Prolonged Formalin Exposure on Liver and Kidney Function Associated with TP53 Gene Expression in Quail Birds (*Coturnix coturnix*)

Iman Ibrahim Al Hacham 1, Abdulrazzaq B. Kadhim 2*, Eman F. Albaghdady 3

Abstract
Background: This study was the first to highlight the deleterious effect of formalin gas on gene expression of the tumor protein TP53 in the digestive and urinary system. This experiment was conducted according to animal ethics and regulations of the College of veterinary medicine/the University of Al-Qadisiyah. Method: 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. 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, Conclusion: that P53 might be considered a suitable biomarker of molecular for formalin impaction in humans and animals especially the birds.

Keywords: Formaldehyde exposure, TP53 gene expression, Liver and kidney function, Quail model, Histopathological changes

Introduction
Formaldehyde is commonly found in various sources such as cigarette smoke, automobile emissions, fuel oil, natural gas, exhaust from vehicles powered by fossil fuels, furniture, and the fumes emitted by chipboard paint. Disruptions in formaldehyde exposure can induce cancer, as the P53 protein, which functions as a transcription factor, controls gene expression in cells and prevents cancer formation (Aggarwal and Garg, 1983; Brown, 1996). Disinfectants and sterilizers containing formaldehyde are frequently used by educational institutions, higher education establishments, and biological, forensic, and pathological laboratories to preserve tissues, surgical specimens, organs, and viscera (Cheney and Collins, 1995; Babar et al., 2001). Studies have shown that broilers exposed to formalin in feed exhibit reduced feed intake and body mass, local necrosis, and crop and intestinal hemorrhage (Rs, 1983; Egwurugwu et al., 2018). Formic acid in the bloodstream can cause significant metabolic acidosis and impair liver function by inhibiting cholinesterase, succinate oxidation, and anaerobic glycolysis (Botsetti et al., 2008; Gupta et al., 2019). The P53 protein, encoded by the TP53 gene in humans, plays a crucial role in regulating cell growth and preventing cancer. This study aimed to identify the importance of TP53 in mitigating the pathological effects of formalin on body tissues, particularly the liver and kidneys, and proposes TP53 as a vital diagnostic criterion.

Significance
This study demonstrated a prolonged formaldehyde exposure in quails. It caused liver and kidney damage and affected gene expression as potential cancer risks.

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Materials and Methods

Study design:
Sixteen quail birds were used for this experiment, conducted in accordance with the animal ethics rules and regulations of the College of Veterinary Medicine at the University of Al-Qadisiyah, under approval number P.G, No. 1890 in 2020. The experiment took place from September to November 2022, following international guidelines for the care and use of animals. Healthy, mature Japanese Quail (Coturnix japonica) were raised in optimal environmental conditions with proper feeding, lighting, and ventilation on the farm.

The quails were divided into four groups. Three groups were exposed to 5% formalin gas twice daily for two hours each in the morning and evening over 30 days. The first group (n=4) was euthanized after ten days, the second group (n=4) after twenty days, and the third group (n=4) after thirty days. The fourth group served as the control, kept under the same conditions without formalin gas exposure.

Serum, liver, and kidney tissue samples were collected from each group throughout the experiment. Serum was analyzed to assess liver and kidney function, measuring Aspartate Aminotransferase (AST), Alanine Transaminase (ALT), and Alkaline Phosphatase (ALP) using a Beckman Coulter AU480 and Biorad D10 (both from California, USA). Major organic compounds such as creatinine, urea, and uric acid were detected using UV kinetic methodology in commercial kits from Human Gesellschaft für Biochemical und Diagnostics mbH (Wiesbaden, Germany), which identified different levels of liver enzymes and kidney function markers.

Liver and kidney specimens were preserved in 10% neutral formalin for histological examination. Additional samples were stored in Trizol (SRCr Green-Zol reagent, Qadisiyah, Iraq) for real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

Liver and kidney enzyme profile
The test tubes containing blood were positioned at an inclined angle and left at 32°C for 30 minutes. Serum was then isolated from the coagulated blood through a centrifugation process at 3000 rpm for 20 minutes, followed by an additional centrifugation at the same speed for 10 minutes. The supernatant was collected using a micropipette and transferred into an Eppendorf tube, which was subsequently stored at -20°C.

In this study, serum samples were used to assess hepatic and renal function by measuring the levels of Aspartate Aminotransferase (AST), Alanine Transaminase (ALT), and Alkaline Phosphatase (ALP). The analysis was conducted using Beckman Coulter AU480 and Biorad D10 instruments, both manufactured in California, USA. The concentrations of major organic compounds, including urea, creatinine, and uric acid, were determined using UV kinetic methodology with commercial kits from Human Gesellschaft für Biochemica und Diagnostica mbH, Germany. These kits effectively detected variations in the levels of liver enzymes and kidney function markers such as creatinine, urea, and uric acid.

Extract of RNA and cRNA synthesis
Total RNA was extracted from liver and kidney tissues using the Trizol reagent kit (SRCr Green-Zol reagent, Qadisiyah, Iraq). A 250 mg sample of liver or kidney tissue was placed in a 1.5 ml Eppendorf tube and homogenized using an electric homogenizer (Fisherbrand, England). Then, 200 μl of chloroform was added, mixed, and the mixture was incubated on ice for 5 minutes. The lysate was then centrifuged at 10,000 x g (approximately 9700 rpm for rotors with a 9.5 cm radius) for 15 minutes at 4°C. The supernatant was transferred to a separate Eppendorf tube, and 500 μl of 99.8% isopropanol (ISA, UK) was added and mixed. The mixture was incubated at 4°C for 10 minutes, while the pellets were neglected. The lysate supernatant was then centrifuged again at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded, and 1 ml of 80% ethanol was added to the pellets and mixed using a vortex. The mixture was centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was discarded, and the pellets were left to air dry.

For cDNA synthesis, the total RNA was incubated with DEPC water to remove any trace amounts of DNA (Promega Company, Madison, Wisconsin, USA) for two hours. The RNA was then reverse transcribed into cDNA following the instructions of the DiaStar™ OneStep RT-PCR Kit (BKMAN Biotechnology Co. Ltd, China). The cDNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific NanoDrop One Microvolume UV-Vis Spectrophotometer, USA), diluted, and normalized to the same concentration for all samples for subsequent RT-qPCR analysis.

RT-qPCR Technique
RT-qPCR was employed to detect the quantification levels of the cosinophil 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_01585941.1, forward primer: AGCCGGCTTTTTAAAACGTGC, and reverse primer: CAAAATGTGCTCTGGAAGAAGG), and for housekeeping gene (size: 77 bp, code: XM_015873412.2, forward primer: TGCTGGCATTTGACGTGGAAT, and reverse primer: CACGGTTGTGTAATCCAAAATC). 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


4°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 60–95 °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 5–6 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 2ΔΔCT
Delta C(T) Method (2 –ΔΔCT method) (Hellemans et al.2007). 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 ≤ 0.05. All the
results were expressed as mean ±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 ± 0.66gm, while the treated groups had average weights of 188
± 1.86gm, 154 ± 0.04gmgm, 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 that at
the end of the third week, 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±192.2, 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±659.5, 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.2).

**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
(Fig.1,2) at different days. The melting peaks analysis of the RT-
quPCR primer were shown high specificity of p53 gene expression
without non-specific products, and the melting peak ranged from
70°C to 80°C. (Fig.4,5). 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.

**Histological features**
The histological features of the kidneys in Japanese quail were like
those observed in poultry and other species, 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 corpuses. 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 5, E.

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 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
Table 1. The biochemical Measurements of Blood kidney and liver function parameters of quails after exposure to formalin vapor. The values with different letters refers to the significant differences (P<0.05).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>group1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>10 Days</td>
<td>20 Days</td>
<td>30 Days</td>
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<tr>
<td>Urea</td>
<td>4.13±0.23 a</td>
<td>4.66±0.3 b</td>
<td>5.08±0.80 c</td>
<td>6.23±0.68 d</td>
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<td>Creatinine</td>
<td>0.2±0.1 a</td>
<td>0.1±0.0 b</td>
<td>0.1±0 b</td>
<td>0.1±0 b</td>
</tr>
<tr>
<td>Uric acid</td>
<td>10.36±1.50 a</td>
<td>8.76±0.70 b</td>
<td>8.26±0.6 c</td>
<td>8.20±0.4 c</td>
</tr>
<tr>
<td>ALP</td>
<td>275.77±17.21 a</td>
<td>586±55.7 b</td>
<td>622±69.5 c</td>
<td>999.6±87.6 d</td>
</tr>
<tr>
<td>ALT</td>
<td>274.44±8.23 a</td>
<td>328±60.3 b</td>
<td>411±84.4 c</td>
<td>785±265 d</td>
</tr>
<tr>
<td>AST</td>
<td>13.16±0.7 a</td>
<td>10±5.7 b</td>
<td>5.33±0.7 c</td>
<td>3.33±1.5 d</td>
</tr>
</tbody>
</table>

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).

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

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

Figure 7. Histopathological section of kidneys in formalin-vapor-exposed quails. A. Congestion of capsule (yellow line) and parenchyma (blue arrow). H&E 100X. B. Hypertrophy and irregular epithelial cells of proximal and distal convoluted tubules. H&E 200X. C. Thickness of renal corpuscles and glomeruli (yellow arrow). H&E 400X. D. Thickness of the Henle tubules (yellow arrow). H&E 400X.
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&E 40X. B. Increases in Kuffer cells (blue arrow). H&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&E 1000X.
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 (Figure 6D and E).

**Histopathologic Features**

In the FVEQ groups, the most frequently observed kidney features were shrunken and ruptured glomeruli with leukocyte infiltrations in renal tubules, degenerated tissue, and congestion of renal glomeruli 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). 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 vain, tissue degeneration, sinusoid enlargement, minor hemorrhages, and increased Kuffer cells (Figure 8A and B). There were amyloid depositions in hepatocyte spaces, which were squeezed and atrophied, creating intercellular gaps, and reducing cell compactness (Figure 8C).

**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 (Koppel, et al., 1990). 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 (Mathai, et al., 1995). 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 (Peckham, 1980), 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. The increased activity of these enzymes in the liver is considered a sensitive indicator of liver damage (Restani, and Galli, 1991), which agrees with our findings. 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, (Roche, et al., 1996) 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. So, this previous study and our study delivered that the different routes of exposure to formalin liquid and gas would affect 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 (Saclarides et al., 1996) and leads to dehydration, and fever, which stimulate tubular sodium reabsorption, increasing urea/creatinine ratio and uric acid (Sarnak et al., 1996). 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 impacts 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 (Soffritti, et al., 2002). 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 Williams, 1980; Usanmaz et al., 2002) 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 quail tissue on different days. The pathways of P53 play a role in trachea and lung cell apoptosis and are accountable for the P53 stage (Heck et al., 1985). 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 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 factors for cancer diseases. Therefore, P53 is the best biomarker for the effect and risk
of formalin gas on humans and animals' digestive and urinary systems.

Conclusion
This study conclusively demonstrates that prolonged exposure to formalin vapor has significant detrimental effects on the liver and kidneys of quails. The experimental findings indicate that formalin exposure leads to marked histopathological changes, including acute hepatitis and nephritis, as well as biochemical disturbances characterized by elevated liver enzymes (ALP, ALT, and AST) and increased levels of urea, creatinine, and uric acid. Moreover, the study reveals that formalin exposure significantly upregulates TP53 gene expression in the liver and kidney, suggesting that TP53 could be a crucial biomarker for formalin-induced cellular stress and potential carcinogenesis. These findings highlight the importance of monitoring formalin levels in environments where it is prevalent to prevent adverse health effects in animals and humans. This research underscores the necessity for stringent regulations and safety measures to mitigate formalin exposure and its associated risks.

Author contributions
I.A.H., A.B.K., E.F.A. wrote, drafted, reviewed, and edited the paper. All authors have read and agreed to the published version of the manuscript.

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Competing financial interests
The authors have no conflict of interest.

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