

Influences of Formalin Gas on Gene Expression Level of Tumor Protein *TP53* in the Liver and Kidney of the Quail (*Coturnix coturnix*)

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ABSTRACT

This study was the first to highlight the deleterious effect of formalin gas on gene expression of the tumor protein *TP53* in the liver and kidney. This experiment was conducted according to the animal ethics and regulations of the college of veterinary medicine and the University of Al-Qadisiyah. Quails were divided into four groups: Three groups were exposed to two hours of formalin gas twice daily, two hours in the morning and evening, for 30 days and the fourth group was the control with the same environmental conditions except for formalin. Then, quails were killed on different days (10, 20 and 30 days) and liver, kidney and blood specimens were collected for gene expression, histological and biochemical studies. The result displayed that formalin gas caused by disturbances in liver and kidney functions, in addition to the great pathological liver and kidney changes, showed acute hepatitis and nephritis. 5% of the formalin gas significantly increased the expression of *TP53* in the liver and kidney regularly with a period of exposure, which confirmed that *P53* might be considered a suitable biomarker for formalin impaction in humans and animals. This study noticed that 5% of formalin gas significantly increased the expression of regular in the liver and kidney, depending on the exposure time. Therefore, this study considered a *P53* determinant biomarker for assessing the effect and risk of formalin gas on cancer diseases in humans and animals' digestive and urinary systems.

Keywords: Formalin exposure; *TP53*; Kidney; Liver damage

INTRODUCTION

Formalin exhibits the properties of being flammable, lacking odor, possessing toxicity and displaying high solubility in water. Formaldehyde is commonly found in various sources such as cigarette smoke, emissions from automobiles, fuel oil, natural gas, exhaust from vehicles powered by fossil fuels, furniture and the fumes emitted by chipboard paint. Disinfectants and sterilizers are commonly employed by educational institutions, higher education establishments, as well as biological, forensic and pathological laboratories to safeguard tissues, surgical specimens, organs and viscera [1]. Hence, formaldehyde is classified as a carcinogenic substance due to its propensity to induce cancer in animals, particularly in the nasal region, when exposed to elevated levels over an extended period [2].

Occupational exposure to formaldehyde has been associated with multiple factors, such as the dosage and concentration of formalin, insufficient ventilation, suboptimal temperature and

inadequate relative humidity levels [3]. The inhalation of formalin has been observed to impact plasma proteins, leading to their conversion into formic acid by the liver and red blood cells. This process has been associated with the development of severe toxicity and increased mortality rates. The presence of formic acid in the bloodstream leads to the development of a significant metabolic acidosis, while also impairing liver function through the inhibition of cholinesterase, succinate oxidation and anaerobic glycolysis [4].

Formic acid induces tissue hypoxia and elevates acid burden through its inhibitory effect on mitochondrial cytochrome c oxidase [5,6]. The livestock industry employs formalin mists and fumigants as means of disinfecting, incubating eggs and sterilizing equipment in order to mitigate the risk of disease and minimize the presence of bacterial and parasitic organisms [7,8]. In order to mitigate the presence of pathogens, the United States allows for a maximum formalin content of 2.5 kilograms per ton

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in poultry feed [9]. It was reported that broilers at eight weeks old revealed a reduction in feed intake and body mass, occurrence of local necrosis, as well as crop and intestinal hemorrhage after the administration of formalin in feed at different concentrations [10].

The P53 protein, also known as the protein *TP53*, is a tumor suppressor protein encoded by the *TP53* gene in humans so, this protein plays a crucial role in regulating cell growth and preventing the formation of cancerous tumors. Any disorders of its expression would induce cancer diseases, the reason is that the P53 protein functions as a transcription factor, which is normally regulated and controls the expression of genes in cells and prevents cancer formation [11].

Previous studies reported that any stress on cells might impact cells and form DNA damage; the P53 protein is activated and can trigger several cellular responses, making tumors possible. Consequently, these responses include cell cycle arrest, DNA repair, apoptosis (programmed cell death) and senescence (permanent cell growth arrest). P53 would induce these cellular responses to help prevent the proliferation of damaged or abnormal cells, which can lead to cancer formation [12].

Importantly, mutations in the *TP53* gene can lead to the loss of *TP53* function, increasing the risk factor of cancer development. Unfortunately, many researchers, have detected mutations in *TP53* among the most common genetic alterations in human cancers. Therefore, the role of the P53 protein in cancer development and progression was studied to develop new cancer treatments and therapies [13]. Previous research on formalin and P53 pushed us to study the effect of formalin gas on the liver and kidney of quail birds in relation to *TP53* expression and activity. Accordingly, it might be selected as a biomarker and indicator for risk factors of formalin gas in humans and animals, particularly regular exposure to formalin gas and the impact of formalin gas on liver and kidney function at different times. Therefore, this study aimed to study the functions of the liver and kidneys in relation to *TP53* gene expression in the digestive and urinary systems of quails after exposure to 5% of the formalin gas at different periods and days.

MATERIALS AND METHODS

Experimental procedure

Sixteen quail birds were used for this experiment. This experiment was conducted according to the rules and regulations of animal ethics at the college of veterinary medicine and the University of Al-Qadisiyah with approval number (P. G, No. 1890 in 2020) during the period September to November 2022 according to the international guidelines for the care and use of animals. The healthy mature Japanese Quail (*Coturnix japonica*) were grown in suitable environmental conditions with good feeding, light and ventilation on the farm. Quails were divided into four groups; three groups were exposed to 5% of formalin gas twice daily, two hours morning and two hours evening for 30 days and every ten days, the first group (n=4) was killed, then group two (n=4) killed on the twentieth day. Finally, group three was killed on the thirtieth day. The fourth group

was the control with the same conditions and environment except for formalin gas. As a result, serum and liver and kidney tissue were collected for each group during the experiment. Serum was used to identify the liver and kidney function, including Aspartate Aminotransferase (AST), Alanine Transaminase (ALT) and Alkaline Phosphatase (ALP), Beckman Coulter AU480 and Biorad D10, both from California, US. Major organic composites in urea, creatinine, urea and uric acid concentration, were detected *via* using UV kinetic methodology in commercial kits (Human Gesellschaft for Biochemical und Diagnostics mbH, Wiesbaden, Germany), which clearly detected the different levels of liver enzymes and creatinine, urea and uric acid. The specimens of the liver and kidney were saved in 10% neutral formalin for histological sections and others were saved in Trizol (SRCr Green-Zol reagent. Iraq). For Real-Time quantity Poly Chain Reaction (RT-qPCR).

Liver and kidney enzyme profile

The test tubes containing the blood were positioned at an inclined angle and left at a temperature of 32°C for 30 mins. The serum was isolated from the coagulated blood through a centrifugation process at a speed of 3000 rpm for 20 mins, followed by additional centrifugation at the same speed for 10 mins. The supernatant was subsequently collected using a micropipette and transferred into an Eppendorf tube. The tube was then stored at -20°C. In this study, serum samples were employed to assess the hepatic and renal function, specifically measuring the levels of Aspartate Aminotransferase (AST), Alanine Transaminase (ALT) and Alkaline Phosphatase (ALP). The analysis was conducted using the Beckman Coulter AU480 and Biorad D10 instruments, both of which are manufactured in California, United States. The concentrations of major organic compounds, namely urea, creatinine and uric acid, were determined using UV kinetic methodology in commercially available kits (Human Gesellschaft for Biochemical und Diagnostics mbH, Germany). These kits effectively detected variations in levels of liver enzymes, as well as creatinine, urea and uric acid. Furthermore, the serum samples from the four experimental groups were employed to assess the concentration of chicken IgG using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Zeptomatrix, United States).

Extract of RNA and cRNA synthesis

Total RNA was extracted from the liver and kidney using the trizol reagent kit (SRCr Green-Zol reagent. Qadyisih. Iraq). 250 mg of the liver and kidney tissue was placed in 1.5 Eppendorf and homogenized by electric homogenizer (Fisherbrand. England), then added 200 µl chloroform was mixed, then incubated on ice for 5 minutes. Next, the lysate was spun at 10,000 × g (approximately 9700 rpm for rotors of a 9.5 cm radius) for 15 minutes at 4°C. The supernatant was taken off in separated Eppendorf and added 500 µl isopropanol (ISA) 99.8% UK and mixed; then the supernatant was incubated at 4°C for 10 minutes while the pellets were neglected. Afterward, the lysate supernatant was spun at 10,000 × g (approximately 9700 rpm for rotors of a 9.5 cm radius) for 10 minutes at 4°C at left; the supernatant was, add 1 ml of 80% ethanol and mixed

with vortex again. Lysate was centrifuged at $10,000 \times g$ (9700 rpm for rotors of a 9.5 cm radius) for 15 minutes at 4°C , the supernatant was discarded and pellets were left to air to dry. As a final point, 50 μl DEPC water (non-specific inhibitor of RNases/BKMAN Biotechnology Co. Ltd, China) was added to the pellets of RNA until dissolve the RNA pellet and saved at -20°C .

For cDNA synthesis, the total RNA was incubated with DNase I to remove the trace amount of DNA (Promega Company, Madison, Wisconsin, USA) for two hours. Then, RNA was translated into cDNA following the instructions of the DiaStar™ OneStep RT-PCR Kit (BKMAN Biotechnology Co. Ltd, China). After that, cDNA was measured by Nanodrop spectrophotometer (Thermo Scientific NanoDrop One Microvolume UV-Vis Spectrophotometer, USA) and diluted and normalized into the same concentrations for all samples for the RT-qPCR technique.

RT-qPCR technique

RT-qPCR was employed to detect the quantification levels of the Eosinophil Cationic Protein (ECP) mRNA transcript of *TP53* and the housekeeping gene of the quail. RT-qPCR primers were applied for *TP53* (size: 136 bp, code: XM_015855944.1, forward primer: AGCCGCGTTTTAACTGTGC and reverse primer: CAAAGTGGCTCTGGAAAGAAGC) and for housekeeping gene (size: 77 bp, code: XM_015873412.2, was forward primer: TGCTGGCATTGCACTGAATG and reverse primer: CACGGTTGCTGTATCCAACTC) (Scientific Researcher Co. Ltd. in Iraq). Consequently, amplification and normalization of the GAPDH housekeeping gene and *TP53* were distinguished by the SYBER green dye qPCR master mix to determine the gene expression level. A real-time PCR system (BioRad, 2000 Alfred Nobel Drive Hercules, CA, US 94547, USA) was used for this experiment. Thermocycler conditions were settled as the following: Initial denaturation was at 50°C for an hour, repeat cycle was at 95°C for 20 seconds, annealing\extension detection (scan) was at 60°C for 30 seconds at 45 repeat cycles and finally, the melting temperature was 60°C - 95°C , for 0.5 seconds and repeated the cycle for once.

Histological procedure

The liver and kidney sections were fixed in neutral formalin 10% for 48 hours; after that, the histological protocol was applied for making histological sections. The specimens were passed in a serious ethanol concentration and transparent by xylene. Afterward, specimens were embedded in wax for blocks. Next, blocks were cut at 5 μm -6 μm thick and stained with hematoxylin and eosin routine stains to identify the histological structures of the liver and kidney tissue of all groups from the experiment. All tissue sections of the groups and control were examined and imaged using a light microscope (Olympus CH-2 Phase Contrast Microscope, Japan)

Statistical analysis

The raw data of the gene expression was assessed by the 2(-Delta Delta C(T)) Method (2-CT method). All the obtained and raw data were analyzed and statistically evaluated using one-way ANOVA (IBM SPSS Statistics 23.0) and meant differences were analyzed at significance at the $P \leq 0.05$. All the results were expressed as mean $\pm$ SE.

RESULTS

Clinical findings

During the exposure period to formalin, the treated group of quail exhibited various clinical signs. These signs included nervousness, depression, anxiety, persistent coughing, reduced appetite and water intake, dullness, an unsteady gait, sitting with closed eyes and decreased responsiveness to disturbances. The observed signs exhibited greater prominence during the morning and evening periods immediately following exposure to FA compared to the rest of the day. Consequently, the treated quail's weight gradually decreased in all experimental groups compared to the control group. The average weight of the control group's normal birds was 200 ± 0.66 gm, while the treated groups had average weights of 188 ± 1.86 gm, 154 ± 0.004 gm and 130 ± 0.43 g, respectively. Significant differences were observed in all groups at a significance level of $p < 0.5$.

Findings of serum analysis

The renal and hepatic function parameters results revealed, at the end of the third week, that all groups showed significant ($p < 0.05$) increases compared with the control group. In the control group, the urea, creatine, uric acid, ALP, ALT and AST values were 4.06 ± 2.30 , 0.2 ± 0.50 , 10.36 ± 6.08 , 275.66 ± 166.3 , 274 ± 162.2 and 13.16 ± 7.50 , respectively. In the FVEQ1 group, the urea, creatine, uric acid, ALP, ALT and AST were 5.03 ± 2.48 , 0.1 ± 0.05 , 8.76 ± 5.42 , 588 ± 333.3 , 328.66 ± 173.20 and 10 ± 5.56 , respectively. In the FVEQ2 group, the urea, creatine, uric acid, ALP, ALT and AST were 4.66 ± 2.51 , 0.1 ± 0.05 , 8.26 ± 5.19 , 622 ± 346.3 , 411 ± 192.22 and 3.66 ± 1.15 , respectively. In the FVEQ3 group, the urea, creatine, uric acid, ALP, ALT and AST were 6.23 ± 3.46 , 0.01 ± 0.09 , 10.5 ± 5.77 , 999.6 ± 569.5 , 785 ± 545.03 and 5.33 ± 2.88 , respectively. The findings showed significant ($p < 0.05$) increases in blood creatinine, urea and uric acid levels in the FVEQ groups. Liver enzymes, ALP, ALT and AST, revealed significant ($p < 0.05$) increases in the FVEQ groups (Table 1).

Table 1: Blood kidney and liver function parameters of quails after exposure to formalin vapor.

Parameters	Control group	FVEQ1 group	FVEQ2 group	FVEQ3 group
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	Mean $\pm$ SEM	10 days	20 days	30 days
Urea	4.13 $\pm$ 0.23	4.66 $\pm$ 0.3	5.08 $\pm$ 0.80	6.23 $\pm$ 0.68
Creatinine	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0	0.1 $\pm$ 0
Uric acid	10.36 $\pm$ 1.50	8.76 $\pm$ 0.70	8.26 $\pm$ 0.6	8.20 $\pm$ 0.4
ALP	275.77 $\pm$ 17.21	586 $\pm$ 55.7	622 $\pm$ 69.5	999.6 $\pm$ 87.6
ALT	274.44 $\pm$ 8.23	328 $\pm$ 60.3	411 $\pm$ 84.4	785 $\pm$ 265
AST	13.16 $\pm$ 0.7	10 $\pm$ 5.7	5.33 $\pm$ 0.7	3.33 $\pm$ 1.5

RT-qPCR result

Our study revealed that the RT-qPCR amplification plots of the *p53* gene of the liver and kidney tissue were differently observed. Threshold Cycles (Ct) numbers of expression between groups of three groups that were exposed to 5% of formalin gas and control (Figures 1 and 2) at different days. The melting peaks analysis of the RT-qPCR primer were shown high specificity of *p53* gene expression without non-specific products and the melting peak ranged from 70°C to 80°C (Figures 3 and 4). This result indicated that 5% of formalin gas potentially increased the *TP53* expression in the liver and kidney tissue ascending with exposure days. The liver and kidney tissue in the third group illustrated that expression of *TP53* is significantly ($P < 0.05$) higher than the other groups.

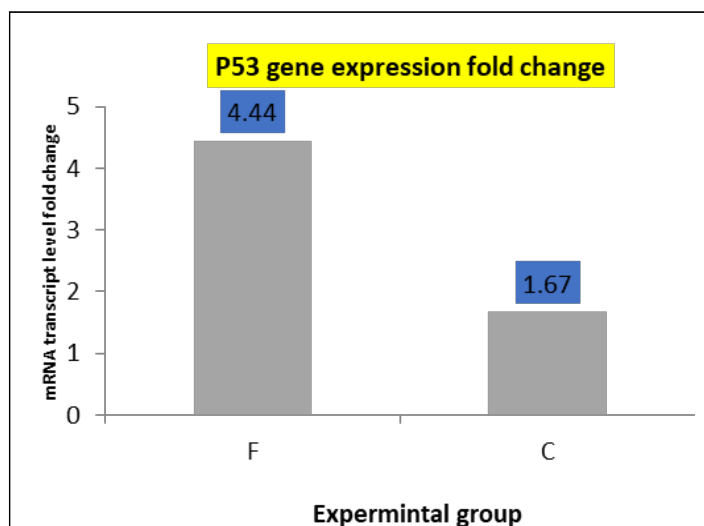


Figure 1: Gene expression of *TP53* of the kidney shows the mRNA *p53* levels in the exposure group (F) was the highest levels compared to the control group (C).

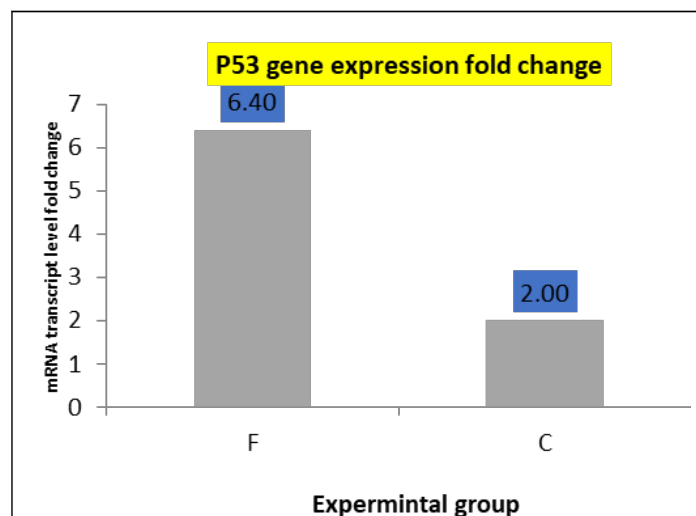


Figure 2: Gene expression of *TP53* of the liver shows the mRNA *p53* levels in the exposure group (F) was the highest levels compared to the control group (C).

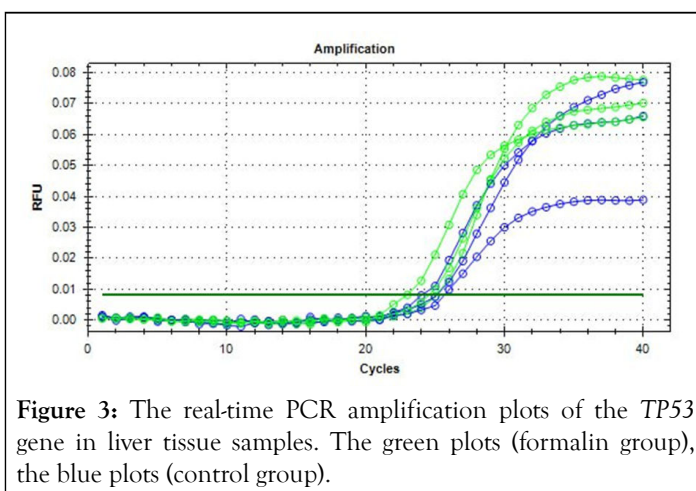


Figure 3: The real-time PCR amplification plots of the *TP53* gene in liver tissue samples. The green plots (formalin group), the blue plots (control group).

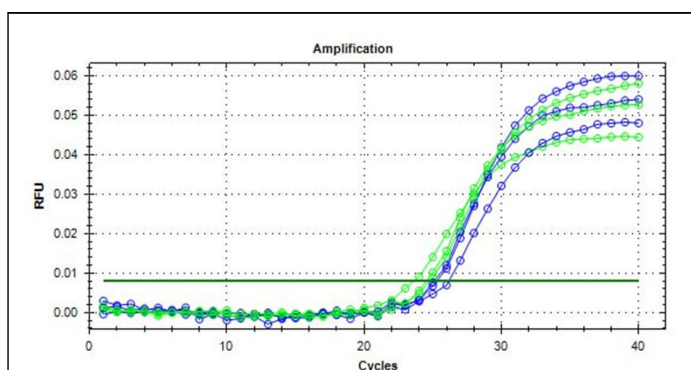


Figure 4: The real-time PCR amplification plots of the *TP53* gene in kidney tissue samples. The green plots (formalin group), the blue plots (control group).

Histological features

The histological features of the kidneys in Japanese quail were found to be like those observed in poultry and other species, both in terms of general histological details and histochemical properties. The control group of quail exhibited kidney tissue sections that displayed a consistent structure, with an enclosing capsule and a well-distributed arrangement of glomerulus and tubules (Figures 5A and 5B). The illustration in Figure 5C demonstrates the anatomical structures of Bowman's capsule and Bowman's fissure, which enclose the glomerulus within the renal corpuscles. The morphology of the proximal convoluted tubule exhibited a longitudinal and rounded configuration. On the other hand, the distal convoluted tubule (Figure 5D) exhibits a convex shape. The collecting ducts serve as the boundary point between the dense and thin limbs of Henle, as depicted in Figure 5E.

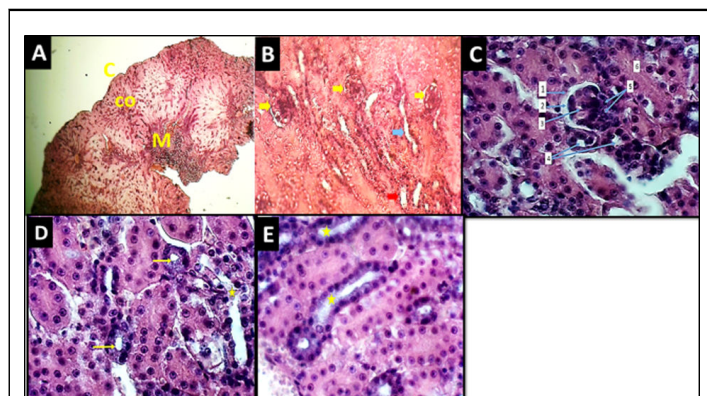


Figure 5: Microscopic section of healthy quail kidney (control group). A) It shows capsule, cortex and medullary con. H and E stain 40X; B) Renal corpuscle (yellow arrow), proximal convoluted tube (red arrow), distal convoluted tube (blue arrow) H and E 100X; C) 1-renal corpuscles, 2-space, 3-glomerula, 4-DCT, 5-mesangial cells and 6-PCT H and E 200x; D) Proximal convoluted tubules (yellow arrow) and Henle loop (yellow star) H and E 200X; E) Collecting tubules (yellow star) H and E 400X.

In the control group, the microscopic liver sections revealed normal hepatic tissue structure. There was no evidence of degenerative or necrotic changes. The parenchyma of the quail

liver appeared to lack lobular structures. It was surrounded by a slender capsule of loose connective tissue and mesothelium (Figure 6A). In a longitudinal micrograph of the liver tissues, the hepatocytes appeared as hepatic lines and were arranged in pairs between the hepatic sinusoid and the central vein (Figure 6B). There are numerous sizes and shapes of hepatocytes, with polyhedral being the most common. Each hepatocyte contained one or two typically large, spherical and eccentric nuclei and possessed a dark ovoid nucleolus. Figure 6C depicts sinusoids lined with flattened endothelial cells, including erythrocytes and macrophages (Kuffer cells). Four to six hepatocytes, bile canaliculi and intralobular bile ducts appeared in the transverse orientation (Figure 6D). The portal region of the liver displayed interlobular arteries, veins and the bile duct. Extracts of cuboidal tissue bordered the interlobular bile duct. The portal area branches were surrounded by smooth muscle fibers and lined with endothelial cells. Abundant connective tissue supported the portal tracts (Figures 6D and E).

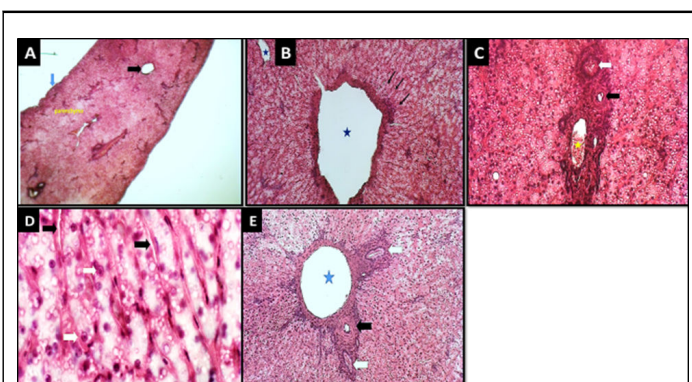


Figure 6: Microscopic section of healthy quail liver (control group). A) Capsule (blue arrow), central vein (black arrow) and parenchyma. H and E 40X; B) Central vein (blue star) and hepatocytes (black arrow). H and E 200X; C) Central vein (yellow star), hepatic artery (black arrow) and intralobular duct (white arrow). H and E 200X; D) Nucleus of hepatocyte (white arrow), sinusoid (black arrow) and Kuffer cells (yellow arrow). H and E 400X; E) Portal area; central vein (blue arrow), hepatic artery (black arrow) and interlobular duct (black arrow).

Histopathologic features

In the FVEQ groups, the most frequently observed features in kidneys were shriveled and ruptured glomeruli with leukocyte infiltrations in renal tubules, degenerated tissue and congestion of renal glomerulus with hemorrhage in the capsule (Figure 7A). In addition, there were epithelial hyperplasia, crowding of epithelial nuclei, hypereosinophilia, degeneration and epithelial cell loss in the proximal and distal tubules (Figure 7B). Moreover, there were thickness in the renal corpuscles and glomeruli (Figure 7C) and Henle loop of the medullary cone (Figure 7D).

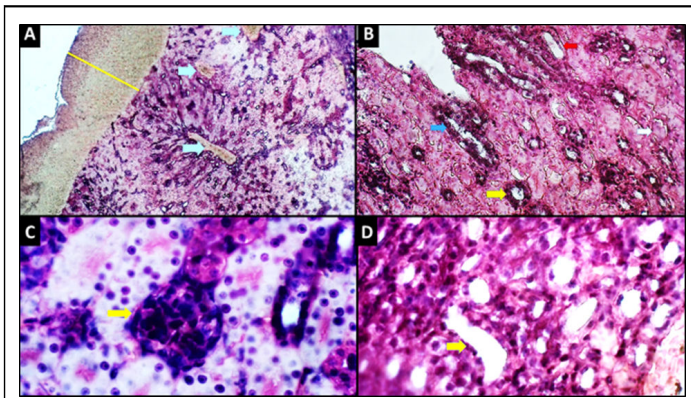


Figure 7: Histopathological section of kidneys in formalin-vapor-exposed quails. A) Congestion of capsule (yellow line) and parenchyma (blue arrow). H and E 100X; B) Hypertrophy and irregular epithelial cells of proximal and distal convoluted tubules. H and E 200X; C) Thickness of renal corpuscles and glomeruli (yellow arrow). H and E 400X; D) Thickness of the Henle tubules (yellow arrow). H and E 400X.

In the FVEQ groups, the most frequently observed features in liver sections were increases in the parenchymal aggregation of lymphoid cells. There were lesions, such as congestion in the central vein, tissue degeneration, sinusoid enlargement, minor hemorrhages and increases in the Kuffer cells (Figures 8A and B). There were amyloid depositions in hepatocyte spaces, which were squeezed and atrophied, creating intercellular gaps and reducing cell compactness (Figure 8C).

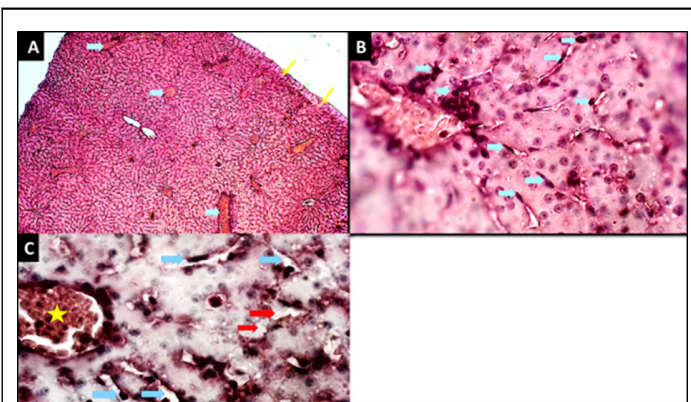


Figure 8: Histopathological section of liver in formalin-vapor-exposed quails. A) Hypertrophy and congestion of capsule (yellow arrow) and hepatocytes and central vein (blue arrow). H and E 40X; B) Increases in Kuffer cells (blue arrow). H and E 400X; C) Dilated central vein (yellow star) and sinusoid (blue arrow), deposition of amyloid (red arrow) and spaces between hepatocytes is compressed and atrophied. H and E 1000X.

DISCUSSION

Many previous studies stated that exposure to formalin at different concentrations with little ventilation and closed places caused reduced oxygen levels in the blood and led to the body's increased metabolic activity, which would require more energy [14]. Formalin exposure for a long time would be produced a significant expansion of blood vessels to resist the lack of oxygen within the body's tissues. Therefore, our study was designed to

identify the effect of 5% of formalin gas on gene expression of *TP53* in the liver and kidney of quail.

Our result found that the quails under the effect of 5% of formalin gas conditions showed clear clinical signs of anxiety, depression and high stress, which agreed with other research [15], who confirmed that exposure of the formalin gas could lead to lack of oxygen in the tissue of the body and depression signs during the exposure period of formalin.

Our findings determined that there was a significant increase in the common enzymes of the ALP ALT concentrations and a decrease in the AST concentrations compared to the control and this might be an indicator of liver damage to varying degrees. These increased activities of these enzymes of the liver are considered a sensitive indicator of liver damage, which agrees with our findings [16]. Also, these changes in liver enzymes might be reflected in the deleterious effects of formalin gas on the hepatocyte function of the liver, which were absent in the control group.

Moreover, reported a decrease in the activity of liver enzymes, including ALP, AST and ALT, in quail during given liquid formalin in drinking water at higher doses [17]. So, this previous study and our study delivered that the different routes of exposure the formalin liquid and gas would be affected liver functions involving ALP, AST and ALT enzymes. Consequently, our result observed significant differences between experimental groups of liver enzymes and control.

A previous study described that pathological changes in the glomeruli of kidneys of the quails would be affected filtration rates and impaction renal tubules reabsorb the urea and leads to dehydration and fever, which stimulate tubular sodium reabsorption, increasing urea/creatinine ratio and uric acid [18]. This result was detected by our study, which detected the disturbances of kidney functions after increasing exposure days of 5% of formalin gas and strong disturbances in the levels of the urea, uric acid and creatinine level of blood in the experimental groups of quails. Then this is definitely impaction of formalin gas on kidney functions.

Also, the high toxicity of formalin resulting from directed exposure or mixed with some formalin products could affect many organs and systems in the body, especially the trachea and lungs of the respiratory system [19]. Thus our findings found great pathological changes in the tissue of the trachea and lungs of the quails.

As a result, continuous exposure to formalin gas regularly may lead to an increased risk of lung cancer. This is the same as the findings of in humans, animals and quail. Therefore, this study has studied the effect of 5% of formalin gas on gene expression *TP53* gene in the trachea and lung quails tissue on different days. The pathways of P53 play a role in trachea and lung cell apoptosis and are accountable for the P53 stage [20]. These results indicated that formalin-dependent apoptosis causes liver and kidney cells' *TP53-independent* phase of apoptosis because there was a significant increase in level concentrations of the P53 in the liver and kidney tissue of the quails consistent with days' exposure of the formalin gas. Moreover, these outcomes highlight the physiological effects of time-dependent formalin

gas irritation, including the specific time intervals between two exposure times for a long period.

This study observed that 5% of the formalin gas would obviously lead to large liver and kidney function disturbances. Interestingly, a long exposure time of 5% of formalin gas would significantly increase the expression of *TP53* regularly in the liver and kidney, which could be evidence of abnormal cellular responses in tissue and risk factor for cancer diseases. Therefore, *P53* is the best biomarker for the effect and risk of formalin gas on the digestive and urinary system of humans and animals.

CONCLUSION

The findings of this study indicate that prolonged exposure to formalin vapor may result in adverse effects on the histological composition and functional performance of the kidney and liver in quail subjected to formalin vapor treatment. Hence, it is advisable that all individuals who handle formalin adhere to time-limited exposure guidelines and work in adequately ventilated environments.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the current study.

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