Early Investigation of Hepatocarcinogenic Effect of Polyvinyl Chloride in Rat

Radwa A. Barakat, H.H. Bakery, M.E. Abuo Salem and Nabila M. Abdelaleem

Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Benha University, Benha, Egypt

Abstract: The present study aimed to early investigation of hepatocarcinogenic effect of poly vinyl chloride on male albino rats in order to improve cancer therapy through studying the toxic effect of PVC on some biochemical changes as ALT, AST, ALP and alfa-fetoprotein as tumor marker. Also study the changes in m-RNA expression of cytochrome P450 2E1 (CYP2E1) and tumor suppressor gene (TP53) as early cancer biomarker. Sixty male albino rats used in this study and classified to 3 groups: Group I used as control, while Group II (1/20 LD₅₀ of PVC) and Group III (1/10 LD₅₀ of PVC) were given PVC orally via stomach tube three times per week for 2 months. The results were more pronounced on increase of some biochemical parameter (ALT, AST, ALP and alfa fetoprotein) and m-RNA expression of tumor suppressor gene (TP 53) at the first and second month of the experiment. Also found an increase of m-RNA expression of cytochrome2E1 gene (CYP2E1) at first month and decrease at second month. This study provided strong evidence that application of gene expression profiling to preneoplastic liver diseases and hepatocellular carcinoma is particularly important in early investigation of carcinogenic risk by toxic agent like PVC.

Key words: Poly Vinyl Chloride • Hepatocellular Carcinoma • Gene Expression • Alfa Fetoprotein

INTRODUCTION

The manufacture, use and disposal of various plastics can pose numerous health risks, including the risk of cancer. A model example of carcinogenic risk from plastic is provided by poly vinyl chloride (PVC), since it is composed of vinyl chloride which known as human carcinogen [1]. Poly vinyl chloride is a poison plastic that has a destructive effect during all steps of production and uses, Vinyl chloride has been identified as a cause of hepatocellular carcinoma [2]. PVC is used for many consumer articles such as credit cards, furniture, floor coverings and toys. It also used for food packaging such as plastic trays in boxed cookies or chocolates, candy bar wrappers and bottles. PVC is also commonly used in teethers, in hospitals for IV bags, bath toys, raincoats and electrical applications [3]. Exposure to polyvinyl chloride can occur in many different settings, the most significance exposure occurs in the petrochemical and plastics industry. The PVC manufacturing workers were found to be at greater risk of liver cirrhosis as compared with control subjects [4]. General population exposures may occur from drinking water from PVC pipe, food beverage from PVC packaging and bottles [5]. The migration of VC and other additives from unplasticized polyvinyl chloride (uPVC) water pipes into potable drinking water has become an interesting area of research [6]. Egyptian Environmental Law recorded that the maximum permissible limit of vinyl chloride is 5ppm; while the American conference of Government Industrial Hygienists recorded that the threshold limit value (TLV) of vinyl chloride is 1ppm [7]. PVC is primarily metabolized in the liver by cytochrome P450 2E1 (CYP2E1) into chloroethylen oxide (CEO) and chloroacetaldehyde (CAA), both of which may be reactive with DNA to form DNA adducts that leading to hepatocarcinogenic effect [8]. There are many key events in the pathway of vinyl chloride-induced hepatocarcinogenesis, these include the metabolic activation to chloroethylene oxide, the DNA binding of the reactive metabolite (characteristic exocyclic etheno-adducts), the promutagenicity of these adducts that lead to G-A and A-T transitions and the effects of such mutations on proto-oncogenes and tumor-suppressor genes at the gene
and gene product levels, with tumorigenesis as the final outcome [9]. The confirmatory evidence between exposure to vinyl chloride and hepatocellular carcinoma has been reported in a recent meta-analysis combining the European and North American of VC workers [10]. Hepatocellular carcinoma (HCC) is a common cancer worldwide, has a dismal outcome partly due to the poor identification in early stage. Currently, one third of HCC patients present with low serum alfa-fetoprotein (AFP) levels, the only clinically available diagnostic marker for HCC [11]. So the most recent study works to identify new diagnostic molecular markers for HCC, especially for individuals with low serum AFP and those with normal serum AFP with smaller-sized tumors [12]. Hepatocellular carcinoma is a multi-step process associated with changes in gene expression. Currently, several technologies enable global gene expression profiling. The number of studies to preneoplastic chronic liver diseases has increased exponentially in recent years. These studies have quickly provided rich information and some additional clues to the genesis of liver cancer. The application of gene expression profiling to preneoplastic liver diseases and HCC is growing in importance and practicality [13]. Our study aimed to early investigation of hepatocarcinogenic effect of PVC on albino rats thought studying changes in the m-RNA expression of cytochrome P450 2E1 (CYP2E1) and tumor suppressor gene (TP53) as early cancer biomarker.

**MATERIALS AND METHODS**

**Experimental Animals:** Sixty apparently healthy male albino rats (western strain) 5-6 weeks old weighted (120-140 gm) obtained from Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt, were used in this study. The rats housed in stainless steel wire bottom cages and kept under constant environmental conditions and fed on fresh standard pellet and given tap water ad libitum throughout the study, with 12 hours light/dark cycle. All animals were acclimatized for 14 days before the beginning of the experiment.

**Tested Substance:** Poly vinyl chloride purchased from Sigma-Aldrich Company, Egypt. PVC is fine white powder, odorless, stable under normal condition, insoluble in water and soluble in oil and fat and most organic solvents. Its chemical formula C₂H₃Cl. Median lethal dose (LD₅₀) of PVC reported to be 5000 mg/kg for rats [14].

The PVC powder was dissolved in corn oil as a vehicle. Rats were divided into 3 groups each one contains 20 rats (Group I kept as positive control given corn oil only, group II given 1/20 LD₅₀ and group III given 1/10 LD₅₀). All rats dosed one dose/3 times /week (day after day), orally by using stomach tube for 2 months.

**Sampling:** At the end of first and second month, ten rats were sacrificed from each group and samples were collected. whole blood collected in clean dry centrifuge tubes, allowed to stand for one hour at room temperature till clotted and centrifuged at 3000rpm for fifteen minutes, for serum separation, then kept in (-20°C) for biochemical analysis. Tissue Specimens from liver were taken and immediately placed in clean sterile Eppendorf tube and preserved at (-80°C) for molecular study.

**Biochemical Analysis:** Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to Schumann and Klauke [15]. Alkaline phosphatase (ALP) was determined according to Belfield and Goldberg [16]. Alfa feto-protein as tumor marker was determined according to Chan and Miao [17].

**Molecular Study**

**RNA Extraction:** Pure RNA was extracted using total RNA purification kits following the manufacturer protocol. Liver samples are homogenized in lysis buffer. The lysate is then mixed with ethanol and loaded on a purification column. The chaotropic salt and ethanol cause RNA to bind to the silica membrane while the lysate is spun through the column. Subsequently, impurities are effectively removed from the membrane by washing the column with wash buffers. Pure RNA is then eluted by nuclease-free water.

**Reverse Transcription of RNA into cDNA:** 5µg of template RNA was added into sterile nuclease-free tube then 0.5 µg Oligodt primers was added and then the volume was completed to 12.5 µl using DEPC-treated water. The mixture was incubated at 70°C for 15 minutes. Put in another tube 4 µl of reaction buffer mixed with 0.5 µg of RNase inhibitor and then add 2 µl of dNTP and 1µl of reverse transcriptase enzyme. Then add all components on the nuclease-free tube of first step. After gentle mixing, the mixture was incubated for 110 min at 55°C. The reaction was terminated by heating at 70°C for 10 minutes for inactivation of reverse transcriptase enzyme.
Semi-Quantitative Polymerase Chain Reaction (PCR):
The isolated c-DNA was amplified using dream taq green PCR master mix kit following the manufacturer protocol. The polymerase chain reaction mixture was carried out in a 50 µl which contain the following mixture showing in (Table 1). Then gently mix the components, cycling conditions have to be optimized for each primer and then transferred to the thermal cycler.

Cycling Instructions: According to Morris and Davila [18] and Watanabe et al. [19].

- The primer used to amplify cytochrome P450 2E1 gene (CYP2E1) (473 bp) is:
  
  F: 5-CTCCTCGTATATCATCTCTG-3,
  R: 5-GCAGCCAATCAGAATGTGG-3

  The amplification conditions for CYP2E1 gene showed in (Table 2).

- The Primer Used to Amplify Tumor Suppressor Gene (TP53) (575 bp) is:
  
  F: 5-ATGGCTTCCATCCTGGCTTC-3,
  R: 5-TGACCCACACTGCAAGGG-3

  The amplification conditions for TP53 gene showed in (Table 3).

- The primer used to amplify cyclophilin gene (CYC) (265 bp) as internal control is:
  
  F: 5-CTTCGACATCACGGCTGATGG-3,
  R: 5-CAGGACCTGTATGCTTCAG-3

  The amplification conditions for CYC gene showed in (Table 4).

- The PCR products were separated by 1% agarose gel electrophoresis.
- Agarose Gel Electrophoresis: Agarose Gel electrophoresis was used to detect RNA fragments after RNA extraction and to determine the size of the PCR products.

Statistical Analysis: The data were statically analyzed by using the statistical software package SPSS for windows (Version 18). The significance of differences between more than two groups was evaluated by one way analysis of variance (ANOVA) test and DUNCAN test as described [20]. The values were statically analyzed for obtaining mean ±, standard deviation (SD) and statistical comparisons between means of different groups. P-value of less than 0.05 was considered significant.

RESULTS

Clinical Signs and Post Mortem Changes: The rats treated by PVC suffered from lethargy, anorexia, depression, rough hair coat, humped back posture, rapid weight loss and emaciation as shown in (Figure 1). That more observed on group that administered high dose of PVC (1/10 LD₅₀) in compared with control. No clinical signs were noticed in normal control group throughout the experimental period. Treated groups by PVC showed congestion on and enlargement of liver as shown in (Figure 2).
Fig. 1: Humped back posture, rough hair coat and emaciation in rat treated with high dose of PVC

Fig. 2: Showing enlarged liver in group administered PVC after 2 months

Fig. 3: The electrophoretic phototograph of mRNA expression of CYP2 E1 gene (473 bp) in liver of rats in different groups at 4th week.

-L: 100bp-1000bp DNA-ladder, Lane 1: Group I (Control), Lane 2: Group II (1/10 LD sub 50 of PVC), Lane 3: Group III (1/10 LD sub 50 of PVC). Molecular weight was 473 bp refers to specific amplified product for CYP2E1 gene.

Table 5: Showing the effect of PVC on alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) on serum of rats in different groups (mean ± SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Weeks</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>4th</td>
<td>17.8±0.66a</td>
<td>25.2±0.70ab</td>
<td>32.1±0.70c</td>
</tr>
<tr>
<td></td>
<td>8th</td>
<td>20.3±0.83a</td>
<td>30.0±1.01b</td>
<td>43.2±1.50c</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>4th</td>
<td>28.1±1.01a</td>
<td>40.1±1.41b</td>
<td>60.2±0.70c</td>
</tr>
<tr>
<td></td>
<td>8th</td>
<td>30.3±0.70a</td>
<td>59.2±0.80b</td>
<td>78.3±0.31c</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>4th</td>
<td>42.1±0.80a</td>
<td>59.0±0.71b</td>
<td>77.8±0.37c</td>
</tr>
<tr>
<td></td>
<td>8th</td>
<td>46.2±0.60a</td>
<td>71.1±0.50b</td>
<td>89.0±0.56c</td>
</tr>
<tr>
<td>Alfa feto protein (ng/ml)</td>
<td>4th</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>8th</td>
<td>4.6 ± 2.5c</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Mean with different letters at the same raw differ significant (P < 0.05).

Fig. 4: The electrophoretic phototograph of mRNA expression of CYP2 E1 gene (473 bp) in liver of rats in different groups at 8th week.

-L: 100bp-1000bp DNA-ladder, Lane 1: Group I (Control), Lane 2: Group II (1/10 LD sub 50 of PVC), Lane 3: Group III (1/10 LD sub 50 of PVC). Molecular weight was 473 bp refers to specific amplified product for CYP2E1 gene.

Effect of PVC on ALT, AST and ALP Levels: There was significant increase on ALT, AST and ALP levels at 4th weeks and 8th weeks of experiment that more clear on group received high dose (1/10 LD sub 50) of PVC in comparison with their corresponding control group as shown in (Table 5).

Detection of Alfa-Feto Protein Levels: Alfa-feto protein was not detected throughout the experiment, except at 8th weeks it detected at low level in serum of group receive large dose of PVC (Group III) as shown in (Table 5).

Effect of PVC on m-RNA Expression of CYP2 E1, TP53 and CYC: The expected product size of CYP2E1 is 473 bp refers to molecular weight of specific amplified product according to the synthesized primer. The molecular result
Table 6: Analysis of PCR product of CYP2 E1 gene in liver tissue of albino rats in different groups (mean± SD)

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Mean total RNA expression of CYP2E1 at 4th week</th>
<th>Mean total RNA expression of CYP2E1 at 8th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>155±1.21</td>
<td>153±0.41</td>
</tr>
<tr>
<td>Group II</td>
<td>185±0.83</td>
<td>145±0.60</td>
</tr>
<tr>
<td>Group III</td>
<td>206±0.95</td>
<td>132±1.14</td>
</tr>
</tbody>
</table>

Mean with different letters at the same raw differ significant ($P < 0.05$)

Table 7: Analysis of PCR product of TP 53 gene in liver tissue of albino rats in different groups (mean± SD)

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Mean total RNA expression of TP 53 at 4th week</th>
<th>Mean total RNA expression of TP 53 at 8th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>128±0.60</td>
<td>129±0.81</td>
</tr>
<tr>
<td>Group II</td>
<td>166±1.32</td>
<td>180±1.10</td>
</tr>
<tr>
<td>Group III</td>
<td>204±2.5</td>
<td>250±0.34</td>
</tr>
</tbody>
</table>

Mean with different letters at the same raw differ significant ($P < 0.05$)

Table 8: Analysis of PCR product of CYC gene in liver tissue of albino rats in different groups (mean± SD)

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Mean total RNA expression of CYC at 4th week</th>
<th>Mean total RNA expression of CYC at 8th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>95.1±1.32</td>
<td>95.3±2.15</td>
</tr>
<tr>
<td>Group II</td>
<td>95.3±0.71</td>
<td>95.2±1.86</td>
</tr>
<tr>
<td>Group III</td>
<td>95.2±0.87</td>
<td>95.4±0.40</td>
</tr>
</tbody>
</table>

Mean with different letters at the same raw differ significant ($P < 0.05$)

Fig. 5: The electrophoretic photograph of mRNA expression of TP 53 gene (575 bp) in liver of rats in different groups at 4th week

Fig. 6: The electrophoretic photograph of mRNA expression of TP 53 gene (575 bp) in liver of rats in different groups at 8th week

revealed up-regulation in mRNA expression of CYP2E1 gene at 4th week of experiment (Figure 3), while at 8th week of experiment the mRNA expression of CYP2E1 gene showing down-regulation in its level in groups treated with PVC if compared with control group as shown in (Figure 4 & Table 6).

The expected product size of TP53 gene is 575 bp refers to molecular weight of specific amplified product according to the synthesized primer. The molecular result revealed up-regulation in mRNA expression of TP53 gene at 4th week of experiment (Figure 5), at 8th week of experiment the mRNA expression of TP53 gene showing highly significance over expression in groups treated with PVC if compared with control group as shown in (Figure 6 & Table 7).

The expected product size of CYC gene is 265 bp refers to molecular weight of specific amplified product according to the synthesized primer. The mRNA expression of CYC gene as internal control gene showed...
Fig. 7: The electrophoretic photograph of mRNA expression of CYC gene (265 bp) in liver of rats in different groups at 4th week

-L: 100bp-1000bp DNA-ladder, Lane 1: Group I (Control), Lane 2: Group II (1/20 LD₅₀ of PVC), Lane 3: Group III (1/10 LD₅₀ of PVC). Molecular weight was 265 bp refers to specific amplified product for CYC gene.

Fig. 8: The electrophoretic photograph of mRNA ALP activity at 4th and 8th weeks in both treated groups by PVC if compared with control group. This result agreed with Du et al. [23] and Saad et al. [24] and Hsieh et al. [25]. This significant increase on ALT and AST levels throughout the experimental period is directly related to the toxic effect of PVC on liver causing hepatocytic degeneration and necrosis and release of cellular enzymes. That also attributed to progressive liver damage leading to liberation of these enzymes or due to extensive break down of body tissue. So, it was suggested to monitoring of serum liver enzyme in the regular health screening of PVC workers.

DISUSSION

Poly vinyl chloride is a poison plastic that has a destructive effect during all steps during manufacturing, using and disposal. It also has widespread uses especially in PVC pipe and food beverage. VC is a well-established animal and human carcinogen. It also been identified as a cause of hepatocellular carcinoma.

This study revealed that the rats in both treated groups by PVC suffered from lethargy, anorexia, depression, rough hair coat, humped back posture and emaciation as shown in (Figures 1&2). These results similar to result obtained by Bi et al. [21] and Lee et al. [22]. These clinical signs may be attributed to toxic effect after administration of PVC and difference in the dose that high dose induced more rapid changes in the degree of toxicity.

Concerning to the effect of poly vinyl chloride on the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels on serum as shown in (Table 5) revealed significant increase on ALT and AST levels at 4th weeks and highly significant increase at 8th weeks of experiment, that more clear on group received high dose (1/10 LD₅₀) of PVC in comparison with their corresponding control group. Our result was similar to results obtained by Du et al. [23] and Saad et al. [24] and Hsieh et al. [25].

An increase in ALP activity indicates lesions in the hepatobiliary tract and leakage of it from the bile caniculi, which could serve as a further indication on liver injury.

Regarding to the effect of PVC on alkaline phosphatase (ALP) activity on serum as shown in (Table 5) founded that there was significant increase in ALP activity at 4th and 8th weeks on both treated groups by PVC if compared with control group. This result was highly significant at group received high dose of PVC (Group III) in comparison with their corresponding control group. This result agreed with Winell et al. [26] and Mir et al. [27].

An increase in ALP activity indicates lesions in the hepatobiliary tract and leakage of it from the bile caniculi, which could serve as a further indication on liver injury.

Regarding to the effect of PVC on alfa-feto protein level on serum as shown in Table (5) founded that alfa-feto protein was not detected throughout the experiment, except at 8th weeks detected at low level (4.6 ng/ml) in serum of group receive large dose of PVC (Group III). This result agreed with Du et al. [28]. Slight statistically significant increases in the alfa-feto protein content in serum at 8th week may indicate the presence of......
foetoglobulin-producing neoplastic or preneoplastic cells in the liver of rats exposed to VCM for a prolonged period. The slight increase in alfa-feto protein content may also have been indication of PVC injury to the hepatic parenchymal cells, since it is known that tissue injury followed by repairing process that may stimulate tumor formation in a long period. It was indicated that there was significant dose-response relationship exists between plasma oncoprotein and VCM exposure [29]. AFP is the main serologic biomarker for liver cancer in clinical practice. With its low sensitivity and nonspecific elevation in nonmalignant hepatic diseases, there is an urgent need to identify additional diagnostic biomarkers that can assist in early diagnosis of liver cancer, especially in alpha-fetoprotein-normal cases and in tumors with smaller sizes [30].

The molecular result of the present study revealed up-regulation in mRNA expression of CYP2E1 gene at 4th week of experiment, While at 8th week of experiment the mRNA expression of CYP2E1 gene showing down-regulation in its level in groups treated with PVC if compared with control group as shown in (Table 6) and (Figures 2&3). The expected product size is 473 bp refers to molecular weight of the specific amplified product for CYP2E1 gene according to the synthesized primer. These results were more clear at group receive large dose of PVC (1/10 LD50 of PVC) if compared with other group. Our result similar to results obtained by Wang et al. [31] and Ji et al. [32]. These results disagreed with Huang et al. [33]. These changes in mRNA expression of CYP2E1 gene may attributed to the effect of PVC that mediates liver damage by the up regulation of CYP2E1 expression; the elevated CYP2E1 mRNA may play a critical role in the induction of liver damage, so that can be followed by destruction of CYP2E1 after liver affection; leading to down regulation on it is expression. It also attributed that the loss of CYP2E1 has been suggested to result from the production of reactive intermediates during the metabolism of PVC [34].

The molecular result of the present study revealed up-regulation in mRNA expression of TP 53 gene at 4th week of experiment, while at 8th week of experiment the mRNA expression of TP 53 gene showing highly significance over expression in groups treated with PVC if compared with control group as shown in (Table 7) and (Figures 4 &5). The expected product size is 575 bp refers to molecular weight of specific amplified product for TP53 gene according to the synthesized primer. These results were more clear at group receive large dose of PVC (1/10 LD50 of PVC) if compared with other group. Our result similar to results obtained by John Luo et al. [35] and Zhu et al. [36]. These changes in mRNA expression of TP 53 gene may attributed to the effect of PVC that lead to genotoxic stress, So the p53 protein is activated by specific phosphorylation events that lead to increased levels of p53 resulting in the cell cycle being interrupted and the inability of damaged DNA to be replicated [37]. It also may attribute to DNA damage that occurs after exposure to a carcinogen, leads to an up-regulation of p53.

The effect of PVC on mRNA expression of CYC gene as internal control gene showed almost stable pattern of expression in all groups of rats along the experimental period, as shown in (Table 8) and Figures (6&7). At 4th week of experiment the mean relative density of CYC gene in groups I, II and III was 95.1± 1.32, 95.3± 0.71 and 95.2± 0.87, respectively. At 8th week of experiment the mean relative density of CYC gene in groups I, II and III was 95.3± 2.15, 95.2± 1.86 and 95.4± 0.40, respectively. The expected product size is 265 bp refers to molecular weight of specific amplified product for CYC gene according to the synthesized primer. This study provided strong evidence that application of gene expression profiling to preneoplastic liver diseases and hepatocellular carcinoma is particularly important in early investigation of carcinogenic risk of PVC.

REFERENCES


