 1.7). Disappearance of NADPH was measured at 10 sec interval for 3 min at 340 nm against blank containing all components expect enzyme in a Systronics Spectrophotometer. Specific activity of the enzyme was expressed as nmol of NADPH oxidized per min per mg protein.
3.2.6.5 **Hydrogen peroxide generation assay**

Hydrogen peroxide generation was assayed as described by Pick and Keisari (1981). The incubation mixture containing 1.64 ml phosphate buffer (50 mM, pH 7.6), 54 µl of horseradish peroxidase (8.5 units/ml), 30 µl of 0.28 nM phenol red, 165 µl of dextrose (5.5 nM) and 600 µl of enzyme source were incubated at 32 °C for 30 min. The reaction was terminated by addition of 60 µl of 10 N sodium hydroxide (see Appendix 1.8). The absorbance was read at 610 nm against blank on a Systronics Spectrophotometer. The quantity of H$_2$O$_2$ produced was expressed as nmol H$_2$O$_2$ generated per min per mg protein. For the preparation of standard curve, known amount of H$_2$O$_2$ and all the above ingredients except enzyme source were incubated at 32 °C for 30 min. Subsequently, 60 µl of 10 N sodium hydroxide was added and optical density was read at 610 nm.

3.2.6.6 **Estimation of lipid peroxidation**

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS) was measured as described by Ohkawa et al. (1979). Briefly, the stock solution contained equal volumes of trichloracetic acid 15% (w/v) in 0.25 N hydrochloric acid and 2-thiobarbituric acid 0.37% (w/v) in 0.25 N hydrochloric acid (see Appendix 1.9). One volume of tissue homogenate and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min in a boiling water bath. After cooling on ice, the flocculent precipitate was removed by centrifugation at 1000x $g$ for 10 min and absorbance was measured at 535 nm against blank. The values were expressed as µmol malondialdehyde produced per min per mg protein. A standard curve was prepared with known amount of malondialdehyde and all the above reagents expect the sample. The optical density was read at 535 nm.

3.2.7 **Determination of plasma amylase and lipase**

Plasma amylase and lipase were assayed using kits from BioVision incorporated, Milpitas, USA. One unit of amylase is defined as amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 µmol of nitrophenol per minute at pH 7.2 at
25 °C. One unit of lipase is defined as the amount of lipase that hydrolyzes triglyceride to yield 1.0 µmol of glycerol per min at 37 °C.

3.2.8 Determination of plasma AST and ALT

Activities of plasma aspartate transaminase (AST) and alanine transaminase (ALT) were assayed by the method of Reitman and Frankel, (1957). In brief, to 0.2 ml of plasma, 1 ml of substrate (aspartate and α-ketoglutarate for AST and alanine and α-ketoglutarate for ALT, in phosphate buffer pH 7.4) were added and incubated for an hour for AST and 30 min for ALT. One ml of DNPH (2, 4-dinitrophenyl-hydrazine) solution was added to stop the reaction and kept at room temperature for 20 min. One ml of 0.4 N NaOH was added (see Appendix 1.10) and absorbance was read at 540 nm. Enzyme activities were expressed as U/mL.

3.2.9 Quantitative determination of plasma glucose, insulin and estradiol levels

Plasma glucose levels were estimated by using a commercially available kit (Autospan, Span Diagnostics, Surat, India) based on glucose oxidase/ peroxidase method as described by Trinder (1969). Plasma insulin concentration was measured using a rat ELISA kit (Mercadia AB, Uppsala, Sweden). Plasma levels of estradiol was determined by ELISA using kits from Diagnostic Systems Laboratories Inc. Webster, Texas, USA.

3.2.10 Determination of glucose metabolism enzymes in liver

Activities of glycolytic enzymes such as hexokinase and phosphofructokinase and the activity of glycogen phosphorylase in liver were assayed by the methods as described below.

3.2.10.1 Assay of hexokinase

The activity of Hexokinase (2.7.1.1) was assayed as described by Bergmeyer and Walter (1983). The reaction mixture contained 1 ml of 0.05 M triethanolamine buffer (pH 7.6), 1 ml of 0.555 M D-glucose, 200 µl of 0.1 M magnesium chloride, 200 µl of 0.014 M NADP, 20 µl of 125 U/ ml glucose-6-phosphate dehydrogenase and
200 µl of tissue homogenate (see Appendix 1.11). The reaction was initiated by addition of 100 µl of 0.019 M ATP. Specific activity of the enzyme was expressed as nmol of NADP reduced per min per mg protein.

### 3.2.10.2 Assay of phosphofructokinase

The activity of phosphofructokinase (2.7.1.11) was assayed after modification of the method of Narabayashi et al. (1985). The assay mixture contained 2 ml of buffer containing 50 mM Tris/HCl (pH 8.0), 1 mM Fructose-6-Phosphate, 1 mM ATP, 2 mM MgCl₂, 0.16 mM NADH, 2.6 mM dithiothreitol, 1 mM EDTA, 5 mM ammonium sulphate, 0.4 units aldolase, 2.5 units triose-P isomerase and 0.4 units glycerophosphate dehydrogenase (see Appendix 1.12). The reaction was initiated by the addition of 100 µl of tissue homogenate and the decrease in absorbance was measured at 340 nm. Specific activity of enzyme was expressed as nmol of NADH oxidized per min per mg protein.

### 3.2.10.3 Assay of glycogen phosphorylase

The activity of glycogen phosphorylase (2.4.1.1) was assayed as described by Niemeyer et al. (1961). The reaction mixture contained 0.2 ml of 0.1 M citrate buffer (pH 6.0), 0.2 ml of 0.02 M glucose-1-phosphate, 0.2 ml of 0.0025 M AMP, 0.1 ml of 0.075 M sodium fluoride, 0.2 ml of 1% glycogen and 0.1 ml of tissue homogenate. The reaction mixture was incubated for 60 min at 37 °C and the reaction was stopped by the addition of 1.0 ml of chilled 10% trichloroacetic acid. The inorganic phosphorus liberated in the above reaction mixture was estimated by the method of Fiske and Subbarow (1925). To 1 ml of the reaction mixture above, 0.2 ml of ammonium molybdate, 0.4 ml of ANSA reagent and 1 ml of distilled water were added and the mixture was incubated at room temperature for 5 min (see Appendix 1.13). The absorbance was read at 595 nm. Specific activity of the enzyme was expressed as µmol of Pi liberated per min per mg protein.
3.2.11 Statistical analyses

The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s post-test using SPSS software (student version 7.5, SPSS Inc., UK). Differences were considered significant at $P<0.05$ against control group.

3.3 Results

3.3.1 Effect of various doses of NP on body weight and organ weights

Administration of NP at low doses did not cause any change in the body weight of the rats as compared to the corresponding group of controls. The weights of liver and pancreas also did not show any alteration following administration with various low doses of NP (Table 1).

3.3.2 Effect of various doses of NP on pancreas and liver antioxidant system

The activities of SOD, catalase, glutathione peroxidase and glutathione reductase were decreased significantly in pancreas (Figs. 1-4) and liver (Figs. 5-8) at all tested doses of NP when compared to the controls. A dose-dependent increase in the levels of hydrogen peroxide generation and lipid peroxidation were observed in pancreas (Figs. 9 and 10) and liver (Figs. 11 and 12) of NP-treated groups when compared to the corresponding group of control animals.

3.3.3 Effect of various doses of NP on plasma amylase and lipase

No change in the activities of amylase and lipase were observed in the plasma of rats treated with 15, 150 and 1500 µg/ kg/ day of NP for 7 days when compared to the control group (Figs. 13 and 14).

3.3.4 Effect of various doses of NP on plasma AST and ALT

No change in the activities of AST and ALT were observed in the plasma of rats treated with 15, 150 and 1500 µg/ kg/ day of NP for 7 days when compared to the control group (Figs. 15 and 16).
3.3.5  **Effect of NP on plasma glucose, insulin and estradiol levels of rat**

Administration of NP at doses of 15, 150 and 1500 µg/kg/day to adult male rats decreased the fasting plasma glucose when compared to controls (Fig. 17). The levels of plasma insulin and estradiol showed a significant dose-dependent increase following exposure to NP (Figs. 18 and 19).

3.3.6  **Effect of NP on the glucose metabolism enzymes in the liver of rats**

Administration of NP at doses of 15, 150 and 1500 µg/kg/day for 7 days significantly increased the activities of hexokinase and phosphofructokinase in the liver of adult rats (Figs. 20 and 21). However, the activities of glycogen phosphorylase in liver were decreased in NP-treated rats when compared to the controls (Fig. 22).
Table 1  Effect of various doses of nonylphenol on the body weight and weights of liver and pancreas of rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>15</th>
<th>150</th>
<th>1500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>172.25 ± 2.70</td>
<td>168.00 ± 3.44</td>
<td>174.25 ± 3.21</td>
<td>169.76 ± 3.59</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.84 ± 0.08</td>
<td>0.86 ± 0.06</td>
<td>0.78 ± 0.08</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td>Liver</td>
<td>6.24 ± 0.14</td>
<td>5.98 ± 0.25</td>
<td>6.14 ± 0.18</td>
<td>5.92 ± 0.28</td>
</tr>
</tbody>
</table>

The weights are expressed in gram.
Data are expressed as mean ± SD for six animals/group.
*p<0.05 against the control group
Fig. 1  Effect of nonylphenol on the activity of superoxide dismutase in the pancreas of adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on superoxide dismutase activity.]

Fig. 2  Effect of nonylphenol on the activity of catalase in the pancreas of adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on catalase activity.]

3 Effects of low doses of nonylphenol on liver-A short-term study
Fig. 3 Effect of nonylphenol on the activity of glutathione peroxidase in the pancreas of adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on glutathione peroxidase activity](image)

Fig. 4 Effect of nonylphenol on the activity of glutathione reductase in the pancreas of adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on glutathione reductase activity](image)
**Fig. 5** Effect of nonylphenol on the activity of superoxide dismutase in the liver of adult male rats (Mean ± SD; *p<0.05)

**Fig. 6** Effect of nonylphenol on the activity of catalase in the liver of adult male rats (Mean ± SD; *p<0.05)
Fig. 7  Effect of nonylphenol on the activity of glutathione peroxidase in the liver of adult male rats (Mean ± SD; *p<0.05)

![Graph of glutathione peroxidase activity](image)

Fig. 8  Effect of nonylphenol on the activity of glutathione reductase in the liver of adult male rats (Mean ± SD; *p<0.05)

![Graph of glutathione reductase activity](image)
Fig. 9  Effect of nonylphenol on hydrogen peroxide generation in the pancreas of adult male rats (Mean ± SD; *p<0.05)

Fig. 10  Effect of nonylphenol on the levels of lipid peroxidation in the pancreas of adult male rats (Mean ± SD; *p<0.05)
Fig. 11  Effect of nonylphenol on hydrogen peroxide generation in the liver of adult male rats (Mean ± SD; *p<0.05)

Fig. 12  Effect of nonylphenol on the levels of lipid peroxidation in the liver of adult male rats (Mean ± SD; *p<0.05)
Fig. 13  Effect of nonylphenol on plasma amylase of adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on plasma amylase activity in adult male rats.](image)

Fig. 14  Effect of nonylphenol on plasma lipase of adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on plasma lipase activity in adult male rats.](image)
Fig. 15  Effect of nonylphenol on plasma AST of adult male rats (Mean ± SD; *p<0.05)

Fig. 16  Effect of nonylphenol on plasma ALT of adult male rats (Mean ± SD; *p<0.05)
Fig. 17  Effect of nonylphenol on plasma glucose levels in adult male rats (Mean ± SD; *p<0.05)

Fig. 18  Effect of nonylphenol on plasma insulin levels in adult male rats (Mean ± SD; *p<0.05)
**Fig. 19** Effect of nonylphenol on plasma estradiol levels in adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on plasma estradiol levels.](image)

**Fig. 20** Effect of nonylphenol on the activity of hexokinase in the liver of adult male rats (Mean ± SD; *p<0.05)

![Graph showing the effect of nonylphenol on hexokinase activity.](image)
Fig. 21  Effect of nonylphenol on the activity of phosphofructokinase in the liver of adult male rats (Mean ± SD; *p<0.05)

Fig. 22  Effect of nonylphenol on the activity of glycogen phosphorylase in the liver of adult male rats (Mean ± SD; *p<0.05)
3.4 Discussion

3.4.1 Experimental design

In the present study the effects of various low doses of NP on blood glucose homeostasis in rats were evaluated. The animals were exposed to 15, 15 and 1500 µg/kg bodyweight of NP/day for 7 days and its effects on the activities of antioxidant enzymes in pancreas and liver, and glucose metabolism enzymes in liver of adult male rats were studied. The major route of exposure of NP to humans is through diet at low doses. Therefore, in the present study NP was administered to rats by oral gavage. All the doses selected in the present study are below the reported NOAEL dose for NP in rats (WHO, 2004).

3.4.2 Significance of the parameters studied

The body weights of the rats were monitored throughout the experiment in order to evaluate the effect of xenoestrogens on general health status of the animals during the period of study. Such information is necessary for the interpretation of effects. Reduction in weight gain may reflect a variety of responses, including rejection of feed and water, treatment induced anorexia or systemic toxicity. In the present study, the weights of the animals were recorded to monitor such changes. After necropsy, organ weights of liver and pancreas were recorded.

Antioxidants are located throughout the cells and provide protection against ROS. SOD, Catalase, glutathione peroxidase and glutathione reductase are powerful antioxidant enzymes in maintaining the fine balance between the pro-oxidants/antioxidants in pancreas and liver. SOD is considered first line of defense against oxyradicals in cells by catalyzing dismutation of $O_2^-$ anion to hydrogen peroxides and molecular oxygen. Catalase, glutathione peroxidase and glutathione reductase protect the cells from highly toxic hydrogen peroxides by converting it into water and oxygen. In the present studies the activities of SOD, catalase, glutathione peroxidase and glutathione reductase were determined. The levels of hydrogen peroxide, a product of the dismutation of superoxide and numerous enzymatic reactions were assessed in the
present study. Increased production of ROS can cause the destruction of the cellular structures including the lipids. Hence, in the present study lipid peroxidation is used as index of oxidative stress. Malondialdehyde, a lipid peroxidation product generated in the tissue by free radical injury is measured by thiobarbituric acid reactive substance and has been considered as sensitive index of free radical generation. In order to evaluate hepatic damage, the activities of specific marker enzymes in plasma, ALT and AST were determined and to evaluate pancreatic damage, the activities of specific marker enzymes in plasma, amylase and lipase were assayed.

Glucose plays a very important role in the maintenance of normal functioning of the body. The homeostatic mechanism of the body which keeps the blood glucose levels within the normal range involves several interacting systems, of which, hormonal regulation by insulin is of prime importance. Estradiol, a sex hormone, has also been shown to play a crucial role in the maintenance of normal insulin sensitivity and pancreatic β-cell function (for review see Godsland, 2005). Therefore, in the present study, the plasma levels of glucose, insulin and estradiol were measured to evaluate the endocrine-mediated effects of NP in adult male rats.

Maintaining the blood glucose homeostasis is of paramount importance and the organ which immediately responds to the signals of elevated or decreased levels of insulin or glucose in the body is the liver. When the blood glucose concentrations are altered, the biochemical pathways of the liver such as glycolysis and glycogenolysis intakes or releases glucose so that the blood glucose levels are maintained within a standard range. In the present study, the activities of glycolytic enzymes, hexokinase and phosphofructokinase and the activities of glycogen phosphorylase were measured to assess the effects of NP on liver glycolysis and glycogenolysis.

### 3.4.3 Effect of various doses of NP on body weight and organ weights

The body weights of NP-treated rats did not show any significant change when compared to the control group of animals, which indicates the absence of general systemic toxicity in rats following NP exposure. The weights of pancreas and liver were
also not altered following low doses of NP treatment, which signifies that short-term administration of NP by oral gavage did not impair the functions of pancreas and liver in adult male rats.

### 3.4.4 Effect of various low doses of NP on the antioxidant status of rat pancreas and liver

NP has been shown to inhibit the activities of cytochrome P450-1A (CYP1A) in rat hepatic microsomal fractions. Inhibition of cytochrome P450 has been shown to provoke ROS generation that impairs both pancreas and liver functions (Lee et al., 1997). ROS are considered to be one of the important mechanisms of toxic insult. H$_2$O$_2$ mediate toxic effects through formation of hydroxyl radicals, a potent activator of lipid peroxidation, the index of toxicant induced cellular damage. A major cellular defense against ROS is provided by SOD and catalase which together convert superoxide radicals first to H$_2$O$_2$ and then to water and molecular oxygen (Kaplowitz et al., 1985). A fine balance between ROS production and scavenging is essential for the maintenance of normal pancreas and liver functions. In the present study, we observed a significant decline in the activities of SOD, catalase, glutathione peroxidase and glutathione reductase in the pancreas and liver of NP-treated rats when compared to the corresponding group of control animals. Moreover, the levels of hydrogen peroxide generation and lipid peroxidation increased in pancreas and liver following short-term administration with low doses of NP. Reduction in the activities of antioxidant enzymes may reflect the inability of pancreas and liver to eliminate H$_2$O$_2$ production, which could be due to inactivation of antioxidant enzymes through excess ROS production. These changes indicate that even at low doses NP impairs the pro-oxidant/antioxidant balance in the pancreas and liver.

### 3.4.5 Effect of various doses of NP on plasma amylase and lipase

NP administration at 15, 150 and 1500 µg/kg/day for 7 days did not cause any change in the activities of plasma amylase or lipase. Though NP induced oxidative stress in the pancreas following short-term administration, no sign of damage were
noted in pancreas of NP-treated rats when compared to the control group of animals. This indicates that short-term administration of low doses of NP does not cause damage to pancreas in adult male rats.

3.4.6 Effect of various doses of NP on plasma AST and ALT

NP administration at 15, 150 and 1500 µg/kg/day for 7 days did not cause any change in the activities of plasma AST or ALT. Though NP induced oxidative stress in the liver following short-term administration, no sign of damage was noted in liver of NP-treated rats when compared to the control group of animals. This indicates that short-term administration of low doses of NP does not cause damage to liver in adult male rats.

3.4.7 Effect of various low doses of NP on plasma glucose, insulin and estradiol levels of adult male rats

In the present study, the plasma levels of glucose decreased following NP administration for 7 days. However, the plasma insulin levels increased in NP treated rats when compared to the corresponding group of control animals. The secretion of insulin from pancreatic beta cells is a complex process relating the integration and interaction of multiple external and internal stimuli. Thus, hormones, neurotransmitters, nutrients and drugs all activate or inhibit insulin secretions. The primary stimulus for insulin release is beta cell response to changes in glucose level. Insulin then acts on various cells throughout the body and stimulate the uptake, utilization and storage of glucose. However, several external factors are known to negatively influence pancreatic insulin secretion. A few studies have shown that environmental contaminants have the ability to stimulate insulin secretion from pancreatic islets and affect the blood glucose homeostasis of the body. Exposure to environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin, arsenic and malathion have been reported to induce alteration in the levels of insulin, glucose and the enzymes involved in the glycolytic pathway (Cranmer et al., 2000; Fu et al., 2010; Rezg et al., 2006). Pancreatic islet cells exposed to bisphenol A and NP have been reported to cause a significant increase in
insulin secretion within 24 hours of exposure (Adachi et al., 2005). It has been reported that when an adult mice exposed to a single dose (10 µg/ kg) of either Bisphenol A or 17β-estradiol caused a hypoglycemia with an increase in the plasma insulin levels, indicating that the estrogenic potency of Bisphenol A altered blood glucose homeostasis. Although physiological levels of estradiol is required for maintaining normal insulin sensitivity and β-cell functions, increased levels of estradiol has been shown to affect on glucose tolerance (for review see Godsland 2005). Therefore, in the present study it is possible that the estrogenic potency of NP stimulated insulin secretion from pancreatic islets and thereby decreased the blood glucose levels.

Estrogens maintain various functions in the body and their actions are mediated through binding with specific nuclear estrogen receptors, which regulate the expression of target genes after hormone binding. Various experimental data from in vivo and in vitro studies suggest that insulin and estradiol interact on carbohydrate metabolism. Artificially raised levels of of estrogens in women taking oral contraceptive pill were found to affect glucose tolerance and insulin sensitivity. In the present study an increase in the plasma estradiol levels were observed following administration with low doses of NP. An increase in the serum estradiol levels was noted in rats exposed to various chemicals and it was reported that impaired hypothalamic-pituitary-gonadal axis altered the hormone levels (Fernandez et al., 2009). In the present study, it is possible that NP had a direct impact on hypothalamo-pituitary-gonadal axis and altered the levels of estradiol in the body

### 3.4.8 Effect of various low doses of NP on glucose metabolism enzymes in the liver of adult male rats

Insulin action is mediated by the insulin receptor, a complex muti-subunit cell surface glycoprotein. Trans-phosphorylation of tyrosine residues in receptors itself and other proteins is required for insulin action. As an event of phosphorlation, numbers of other proteins are activated and bring out the various physiological and metabolic processes in cells (Bourbon and Gilbert, 1981; Wu et al., 2005). Insulin suppresses endogenous glucose production, stimulates glucose utilization
by insulin-sensitive tissues and lowers the blood glucose concentration. Insulin can suppress glucose production in liver cells by stimulating glycolysis via increasing the activities of rate limiting enzymes hexokinase and phosphofructokinase, suppressing glycogenolysis by inactivating glycogen phosphorylase and inhibits gluconeogenesis by suppressing the activity of glucose 6 phosphatase (Bourbon and Gilbert, 1981; Nordlie et al., 1999; Wu et al., 2005). Acute increase in insulin levels result in phosphorlation or de-phosphorlation of the enzymes involved in the glycolytic and gluconeogenic pathways, which have rapid but transient effects on their activity. High levels of insulin or prolonged stimulation of insulin results in alteration in rate of transcription of a variety of genes, which could leads to increased or decreased capacity for the processes mediated by those gene products. Increased activities of hexokinase and phosphofructokinase and decreased activity of glycogen phosphorylase in liver following NP treatment reflects increased glycolysis and decreased glycogenolysis in liver. It has been reported that arsenic, a heavy metal, when administered to mice via drinking for 21 days increased the expression of hexokinase II in renal glomeruli (Pysher et al., 2007). Malathion, an organochlorine pesticide has been shown to increase the activity of phosphofructokinase in skeletal muscle when administered at doses of 100, 200 and 400 ppm to rats for 4 weeks (Pournourmohammadi et al., 2005). A reduction in the activities of hexokinase, pyruvate kinase and lactate dehydrogenase were observed when cadmium at a dose of 0.3 mg/ kg body weight was administered to rats for three months (Kielan et al., 1989). These studies provide evidence that environmental toxicants have the ability to alter glycolytic pathway in liver. In the present study, it is possible that the effect of NP on these enzymes could be mediated by hyperinsulinimia as it stimulates glycolysis by increasing the activities of hexokinase and phosphofructokinase, and suppress glycogenolysis by inactivating glycogen phosphorylase in liver to reduce blood glucose.