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Effect of anti-breast cancer agent, PQ1, on normal tissues

(Running Head: Effect of PQ1 on normal tissues)

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Conflicts of Interest: None Declared

Abstract

Gap junctions are intercellular channels connecting adjacent cells, allowing cells to transport small molecules. Loss of gap junctional intercellular communication (GJIC) is one of the important hallmarks of cancer. Restoration of GJIC is related to the reduction of tumorigenesis and increase of drug sensitivity. Previous reports showed that PQ1, a quinoline derivative, increases GJIC in T47D breast cancer cells, and subsequently attenuates xenograft breast tumor growth. Combinational treatment of PQ1 and tamoxifen can lower the effective dose of tamoxifen in cancer cells. In this study, effects of PQ1 were examined in normal C57BL/6J mice, evaluating the distribution, toxicity and adverse effects. Distribution of PQ1 was quantified by HPLC and mass spectrometry. Expressions of survivin, caspase-8, cleaved caspase-3, aryl hydrocarbon receptor (AhR), and gap junction protein, connexin 43 (Cx43), were measured using Western blot analysis. Our results showed that PQ1 absorbed and distributed to all tested organs in 1 hour and the level of PQ1 diminished after 24 hours. PQ1 increased the expression of survivin, whereas decreased the expression of caspase-8 and active caspase-3 in vital organs. Furthermore, expression of AhR increased in the presence of PQ1, suggesting that PQ1 may be involved in AhR-mediated response. Expression of Cx43 decreased after PQ1 treatment, which is contrary to the effect of PQ1 on cancer cells. Hemotoxylin and eosin staining of the tissues showed no histological change between treated and untreated organs (after 1 h or 24 h?). Our studies indicate that PQ1 administration by oral gavage can be achieved with low toxicity to normal vital organs.

Keywords: Adverse effect**,** anti-breast cancer agent, distribution, gap junction, PQ1, toxicity.

Introduction

Gap junctional intercellular communication (GJIC) plays an important role in controlling cell growth, regulating cell differentiation, and maintaining homeostasis in normal cells and tissues [1, 2]. Gap junction is a hydrophilic channel which is formed by transmembrane proteins, connexins [3]. Six connexins oligomerize into a hexameric structure known as connexon. Connexon at the plasma membrane may stand alone as a hemichannel or may dock with another connexon of an adjacent cell to form a gap junction [4]. The gap junction channel allows cells to exchange small molecules of less than 1.2 kDa in size including small metabolites, electrical signals, and secondary messengers [5]. This maintenance of communication keeps cells at homeostasis. Collective information shows that mutations in connexin genes or deficiency in GJIC are related to various human diseases, such as deafness, peripheral neuropathy, skin disorders, cataracts, and even cancers [6, 7].

Diminished connexin expression and deficiency in GJIC are considered to be two characteristics of tumorigenesis [8, 9]. Although it is still controversial about the facilitative function of connexins in invasion, intravasation, extravasation and metastasis, it has been widely accepted that connexins are tumor suppressors due to both the GJIC-dependent and GJIC-independent mechanisms [10-14]. Restoration or/and activation of GJIC in cancer cells are suggested to have the ability to reduce cancer cell proliferation and tumor growth [15, 16]. In addition to this directly suppressive function, upregulation of GJIC in cancer cells is also important to increase efficacy of anticancer drugs in cancer combinational treatment. Re-establishment of GJIC is helpful for drug or pro-drug delivery throughout a tumor, and kill more cells by the way of so-called ‘bystander effect’, a mechanism by which cytotoxic molecules are transported from a treated cell to a neighboring cell [13]. This mechanism has demonstrated to be

an effective way to potentiate drug effect. The application of bystander effect in gene therapy showed that after enhancing connexin 43 (Cx43) and GJIC by 8-bromo-cyclic-AMP treatment, gene therapy effect was strengthened by herpes simplex virus thymidine kinase/gancyclovir (HSV-TK/GCV) system [17]. Besides gene therapy, bystander effect is also responsible for improving radiation therapy and chemotherapy [18, 19]. Therefore, developing novel agent or method to enhance or restore GJIC in cancer cells is a new research strategy in cancer treatment.

PQ1 (Fig. 1), a quinoline derivative, was reported as a gap junction enhancer in T47D breast cancer cells. PQ1 increases GJIC in T47D cells, whereas it has no effect on GJIC in normal human mammary epithelial cells (HMECs) [20]. One μM of PQ1 decreased cell viability to 50% in T47D cells and attenuated 70% of xenograft tumor in nude mice [20]. Combinational treatment of PQ1 and tamoxifen showed that PQ1 potentiated the effect of tamoxifen in T47D cells [21]. All these studies implied therapeutic potential of PQ1 in breast cancer treatment. However, data of PQ1 on normal tissues are needed prior to preclinical trial of PQ1.

In this study, effect of PQ1 was evaluated in healthy C57BL/6J mice. Drug distribution to vital organs was determined and effect of PQ1 on apoptosis was analyzed by the expression of caspases. We also studied the response of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that regulates transcription and activity of several important drug-metabolizing enzymes. Further analysis using histological observation of PQ1-treated tissues showed no alteration in structure change. Our results showed that the distribution of PQ1 via oral administration in mice can be assessed and low toxicity in vital organs was found.

Material and Methods

PQ1. A quinoline derivative, PQ1, was obtained as described by Shi et al. [22].

Animals. Female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All mice were housed together in a temperature controlled environment (72°F) with a 12-hour light-dark cycle and unlimited access to standard mouse chow and water. Five-week-old mice, with an average weight of 24 grams, were used. Twenty-five mg/kg PQ1 was administered by oral gavage to each animal. Animal care and use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Kansas State University, following NIH guidelines.

Extraction of PQ1 from organs. Organs were cut into small pieces and diluted with 4 ml of deionized water and 10 ml of a solution of 9:1 ratio of ethyl acetate and 1-propanol. Tissue mixture was sonicated for 40 minutes, and the organic layer was separated from a separatory funnel. The aqueous layer was extracted twice with 10 ml of a 9:1 mixture of ethyl acetate and 1-propanol. The organic layers were combined, washed with 5 ml of brine, dried over anhydrous $MgSO_4$, and concentrated to dryness on a rotary evaporator. The residue was diluted with 1 ml of 1-propanol, filtered through a 0.2 μm filter disc (PTFE 0.2 μm, Fisherbrand), and analyzed using high-performance liquid chromatography (HPLC) and mass spectrometry as described below.

Quantification of PQ1 in tissue extracts using HPLC. HPLC analysis was carried out on a Varian Prostar 210 with a UV-Vis detector and a reverse phase column (250 x 21.20 mm, 10 micron, Phenomenex Inc.). A flow rate of 4 ml/min and detection wavelength of 254 nm were used. A gradient elution of solvent A, containing deionized water and 0.01% of trifluoroacetic acid, and solvent B, containing acetonitrile and 0.01% of trifluoroacetic acid, was applied for the

analysis. 1,2,4,5-Benzenetetracarboxylic acid (BTA) was used as an internal standard to quantify the amount of PQ1 in the tissue extracts. Solutions of 100 µl of various mixtures of authentic PQ1 and BTA were injected into a HPLC instrument, the peak areas corresponding to PQ1 and BTA were integrated from the HPLC chromatogram, and the ratios of the peaks were obtained. Results of the ratios of HPLC peak areas and ratios from PQ1 and BTA concentrations were plotted, and a linear correlation line was obtained from the graph. Hence using this correlation diagram, the ratio of HPLC peak areas of PQ1 and BTA from tissue extract and the added known amount of BTA to the tissue extract, the amount of PQ1 in the tissue extract was determined. Moreover, the peak that has the same retention time as that of PQ1 from the injection of the tissue extract was collected, and its mass was determined using a mass spectrometer. The mass spectrum acquired from collected peak of PQ1 from the tissue extract was identical to that of the authentic PQ1 mass spectrum. Hence, the molecular identity of PQ1 in the tissue extract was verified by mass spectrometry.

Mass spectroscopy. An Applied Biosystem API 2000 LS/MS/MS mass spectrometer was used in the analysis. The eluent corresponding to PQ1 peak from the HPLC was collected and injected into the mass spectrometer. A mass of 406 corresponding to M+1 of PQ1 was found in the mass spectra, and the fragmentation pattern of this M+1 mass is identical to that of authentic PQ1.

Western blot analysis. Organs from treated or untreated mice were collected and homogenized with cell lysis buffer (Cell Signaling Technology, Inc, Danver, MA) using Vibra-Cell sonicator (Sonics & Materials Inc, Danbury, CT). The mixture was centrifuged at 13,000 rpm for 30 minutes at 4°C, and the supernatant was collected. Total protein concentration was determined by the Bio-Rad protein assay. Forty µg of protein extract were separated by 4 - 20% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 35 minutes at 200 Volts and

protein separation was transferred to nitrocellulose membrane. The membrane was immunoblotted against protein of interest. The goat anti-survivin antibody and mouse anti-caspase-8 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-cleaved caspase-3 and rabbit anti-connexin 43 antibodies were obtained from Cell Signaling Technology (Danvers, MA). The rabbit anti-AhR and rabbit anti-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Immunoreactions using chemiluminescence were visualized by FluoChem E Imaging Instrument (Cell Biosciences, Inc, Santa Clara, CA). Intensities of the bands were digitized using Un-Scan-It software.

Hematoxylin and eosin (H&E) staining. H&E staining was performed on paraffin-embedded tissues by following standard protocol. Five μm sections were dewaxed and rehydrated in xylene and decreasing ethanol concentrations to water. Sections were stained with hematoxylin and eosin and mounted for microscopic imaging.

Statistical analysis. Pixel intensities of protein bands were normalized to pixel intensities of loading control protein, actin or GAPDH. All protein expression data presented were expressed as mean ± S.D. of at least three independent experiments from different animals. Significant differences were analyzed by comparing the data between treated animals and control (untreated) animals. Significance was considered at $p < 0.05$ using student's t-test.

Results

Distribution of PQ1

After one-hour treatment, majority of PQ1, 10% and 5% of total amount administered, was detected in liver and brain, respectively. PQ1 was low, in the heart with 1%, lung with 1.5%, kidney with 1%, and uterus with 2.5% (Fig. 2A). Interestingly, PQ1 distribution changed after 12 hours of administration. The percentage of PQ1 in liver decreased from 10% to 5%, and percentage of PQ1 in brain dropped from 5% to 2%. On the contrary, PQ1 in kidney increased from 1% to 3%, indicating a shift of PQ1 from liver to kidney had occurred. Amounts of PQ1 in heart, lung and uterus remained consistent at 12 hours of administration (Fig. 2B). After 24-hour treatment, no PQ1 was found in brain and heart. Percentage of PQ1 decreased to 3% in liver and 1% in kidney. The average percentage of PQ1 in uterus stayed at 3%. PQ1 in lung had a slight increase from 1.5% to 2.6% at 24-hour time point (Fig. 2C).

Effect of PQ1 on apoptosis in normal tissues

Apoptosis is a programmed cell death, an important event in homeostasis of healthy organs [23, 24]. Drugs, affecting apoptosis in healthy organs, are concerned due to the relevant side effects that they may cause [25]. Cell proliferation or cell death depends on the balance of pro- and anti-apoptotic factors. Thus, expressions of anti-apoptotic factor, survivin, and pro-apoptotic proteins, caspases, were evaluated. Since cleaved caspase-3 is the checkpoint protein of both intrinsic and extrinsic apoptotic pathways and caspase-8 is the key reporter of extrinsic apoptotic pathway [26], these two caspases were examined in the presence of PQ1.

The results showed that level of survivin increased in PQ1-treated organs, whereas both cleaved caspase-3 and caspase-8 decreased in these organs (Fig. 3A, 3B, 3C). The level of survivin increased by 14% in liver, 28% in heart, and 44% in lung at 1 hour after PQ1

administration, compared to controls. These effects are consistent with the detected level of PQ1. Interestingly, the level of survivin in these organs was reduced to the same level as the controls at 24-hour time point. In brain and kidney, there were no detectable changes in survivin expression at any time point. Uterus was the only exception in which survivin decreased more than 25% after PQ1 treatment (Fig. 3A). As for caspase 8 expression, brain, heart, lung, liver, and uterus of the treated animals have a slight decrease expression ranging from 12% to 37% compared to untreated animals; however, there was no significant change in the kidney (Fig. 3B). Cleaved caspase-3 was only detected in the uterus, liver, and lung of untreated animals; thus, the change in cleaved caspase-3 upon PQ1 treatment was measured in these three organs. A significant decrease ranging from 37% to 45% of cleaved caspase-3 at 12-hour dosing was observed compared to control (Fig. 3C). Results of caspases and survivin suggest that PQ1 inhibits pro-apoptotic factors and promotes anti-apoptotic proteins, which accordingly protects normal cells from apoptosis at the early time point from PQ1 exposure.

Effect of PQ1 on AhR levels in normal tissues

Aryl hydrocarbon receptor (AhR) is a transcriptional factor involved in the metabolic pathway of aromatic hydrocarbon compounds [27]. The main adaptive response of AhR is the binding of AhR and hydrocarbon compounds, inducing metabolizing enzymes that are involved in its metabolic pathway [27]. Aromatic hydrocarbon compounds have demonstrated to trigger AhR-mediated pathway for its metabolism; thus, the effect of PQ1, an aromatic hydrocarbon compound (Fig. 1), on AhR expression was examined.

The results showed that the level of AhR in brain, heart, and liver increased significantly at 12-hour point of PQ1 treatment, 161%, 167%, and 124% compared to controls, respectively; however, there was a delay in detecting AhR in the kidney. A 114% AhR was detected in the

kidney at 24-hour point (Fig. 4A). From the drug/tissue distribution data, the amounts of PQ1 peaked at 1 hour in brain, heart and liver, but peaked at 12-hour point in kidney (Fig. 2A, 2B). These suggest that there is a time-delay response in AhR in these organs. Interestingly, the level of AhR fluctuated from 117% at 1-hour of dosing to 63% at 12-hour of dosing. Furthermore, only 57%, 62%, and 55% of AhR were detected in the treated uterus at 1-, 12-, and 24-hour time points, respectively, compared to controls. An early onset of AhR downregulation after PQ1 administration implies that PQ1 might be involved in a different mode of action in the uterus (Fig. 4A). At 1 hour of PQ1 administration, level of AhR proportionally changed along with the amount of PQ1 in liver, indicating a direct dependent function of AhR to PQ1 in liver (Fig. 4B). The data demonstrated that PQ1 can trigger the response of AhR in brain, heart, liver, and kidney, signifying its involvement in the AhR-mediated metabolism pathway.

Effect of PQ1 on connexin in normal tissues

Since PQ1 has been shown to enhance GJIC [20] and increase Cx43 expressions (data not shown) in breast cancer cells, Cx43 in treated- and untreated-PQ1 organs was measured in. Cx43 was detected in heart, brain, and lung in the absence of PQ1 treatment; however, the level of Cx43 diminished in all PQ1-treated organs. A statistically significant decrease of 31% compared to control was found at 24-hour point in the heart. A constant level of Cx43 in the lung was observed at all-time points. Interestingly, level of Cx43 in brain gradually declined over time (Fig. 5). Results are contrary to the function of PQ1 in cancer cells where the lack of GJIC and low expression of Cx43 in T47D breast cancer cells were restored in the presence of 200 nM PQ1.

Histological analysis of normal tissues

Liver is an important organ in drug metabolism. Hematoxylin and eosin (H&E) staining of PQ1-treated organs was performed. All twenty-four mice were assessed grossly or microscopically for histological changes. Histological results showed that PQ1-treated liver remained unchanged compared to control, which indicate no observable toxicity of PQ1 to liver at the treated dosage and time (Fig. 6A). Other tissues including heart, adrenal gland, kidney, and reproductive tract were also examined and no histological change was observed (Fig. 6B). Twenty-one of the histologically PQ1-treated mice had no evidence of hemorrhage or inflammatory cells. These mice had no histologic evidence of lesion compared to control mice without PQ1 treatment at any time point.

Discussions

Since cancer is a complicated disease with multiple deregulation pathways, cancer treatments have to focus on combinational treatments [28]. The deficiency of GJIC in cancer cells adds to the complexity of cancer therapy in which the lack of drug transfer to the surrounding area creates challenges to cancer therapy [14]. Some anticancer drugs are reported to inhibit GJIC and reduce connexin expression, adding to the complexity of cancer therapy [29, 30]. Hence, restoration of GJIC in cancer cells is a focal point in combinational treatment by potentiating the effect of anticancer drugs. In addition to combinational treatment, overexpression of connexin and activation of GJIC also play a suppressive role to tumors [13]. Therefore, the development of molecules and agents modulating the connexin expression and GJIC function is a therapeutic strategy in cancer treatment.

Quinolines are known for their anticancer effects by targeting tumor hypoxia and modulating multidrug resistance [31, 32]. Previous reports showed that a quinoline derivative, PQ1, enhances GJIC, inhibits cell and tumor growth, and increases potential of the

combinational treatment with tamoxifen in T47D breast cancer cells [20, 21]. Therefore, the current study provides data of drug/tissue distribution and possible pathway of PQ1 metabolism in normal mice.

A desirable and safe route of administration, oral gavage, is used in this study. Uptake of any drug is depending on the rate of blood flow; thus, the level of PQ1 was evaluated in five vital organs (brain, heart, lung, kidney, and liver) that have high rate of blood flow. PQ1 was measured in each vital organ after oral administration. Antineoplastic drug such as tamoxifen has been shown to affect tumorigenesis in the uterus; therefore, the effect of PQ1 in this organ was also examined [33]. The effective dosage of PQ1 falls in nM range in cells and xenograft tumors [20]. To investigate the toxicity in normal organs and make this study compatible with the relevant level for therapeutic dose, a higher concentration of PQ1 was administered at 25 mg/kg body weight, which is equivalent to 47.7 μM. With this dosage, the concentrations of PQ1, distributed in tested organs after oral administration, were more than 20-fold higher than the therapeutic dosage. PQ1 was detected in all tested organs after 1-hour treatment and diminished at 24 hours of dosing, suggesting that PQ1 can be eliminated or excreted after 24 hours (Fig. 2). The highest concentrations of PQ1 were found in the liver and kidney at different times (Fig. 2A and 2B). A high percentage of PQ1 was detected in the brain at 1 hour and may be due to the processing of tissue in which PQ1 in the blood vessels could not be excluded during the whole tissue extract (Fig. 2A). Our results show that PQ1 can be absorbed, distributed to vital organs, and metabolized in C57B/6J mice.

Triggering apoptosis pathway in normal cells and tissues is one reason that causes serious side effects of therapeutic drugs. Diarrhea, a common side effect of chemotherapy, is partly caused by induced apoptosis in normal cells of the small intestinal epithelium [25]. It has also

been reported that both chemotherapeutic drugs and irradiation can induce apoptosis in normal thymocytes [34, 35]. In this report, the effect of PQ1 on apoptosis in normal tissues was examined. The presence of PQ1 via oral gavage caused a decrease in cleaved caspase-3 and an increase in survivin of normal tissues, indicating the inactivation of apoptosis (Fig. 3A, 3C). Further study of extrinsic apoptotic pathway, a checkpoint protein of caspase-8, was performed. Decrease of caspase-8 after treatment of PQ1 further elucidated that PQ1 cannot activate the extrinsic pathway of apoptosis in normal tissues (Fig. 3B). The effect on apoptosis in normal organs indicates a minor, apoptosis-related side effect caused by PQ1. Interestingly, PQ1 increases cleaved caspase-3 [20] and caspase-8 in T47D cells and xenograft tumors [data not shown]. The opposing aspect of PQ1 on apoptosis in cancer cells and tumors compared to normal tissues implied that PQ1 may have a different mechanism in cancer cells. The difference between cancer and normal cells is also shown by the function of PQ1 on connexin expression. PQ1 enhances GJIC [20] and increases connexin expression in both T47D breast cancer cells and xenograft tumors; however, it decreases the expression of Cx43 in a normal heart, brain, and lung (Fig. 5). PQ1 mechanism of tumor specificity is not clear. Further studies are needed to clarify the causes of this specificity.

AhR, a ligand-dependent transcription factor involved in the transcription of many important drug-metabolizing enzymes [36], is widely expressed in rodent and human tissues [37]. Increase of AhR protein level in PQ1-treated mice was observed in tested vital organs, indicating the possible involvement of PQ1 in the activation of ligand-dependent transcription of AhR pathway (Fig. 4A). The proportional relation between AhR expression and detected level of PQ1 in liver at 1 hour showed a direct and rapid response of AhR to PQ1. However, AhR was decreased by PQ1 treatment in the lung compared to control. Previous report demonstrated that

increase of AhR was found in the early stage of lung adenocarcinoma [38], suggesting that low level of AhR in PQ1-treated lung is due to tissue specificity. Furthermore, increase of AhR in PQ1-treated organs implies that PQ1 is involved in AhR-mediated response. Further analysis of gene regulation and enzyme activities in AhR-mediated pathways is needed to elucidate the metabolism of PQ1.

Gap junction has been studied for more than forty years. Until recently, the involvement of gap junction in cancer has been reported and widely discussed. Although several molecules have been developed to modulate different levels of gap junctional proteins and GJIC [13], none of these molecules has reached clinical trials for the treatment of cancer. Our present findings support the notion that PQ1 is a promising anti-breast cancer candidate and may serve as a lead compound for drug development.

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Figure Legends

Figure 1. Chemical structure of PQ1.

$C_{21}H_{22}F_3N_3O_2$. Molecular weight is 405.3744. Exact Mass is 405.1431.

Figure 2. PQ1 distribution in mice.

Mice, treated with 25 mg/kg of PQ1, were sacrificed at 1 (A), 12 (B), and 24 (C) hours. Percentages of PQ1, normalized to total amounts of PQ1 in brain, heart, lung, liver, kidney, and uterus, were presented. Data of each experiment were obtained from four mice. Data points represent the percentage of PQ1 in an organ of each mouse, and the dash lines show the average of PQ1 in four mice.

Figure 3. Effect of PQ1 on apoptosis in normal tissues.

Vital organs from PQ1-treated and untreated animals were subjected to Western blot analysis , examining the effect of 1-hour, 12-hour, and 24-hour treatments of PQ1 on the levels of survivin (A), caspase-8 (B), and cleaved caspase-3 (C). Immunblotting images and graphical data are presented. “C” indicates the control animals without treatment and “T” indicates PQ1-treated animals. In the bar graph, pixel intensities of protein bands were normalized to pixel intensities of loading control protein, actin, and the results of treated animals are normalized to the results of control animals. Graphical presentation of three experiments are presented with ±SD and statistical significance, $*P<0.05$.

Figure 4. Effect of PQ1 on AhR levels in normal tissues.

(A) Western blot analysis was performed, examining the effect of 1-hour, 12-hour, and 24-hour treatments of PQ1 on the levels of AhR. Mice without PQ1 treatment were used as control. Immonoblotting images and graphical data are presented. “C” indicates the control animals

without treatment and "T" indicates PQ1-treated animals. In the bar graph, pixel intensities of protein bands were normalized to pixel intensities of loading control protein, actin. Graphical presentation of three experiments are presented with ±SD and statistical significance, $*P<0.05$. (B) The levels of AhR proportionally change along with the amounts of PQ1 in liver after 1-hour treatment. Immnoblotting images are also shown above the graph. In the graph, a line indicates percentage of PQ1 normalized to the amount of PQ1 in the liver of a corresponding animal. AhR level normalized to control group are shown by bar. All the data have been normalized with the body weight of each mouse as well.

Figure 5. Effect of PQ1 on connexin 43 expression in normal tissues.

Western blot analysis was performed, examining the effect of 1-hour, 12-hour, and 24-hour treatments of PQ1 on the levels of connexin 43 in heart, brain, and lung. Mice without PQ1 treatment are used as control. Both immunoblotting images and graphical data are presented. Pixel intensities of protein bands were normalized to pixel intensities of loading control protein, GAPDH, in the bar graph. Graphical presentation of three experiments are presented with ±SD and statistical significance, $*P<0.05$.

Figure 6. A H&E staining of whole organs.

(A) Effect of PQ1 on liver at 1 (B), 12 (C), and 24 hours (D). Livers from untreated animals were used as control (A). Toxicity of PQ1-treated liver was examined by H & E staining using 40X magnification. Histological results showed that PQ1-treated liver had no change compared to control. (B) Histology of PQ1-treated animals for heart (A), adrenal gland (B), and reproductive tract (D) were observed under 4X magnification, and kidney was observed under 10X magnification. The results show no histological alteration in the treated animals compared to control.