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RESEARCH ARTICLE

Potential Risk Assessment using Morphometric and Hepatic histopathological studies on developing Chicken Embryos caused due to Reclaimed water samples

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Abstract

Untreated effluents released from hospitals and industries may contain hazardous toxins that possess a long-term risk to human health and environment. Sometimes, due to negligence of people and inefficacy of treatment plants, effluents find their way to municipal supply of potable water causing significant health effects. To determine the toxicological risk possessed by these effluents, morphometric and histopathological studies were performed in Chicken embryos with an attempt to evaluate and compare the developmental defects caused by four reclaimed water samples collected from their respective treatment plants, in Jaipur, Rajasthan. The fertilized eggs on Day 4 of incubation were immersed in water samples for 60 minutes. On the 18th day of incubation, eggs were break open that showed abnormal morphology such as sparse body hair, gastrochisis, body cysts, edema, etc. while the group treated with tap water showed normal morphology. The histopathology of liver showed damage in the form of necrosis, hemorrhage and dilated blood vessels in treatment groups.The highest mortality was observed in Sewage followed by Hospital, Industry, and Recreational Pond sample. The findings point towards disquality of water and that its monitoring must be ensured from time to time. before it is disposed of into municipal supply.

Keywords: Chicken Embryos, Developmental Defects, Reclaimed Water, Histopathology, Morphometric

Highlights

1. Poor monitoring of reclaimed water before its disposal can cause harmful effects
2. Unmonitored Reclaimed water samples can even cause developmental defects
3. Morphological defects during development and liver toxicity are observable changes of the treated groups
4. Utmost care must be taken to ensure Efficiency of treatment plants.

Introduction

Water is an essential natural resource gifted to us by mother earth and is required by every creature. It is considered as a universal solvent because of its ability to dissolve maximum substances. However, this ability makes it prone to pollution. Any water contaminated by human use is referred to as wastewater. It could be domestic or from commercial, agricultural or industrial sources. It is estimated that approximately 80% of the water returns to the ecosystem without undergoing proper treatment. This wastewater consists of 99.7% water and 0.3% dissolved and suspended particles which majorly consist of decaying organic matter and debris, excessive nutrients such as phosphorous and nitrogen, inorganic compounds such as chloramines, metals such as mercury, lead, etc., disease-causing pathogens, and other substances such as pharmaceuticals.

The overwhelming population growth along with the water crisis is a challenge for economic and human development. Reclaimed water could act as cost effective alternative for water sources and a solution for the growing water crisis. However, if the wastewaters are not properly treated then the environment and the human health both are negatively impacted. The impacts may include harm to aquatic organisms and wildlife populations, eutrophication, restrictions on recreational water usage and contamination of the drinking water.

The pharmaceuticals, nanoparticles, organic and inorganic constituents are not well known in many causes and their unknown profile imposes a significant hazardous risk on public health. Risks to the environment and human health could result from the minute pollutants in reclaimed water (Deng *et al.*, 2019).

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More consideration needs to be given to the ecological risk assessment related to the pollutants in recovered water. However, the existing methods for chemical analysis cannot detect all the pollutants present in reclaimed samples and at the same time it is crucial to assess the combined possible effects of the reclaimed water. Therefore, a thorough examination of biological toxicity is a useful method for assessing the ecological concerns in aquatic systems.

Human consumption of the untreated liquid effluents could lead to ingestion of toxins and disease-causing pathogens which may cause serious illnesses such as diarrhea, cholera, typhoid, and polio. Ingestion of chemical toxins also possess a risk of altered brain function, damage to immune and reproductive systems, hormone disruption, and cardiovascular and kidney problems (Dickin *et al*., 2016; Garg *et al*., 2022).

To address this concern, wastewater treatment and pathogen eradication measures should be adopted that require cooperation in wastewater treatment policy supported by field research, cost-effective analysis, and feasibility studies (Wasi *et al*., 2013).

An ideal technical method that can reflect the overall quality of water is the toxicity evaluation in Chicken. However, the conventional adult chicken toxicity test is time-consuming and labor-intensive, making it unsuitable for real-time monitoring of recycled water. Therefore, the objective of the study was to evaluate the performance of Chicken embryos exposed to different reclaimed water samples;specifically comparing the developmental rates, frequencies of developmental deformities, size and length of embryos and hepatic histopathological effects with reference to embryos that received tap water samples.

Material and Methods

Water Samples

Four samples of reclaimed water were collected from the treatment plants installed to remove pollutants, situated at different locations of Jaipur. Samples were collected from the Sewage plant, Hospital plant, Industry (textile industry) and Recreational Park situated in different locations of the Jaipur city. Samples were taken from a treatment plant discharge point and placed in previously cleaned and sterilised glass bottles at the busiest times for that treatment plant, which is in the premonsoon period between 11:00 A.M. and 1:00 P.M. Normal Tap water was used as a control in the study. The sample waters were used in the same form as they were collected i.e., without any dilution.

In ovo study

The SPF (Specific Pathogen Free) chicken eggs weighing 61± 3 g and age "0" days were procured from Immunetic Lifesciences Private Limited, Himachal Pradesh, which is a leading poultry company and one of the largest veterinary diagnostic laboratories. The eggs were procured after obtaining approval from the Institutional Animal Ethical Committee vide its approval number IISU/IAEC/2021/I/3.

Eggs were wiped with disinfectant and were kept in an incubator maintained at 37.5° C and relative humidity 65-70% with their narrow ends facing downwards and rotated daily manually. Candling was done every alternative day to ensure the proper growth and development.

Chicken embryos in their early stages of development are a flat, discoidal shaped multicellular structure which is positioned at the animal pole on the yolk, enclosed within vitelline membrane and wrapped in thick sheets of albumen (Kotwani, 1998).

Live embryos were divided into two Groups: Group- I: Control and Group II: Treatment group. A clear presentation of the different treatment groups, their dosing and day of termination of experiment is given in Table 1.

On Day 4 of incubation the eggs were dipped for 60 minutes in the test samples respectively, after which they were air-dried and kept in the incubator till 18 days of incubation. In avian embryonic development there occur three critical periods which end approximately at Day 4, 11 and 19 of incubation. Up to Day 4 it is primary stage of organogenesis when initial development of organs has begun and by end of Day 4 most of the organ primordia are developed. In avian developmental studies, it is a standard practice to treat the eggs at or beyond the Day 4 of incubation . At this stage the viability of eggs can be clearly established as the developing vasculature is visible and if the embryos have passed the initial critical period their rate of survivability is high and the results of the study can then be easily obtained.

The eggs after treatment were allowed to develop till Day 18 and then on Day 19 when the final critical period of embryos have finished (Hamburger and Hamilton, 1951) and ready to hatch, the eggs were break open manually ; the prehatched embryos were recovered for developmental studies. The parameters studied were mortality, weight of embryos and crown rump length (CRL). Embryos were examined for any external malformations with respect to abnormalities of the body, head, beak and deformities of limbs under the Stereoscopic dissecting microscope.

They were intermittently checked for mortality using the candle method, and the dead embryos were removed. Weight of the embryos was measured using digital weighing machine and Crown rump length was measured for each embryo by passing a thread from the root of the beak along the back to the tip of the coccyx and then measuring the length of the thread.

The animals were dissected to isolate the specimen of Liver. Equal sized portions of the liver were fixed in 10% neutral buffered formalin solution for histopathological analysis.

Histopathology

The Fixed liver specimen were treated overnight in an automatic tissue processor (Make - Thermo Scientific) for dehydration, clearing, and impregnation. Serial pieces of 5 µm thickness were cut using a microtome (Make - Leica) and implanted in paraffin blocks using an embedding station (Make - Microm). Hematoxylin and Eosin staining of the sections was done using an autostainer (Make - Leica). Under light microscopy, the mounted specimen were examined and scored (Make - Labomed).

Statistical Tests

Statistical analysis was done using SPSS Software Version 22. The wet body weight of the chick was statistically analyzed using Student's t-test, calculations were done at d=0.05 levels and significance of differences were attributed at $P<0.05$, $P<0.01$ and $P<0.001$.

Results and Discussion

Mortality Percentage

The mortality (%) of embryos was determined by counting the number of dead embryos which died normally or due to the exposure of the reclaimed water. The number of both the dead and surviving embryos was recorded (Table 2). Relatively low mortality percentage was observed in the control group eggs as compared to the treatment group eggs. In treatment group eggs the highest mortality was observed in the eggs treated with reclaimed water from Sewage followed by Industry and Hospital and the least mortality was observed in group of eggs that were treated with reclaimed water from Recreational Pond.

Morphological Observations

The morphology of the representative control group chick at Day 18th of incubation is shown in Figure A. At this stage of development, the body of the chick was well covered by down feathers. The head region contained enlarged eyes and a hard beak which had become blunt at its tip. The forelimbs (wings) of the chick were well developed and showed normal skeletal structural parts i.e., humerus, radius, ulna, digits and metacarpus. The hindlimbs (legs) too showed normal parts i.e., femur, tibia, fibula, metatarsus and four digits made up of phalanges.

Treatment of eggs with reclaimed water resulted in retarded growth and development of embryos. The defects that were mostly visible in the chicks of treatment groups were underdeveloped embryo with short length, reduced body weights and underdeveloped or crooked legs (Fig. 1 D-F). Besides these embryos also displayed adermia i.e., absence of body hair or sparse or irregular occurrence of body hair (Fig. 1 D-F). Cyst like structure (Fig. 1 C) were also observed in some embryos. Gastrochisis (Fig. 1 D) a birth defect where an opening develops in the abdominal wall through which body vital organs such as intestine protrude out of the body was also observed in treatment group embryos. Maximum toxicity and poor development of the embryos was found in reclaimed water collected from Sewage.

Table 1. Experimental groups used in the study

Groups	Test substance	Day of commencement of experiment	Day of termination of experiment
I. Control Group	Normal Tap Water	Eggs Immersed in 1 litre of Sample waters for 60 min on day 4 of Incubation	Embryos recovered on day 18th of Incubation
II. Treatment Group			
A	Reclaimed Sewage Water		
B	Reclaimed Hospital Water		
C	Reclaimed Industrial Water		
D	Reclaimed Recreational Pond Water		

As compared to the other samples of reclaimed water, sample collected from Recreational Pond proved to be less toxic. 100% survival rate of the embryos with a slight reduction in the length and weight of the embryos was observed in this group as compared to the control.

Body weight

Fig. 2 displayed the changes in the body weight of embryos in different groups. There was a significant reduction in the body weight of the embryos of the treated group as compared with the control.

Crown –rump Length

Fig. 3 displayed the changes in the crown- rump length of embryos in different groups. The embryos of the Group II A, II B and II C showed highly significant shortening in the body length compared with the control group. However, Group II D embryos showed insignificant difference compared with the control group.

Table 2. Toxicity Assessment of reclaimed water in the chick embryos on 18th day of incubation (Treatment given on "4th" day of incubation)

Groups	Number of eggs/ treatments	Surviving embryos (%)	Mortality (%)	Surviving embryos with malformations		Average Body weight of Chick (gm)	Crown-rump length of Chick (cm)
				Percentage	Number		
I	20	95%	5%	10.5%	2	13.921±0.375	7.9±0.11
II A	20	65%	35%	76.9%	10	6.13±0.240*	5.3±0.06*
II B	20	80%	20%	62.5%	10	7.41±0.35*	4.2±.03*
II C	20	75%	26.6%	66.6%	10	7.717±0.358*	5.02±0.02*
II D	20	90%	10%	50%	9	11.605±0.272	6.6±0.05

*The values are expressed as mean ± standard error (S.E.) for wet body weight and crown rump length

Table 3. Incidence of malformations in surviving chick embryos treated with wastewaters on 18th day of incubation (Treatment given on "4th" day of incubation)

Deformities	GROUPS (n=20 eggs in each group)				
	I	II A	II B	II C	II D
Total number of deformed embryos	2	10	10	10	9
Unutilized yolk sac	0	8	4	0	0
Body cyst	0	5	0	0	0
Adermia	0	3	2	4	0
Sparse body hairs	0	3	2	5	0
Gastrochisis	0	7	3	0	0
Limb size reduction	2	7	0	8	2
Poorly developed claws	0	2	1	3	0
Runt (Underdeveloped embryo)	0	4	3	4	0

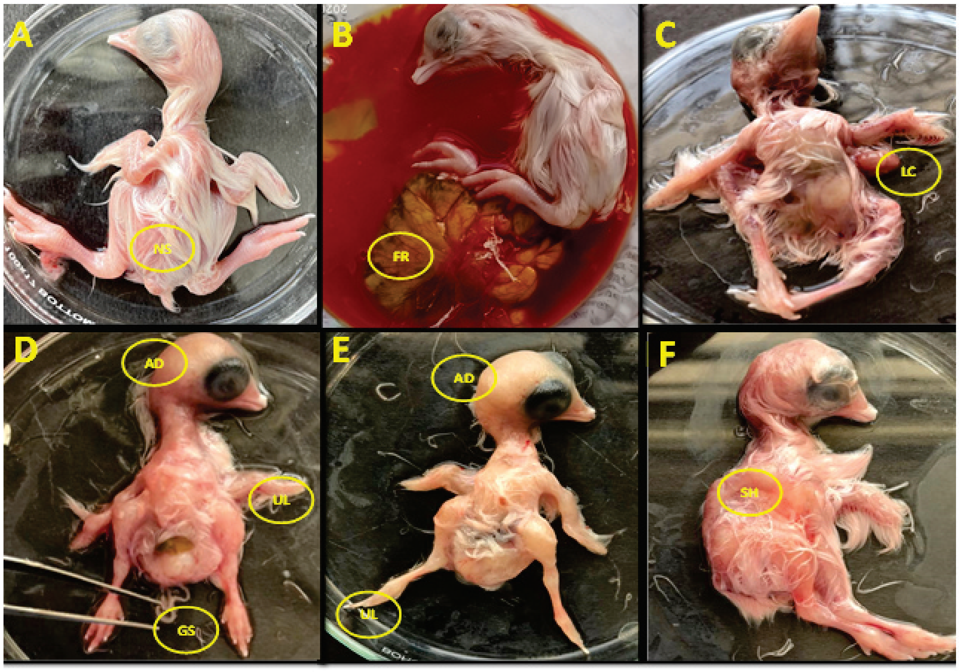

Fig.1. Morphological deformities observed due to Reclaimed water samples

A: Group I embryo showing ND – Normal Growth and Development
B and C: Group II A embryos showing FR – Failure of Yolk Sac Retraction; LC – Cyst like structure on lateral side of body; UL – Underdeveloped limbs
D: Group II B embryo showing AD – Adermia; GS – Gastrochisis; UL – Underdeveloped limbs
E: Group II C embryo showing AD – Adermia; UL – Underdeveloped limbs
F: Group II D embryo showing proper development and SH – Sparse body hair

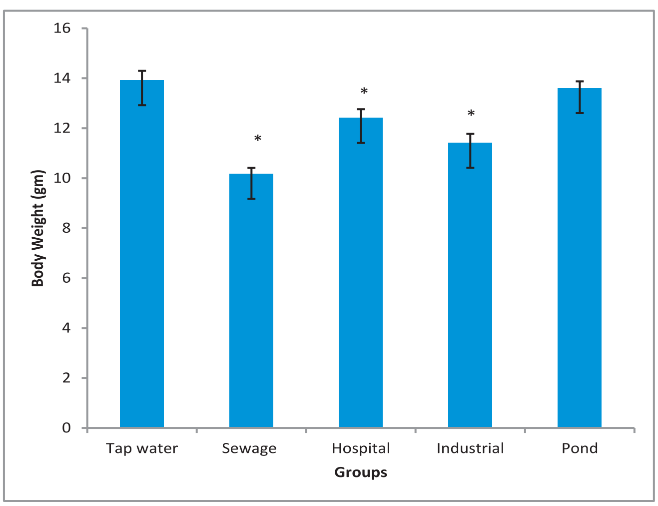

Fig. 2. A Graph displaying the body weight of various groups 18 days old chick embryos. Data is displayed as mean ± SE. The P value in comparison to the control group is indicated by an asterisks (*, **,***).

*⁼p≤0.05 (Significant), **⁼p≤0.01 (Highly Significant) and ***⁼p≤0.001 (Very Highly Significant).

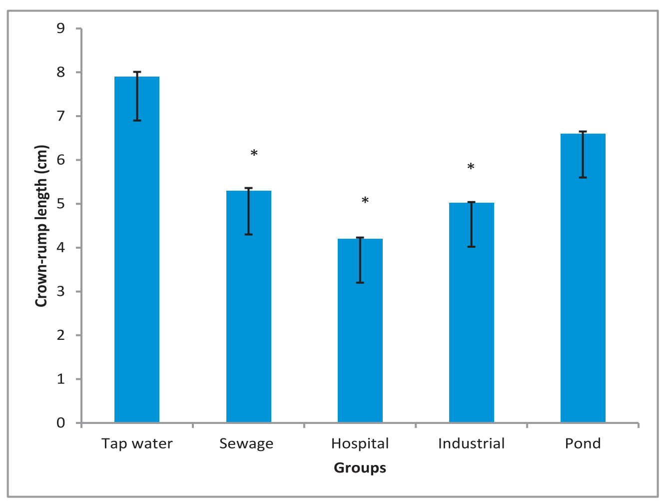

Fig. 3. A Graph displaying the crown-rump length of various groups of 18 days old chick embryos. Data is displayed as mean ± SE. The P value in comparison to the control is indicated as asterisks (*, **,***).

*⁼p≤0.05 (Significant), **⁼p≤0.01 (Highly Significant) and ***⁼p≤0.001 (Very Highly Significant).

Histopathological image of Liver tissue of control group showed no pathological changes. A thin layer of loose connective tissue covered the whole surface of the liver, and below it is a thin capsule of strong connective tissue that separates the liver into lobules and extends into the liver lobes. Hepatic lobules are composed of hepatic artery and portal vein interlobular branches, bile ductules, and less conspicuous lymphatic and nerve branches. The hepatocytes are arranged into these lobules. At the edge of each lobule are portal canals. Every lobule has a sizable central vein in the middle. Hepatic plates, which are irregularly oriented inward from the peripheral edge of each liver lobe towards the central vein, are formed by the hexagonal arrangement of the hepatocytes.

Histopathological images of the Liver tissue in treatment groups show damages like necrosis of hepatocytes, hemorrhage and dilations at some places. The damage to hepatocytes was observed to be maximum in Group II A (Fig. 4 B) followed by less severe damage in Group II B (Fig. 4 C). However, liver sections of Group II C (Fig 4 D) and II D (Fig 4 E) shows that the damage caused to liver was almost negligible in these groups and the hepatic architecture was quite similar to Control group (Group I).

Chicken embryos are considered as a model organism to study the biology of diseases, effects of environmental exposures, toxicological studies, and as a model to study the efficacy of pharmaceuticals. It presents an easily available, inexpensive, and self-sufficient model that displays similarities with human organ systems. (Maci *et al*., 1980; Hill and Hofmann, 1984).

Wastewaters are water that has been contaminated by human use and has the potential to cause severe risks to humans and to the environment. The effect of reclaimed water from different sources on chick embryos has been described in the present report. Studies on other groups of vertebrates have also been done where similar findings have been reported. In one of the studies, exposure to hospital wastewaters in *Xenopuslaevis* and

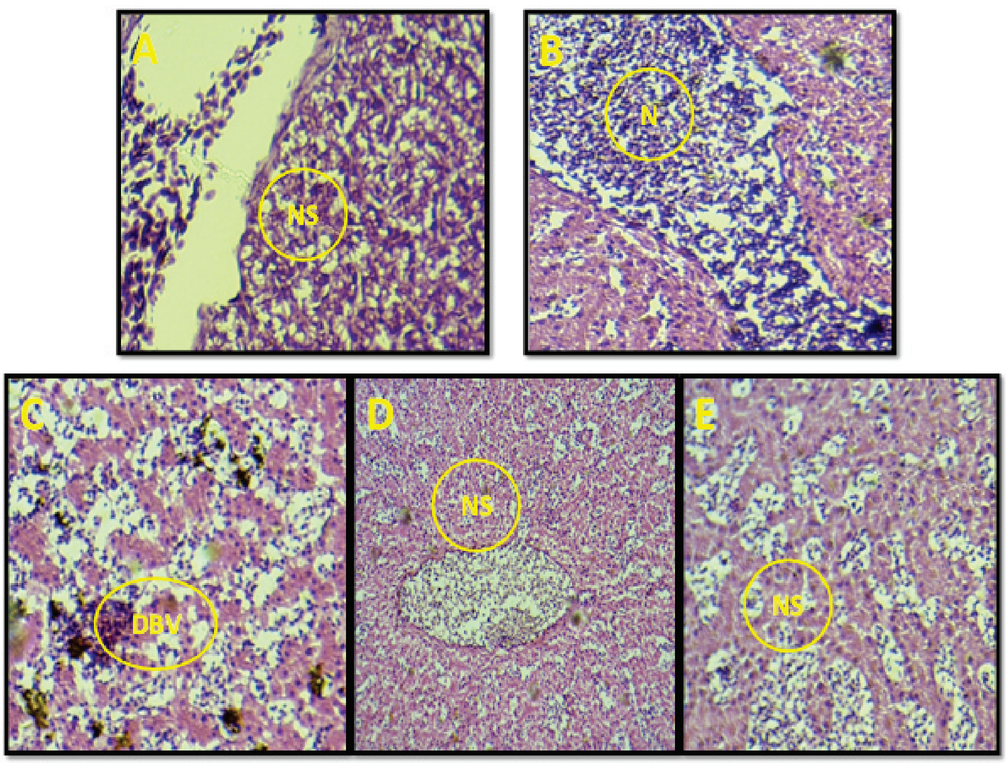

Fig. 4. Histopathology of Hematoxylin –eosin-stained sections of the Chicken embryo liver

A: Group I showing NS - normal hepatic and sinusoidal architectures
B: Group II A showing N - Necrotic areas
C: Group II B showing DBV - Dilated blood vessel
D: Group II C showing NS - near to normal sinusoidal structures
E: Group II D showing NS - restored sinusoidal structures and reduced necrosis

Lithobates catesbeianus, caused growth retardation along with diverse malformation such as microcephaly, cardiac and facial edema, eye malformations, and notochord, tail, fin, and gut damage (Alvarez *et al.,* 2018). Similarly, in embryos of fishes (*Fundulusheteroclitus, Pseudopleuronectes Americanus a*nd *Maronesaxatilis*) exposure to municipal wastewaters caused cardiovascular and skeletal defects and decreased hatch and larval growth (Weis *et al*., 1989).

The growing and widespread use of wastewaters in agriculture and other activities with or without proper treatment presents severe health risks which need to be addressed. Wastewaters pass through industries, homes, and other useful points they contain many pathogenic microorganisms, chemical residues, and other chemical substances such as metals, drugs, organic compounds, etc. (Kumar *et al*., 2014). These compounds in wastewaters possess a risk of acute and chronic toxicity, carcinogenicity, developmental disorders, and neurotoxicity (Koopaei and Abdollahi, 2017). Their concentrations are also increasing in the food chain and the environment. However, there are well-developed and sophisticated techniques for the treatment of wastewaters but they are not viable for a developing country due to technological barriers.

There are a large number of evidences which supports the fact, developing embryos are susceptible to several hazardous toxic substances (Amini *et al*., 2019; Samak *et al*., 2020). The various constituents present in the reclaimed water are one of such stressors, which interferes with the normal developmental phenomenon of the Chick and eventually prevents it from reaching its proper endpoints (Harnett *et al*., 2021). This study illustrates a similar instance.

In our study, the group of embryos treated with reclaimed water samples displayed developmental anomalies such as reduction in body length and weight, sparse body hairs, cysts on the body, gastrochisis, and failure of yolk sac retraction, crooked legs, and stunted growth. This confirms the damage caused by reclaimed water to the developing embryos. The liver's histological changes supported the preceding conclusions. Damage to the liver was observed in reclaimed water samples; necrotic areas and dilated blood vessels are clearly visible. The damage caused may be due to the significant altered levels of AST, ALT, ALP and triglycerides which are responsible for the damage caused to major vital organs (Khandia *et al*., 2020). Similar results were obtained by Bhardwaj *et al*., 2010, they revealed and increase in the levels of ALT, AST and glucose in the rats treated with Imidacloprid.

However, the exact mode of action of the sample water needs to be explored further. It can be speculated that the teratogenic potential of the reclaimed water constituents would likely have altered the cell signaling pathways and must have caused cells to undergo inappropriate apoptosis. It could also be speculated that reclaimed water has constituents that acts as a strong proliver toxicant and can produce methyl and chlorine free radicals after getting into the body via different pathways. This may lead to lipid peroxidation of the cell and endoplasmic reticulum membranes, which would subsequently harm the liver cell. Furthermore, the harmful components have the ability to lessen liver cancer, fibrosis, necrosis, and degeneration. Thus, in liver slices, we can see the infiltration of inflammatory cells, fatty degeneration, hazy swelling, ballooning degeneration, and hepatic necrosis. The soluble enzymes (ALT and AST) become more active when the structure and functional integrity of the liver cell membrane are destroyed. Subsequently, the liver cells release these soluble enzymes into the bloodstream, which raises serum enzyme activity. As a result, the serum levels of ALT and AST activities are a sensitive indicator of liver damage and can reveal the extent of necrosis and liver damage to a certain extent. The various anomalies observed in chick embryos is also due to exposure of toxicant present in reclaimed water which have possibly altered the normal developmental cycle and caused anomalies beginning from stunted growth to serious abnormalities such as gastrochisis, adermia etc.

The exposure to toxicants also interfered with the nutritional demands of the developing chick embryos. It has also been reported that nutritional deficiencies caused coagulative necrosis, congestion of sinusoid and extensive degeneration of the hepatocyte. Besides this nutritional deficiencies are also responsible for many developmental anomalies.

The development of various organs in an organism mainly depends upon its metabolism and any deviation from the normal metabolic pathways, especially during the critical period of growth might affect the overall development of an organism (Sahu and Ghatak 2002). The incidence of limb deformities in chicken embryos is quite often. A number of deformities like crooked legs, polymelia, micromelia, and limbless (complete absence of limbs) etc. are observed in the chicken embryos (Uggini *et al*., 2012). The deformities may be due to exposure to an environmental toxicant, musculoskeletal or may be genetic. Such as the limbless expression has a genetic basis of expression. The chicken embryos that are homozygous for the limbless mutation have limb bud outgrowth initiated in them but due to underdevelopment of distinct apical ridge, limbs are not formed in them. The genetic studies have revealed that the genes Fgf4, Fgf8, Bmp2, Msx2, and Shh are not expressed in the limb bud of chicks that have undergone mutations (Grieshammer *et al*., 1996).

A number of deformities are observed in Chicks which are caused due to alternation or problems of the musculoskeletal system. For instance, the observed crooked legs in chicken could be due to muscular tension at the time of bone ossification. The reason for this might be the failure of the embryo to receive an adequate amount of esterase metabolized products (Upshall *et al*., 1968; DeSosso *et al*., 2018).

Various pharmaceuticals and other organic and inorganic constituents present in the reclaimed water are known to influence neurotransmission. The skeletal defects of the vertebral column such as the stunted growth caused due to improper fusion of the vertebrae are attributed to the decrease in the enzyme acetylcholinesterase and the disruption of the associated cholinergic system (Landauer, 1975). This inhibition during the development of an embryo becomes lethal because, acetylcholine esterase provides signals which regulate the proliferation, differentiation, and migration of cells (Meiniel, 1978). Thus, it can be concluded that a given neurotransmitter at the early stages of development, activates the genes for replication of the target cells, and at later stages of development is responsible for differentiation of the target cells (Slotkin 2004). Hence any deviation in the normal functioning of acetylcholinesterase in the early stages of development would cause severe problems to the developing embryos. This could be the possible reason for the observed malformations in the Chick embryos.

Conclusion

Various developmental anomalies in the developing Chicken embryos due to the exposure of reclaimed water indicates that water even after undergoing a treatment process is still toxic and contains harmful substances that have potential to disrupt normal developmental process. Such drastic effects should not be ignored; besides there must be strict guidelines for treating wastewater in such a way that it does not cause any sort of harm to human and animal life. The study, therefore, advises caution in the extended use of wastewaters without being properly treated. Cost effective practices such as phytoremediation should be adopted to minimize the toxicity to a greater extent.

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Conflict of Interest

The Authors declare no conflict of interest.

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