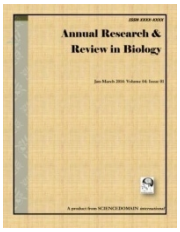

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Cytotoxic Effects of Cadmium and Paraquat on Avian Skin Fibroblasts

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Authors' contributions

This work was carried out in collaboration between all authors. Author HAK analyzed the data and wrote the manuscript, Author IAA managed the literature searches, author AGS did the experiments, and author JBW designed the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Recent studies have shown that wild birds may act as potential barometers of environmental pollution. We studied the effects of two environmental toxicants, cadmium (Cd) and paraquat (PQ), on the skin fibroblasts of three wild birds including, green-winged teal (*Anas carolinensis*), bobwhite quail (*Colinus virginianus*), and house sparrow (*Passer domesticus*) of the orders Anseriformes, Galliformes and Passeriformes, respectively. The toxicities were represented as 50% lethal concentrations (LC_{50}), a dose causing death of 50% of the cells after 24 h exposure of toxicant. The LC_{50} of Cd for the skin fibroblasts from green-winged teal, bobwhite quail, and house sparrow were found to be 0.015 mM, 0.078 mM, and 0.176 mM. The LC_{50}s of PQ for the same cells were 4.58 mM, 25.32 mM, and 36.53 mM, respectively. These results indicated that cells from water fowl may be highly susceptible to Cd and PQ poisoning whereas house sparrow was the least susceptible. Further studies are warranted to investigate the mechanism of differential susceptibility of birds to environmental toxins.

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Keywords: Wild birds; skin fibroblasts; cadmium; paraquat; cytotoxicity; environmental pollution.

1. INTRODUCTION

Recent studies have shown that birds are exposed to environmental toxins such as heavy metals [1] and pesticides [2]. Repeated exposure of heavy metals leads to the accumulation of these pollutants in wild animals including birds [3,4]. The main exposure route of environmental chemicals in wild birds is by ingestion of contaminated food and water [5]. Some birds mainly feed on earthworms in the breeding season, which are known to accumulate heavy metals from the soil [6]. Contaminated fish are another potential source of exposing wild birds to environmental chemicals including heavy metals and pesticides [7]. De Lange et al. [8] evaluated the ecological vulnerability in wildlife due to the presence of heavy metals and pesticides in food chain and terrestrial and aquatic habitats. Increased environmental pollution has exerted an extra pressure on the fitness and survival of wildlife in the situation of limited food and scarcity of favorable habitat for wild animals. Notwithstanding the availability of literature on the toxic effects of environmental chemicals on experimental animals, we know little about how wild animals respond to these toxicants.

Cadmium (Cd) is an environmental pollutant that is difficult to metabolize and tends to accumulate in ecosystems and enters the food chain through environmental contamination. Because there is little to no effective Cd elimination pathway in organisms its biological half-life happens to be as longer as 15–20 years [9]. Cadmium is toxic to several organ systems such as pulmonary, hepatic, renal, reproductive and nervous systems [10,11] and is also regarded as a carcinogen [12]. Elevated levels of Cd have been reported in the kidneys of sea ducks and Cd toxicity has been postulated as a possible cause of population declines in these birds [13]. Lucia et al. [14] assessed the effect of two dietary levels of Cd (1 and 10 mg/kg) in ducks and observed the accumulation of high amounts of Cd (8.1 and 37.7 mg/kg, respectively) after 40 days, especially in kidneys. Interestingly, the high dose exposure caused the repression of genes encoding for antioxidants, whereas the low dose triggered their induction [14]. Thijssen et al. [15] provided evidence that chronic exposure to low concentrations of Cd (10-100 mg/l) in drinking water triggers biphasic defense activation in the kidney that might lead to adaptation and survival in mice. Dietary exposure of Cd (140 and 210 mg/kg) resulted in significant oxidative damage of hens' ovary tissue by altering antioxidant defense enzyme systems, lipid peroxidation, apoptosis and endocrine disturbance which could be the possible underlying reproductive toxicity mechanisms induced by Cd [16]. Ingestion of Cd (150 mg $CdCl_2$ per kg of diet) in chicken significantly reduced antioxidant activities in the liver leading to oxidative stress and liver damage that was ameliorated by selenium supplementation [17].

Paraquat (1,1'-dimethyl-4,4'-bipyridinium) (PQ) is an effective, fast-acting, and non-selective herbicide that is widely used for weed control in agriculture. Chronic exposure to PQ is associated with liver damage, kidney failure, fibrosis, and Parkinsonian lesions [18,19]. The chemical structure of PQ is very similar to the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is commonly used to induce Parkinsonism in experimental animal models [20,21]. Both Cd and PQ are known to cause cellular damage by inhibiting the electron transfer chain in the mitochondria leading to excessive generation of potentially toxic reactive oxygen species (ROS) [22,23]. There are numerous studies suggesting the involvement of ROS in toxic effects of Cd [24,25] and PQ [26,27]. However, mitigation of oxidative stress by antioxidants significantly prevented the cytotoxic effects of both Cd [28,29] and PQ [30,31].

Considering the potential toxicity and environmental occurrence of Cd and PQ, it is important to study the impact of their exposure on wild animals. Traditional toxicological testing relies on utilizing a large number of whole animals to test the potential toxicity of chemicals. However, these in-vivo tests tend to be expensive, time consuming, and provide little information on early, low dose effects on target cells. Harper et al. [32] have pointed out that differences in stress resistance of dermal fibroblast cells may serve as an indicator to cellular properties involved in stress resistance and longevity in intact organisms, though there may not be a direct involvement of skin fibroblasts in lifespan determination. Previous studies have shown important interactions between environmental stressors, metabolic rates, oxidative stress and longevity of wild animals [33-35]. An empirical analysis of the scaling relationship between field metabolic rate and body mass has indicated that the primary taxonomic level at which heterogeneity occurred is the order level although substantial heterogeneity may also occur at the species level [36]. In this study, we used dermal fibroblast to evaluate the cytotoxicity of graded doses of Cd and PQ in three bird species including green-winged teal, bobwhite quail and house sparrow, each belonging to a different order.

2. MATERIALS AND METHODS

2.1 Sample Collection

Green-winged teal (*Anas carolinensis*), bobwhite quail (*Colinus virginianus*), and house sparrows (*Passer domesticus*) (Fig. 1), belonged to the orders Anseriformes, Galliformes and Passeriformes, respectively, were collected from the wild, sacrificed and feathers removed from the ventral (abdominal) region. The abdominal skin was cleaned using antibacterial soap and sprayed with 70% ethanol. Skin pieces of about 1.0 cm^2 were cut and immediately placed in sterile tubes containing complete cell culture medium, Dulbecco's modified Eagle medium (DMEM), high-glucose variant (4.5 mg/ml), with sodium pyruvate (110 mg/l), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% heat-inactivated chicken serum, and antibiotics (1% penicillin/streptomycin).

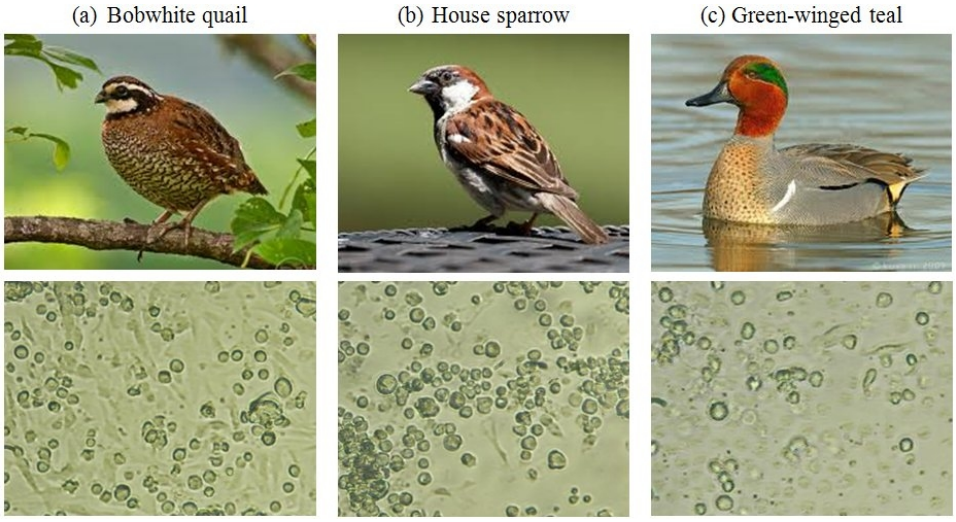

Fig. 1. Images of the birds species used in this study (upper panel). The lower panel shows the trypsinized skin fibroblasts form the respective bird samples

2.2 Isolation of Skin Fibroblasts

Individual skin samples were placed in sterile petri dishes and any associated fat was removed. The sample was exposed to 5 ml of sterile collagenase B solution (0.5%) and finely minced using a sterile scalpel. Pieces of skin were evenly distributed across the dish; the dish covered and then incubated overnight at 37°C in an atmosphere of 5% CO_2 and 5% O_2. After incubation, the following day, we dislodged any adhered cells by repeated pipetting, and then filtered the contents through a sterile mesh into a sterile 15 ml Falcon tube and centrifuged at 1000g for 5 min. The media was removed and the cell pellet was re-suspended in 5 ml of media and the entire contents were transferred into a cell culture flask for overnight incubation.

2.3 Cell Culture and Cryopreservation

The media in the culture plate was replaced after 24 h with 5 ml of fresh media, and thereafter, we replaced media every 4-5 days until the cells reached 80-90% confluence. All incubations were performed at 37°C in an atmosphere of 5% CO_2 and 5% O_2. For passaging, adhered cells were washed with phosphate buffered saline (PBS) (calcium and magnesium free) to remove any residual FBS that may inhibit the activity of trypsin. Cells were detached by addition of trypsin/EDTA solution for 1-2 min and then passaged. At the confluent stage of passage-2, the cells were trypsinized, counted, suspended in 10% DMSO (10^6 cells per vial) and first placed overnight in a -80°C freezer and then cryopreserved in liquid nitrogen.

2.4 Cytotoxicity Assay

The cryopreserved cells were thawed and then cultured, trypsinized, counted, and then we adjusted their concentration to 20,000 cells in 100 μl of complete DMEM media. Then we individually seeded cells in the respective wells of a sterile microplate and allowed cells to attach by overnight. The media was then removed and cells were washed twice with sterile PBS followed by addition of100 μl DMEM devoid of serum and sodium pyruvate but containing 2% bovine serum albumin (BSA) and the plate was incubated overnight. Next day, the media was removed and cells were exposed for overnight to graded concentrations of Cd as $CdCl_2$ (0.1-3.0 mM) and PQ (0.1-100 mM) in 100 μl media. Three wells each were assigned as control (only cells, no toxicant) and blank (no cells, no toxicant). The plate was removed from the incubator and WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) solution (10 μl) was added to each well and the plate was incubated for 2 h followed by measuring the absorbance at 450 nm. The average blank absorbance was subtracted from all the values and then normalized to the control value. This colorimetric assay is based on the reduction of tetrazolium salt WST-1 (faint red) to formazan (dark red) by electron transport across the plasma membrane of dividing cells. The amount of formazan dye formed is directly proportional to the number of metabolically active cells in the culture. Thus, a high absorbance value is a proxy for a large number of viable cells and vice versa. The toxicity of Cd and PQ were represented as 50% lethal concentrations (LC_{50}), a dose causing death of 50% of the cells after 24 h exposure of toxicant. All the measurements were taken in two replicates and average values were reported.

3. RESULTS

The lowest concentration of Cd (0.1 mM) caused differential mortality in skin fibroblasts from three different bird species wherein house sparrow showed a greater tolerance (6.25% cell death) followed by bobwhite quail (35.46% cell death) and green-winged teal (56.69% cell death) (Fig. 2, left panel). Increasing the Cd concentration by a factor of three (0.3 mM) sharply reduced the gap in differential toxicity in the range of 81.06% to 96.47%. Further increase in the concentration of Cd from 0.6 mM to 3 mM produced the same magnitude of lethality in the fibroblasts from the respective bird species (Fig. 2, left panel). The LC_{50} of Cd (in increasing order) for skin fibroblasts from the three bird species were as follows: green-winged teal (0.015 mM), bobwhite quail (0.078 mM), and house sparrow (0.176 mM) (Fig. 2, right panel).

Paraquat concentrations up to 0.3 mM did not produce any cytotoxicity in fibroblasts of any species, but thereafter cells of green-winged teal became susceptible to toxic effects. Fibroblasts from bobwhite quail and house sparrows tolerated PQ concentrations up to 10 mM (Fig. 3, left panel). The highest concentration of PQ (100 mM) caused 97.57% mortality in the fibroblasts from green-winged teal, 89.90% in house sparrows and 86.78% in bobwhite quail (Fig. 3, left panel). The LC_{50} of PQ for skin fibroblasts from green-winged teal, bobwhite quail and house sparrow were found to be 4.58 mM, 25.32 mM and 36.53 mM, respectively (Fig. 3, right panel).

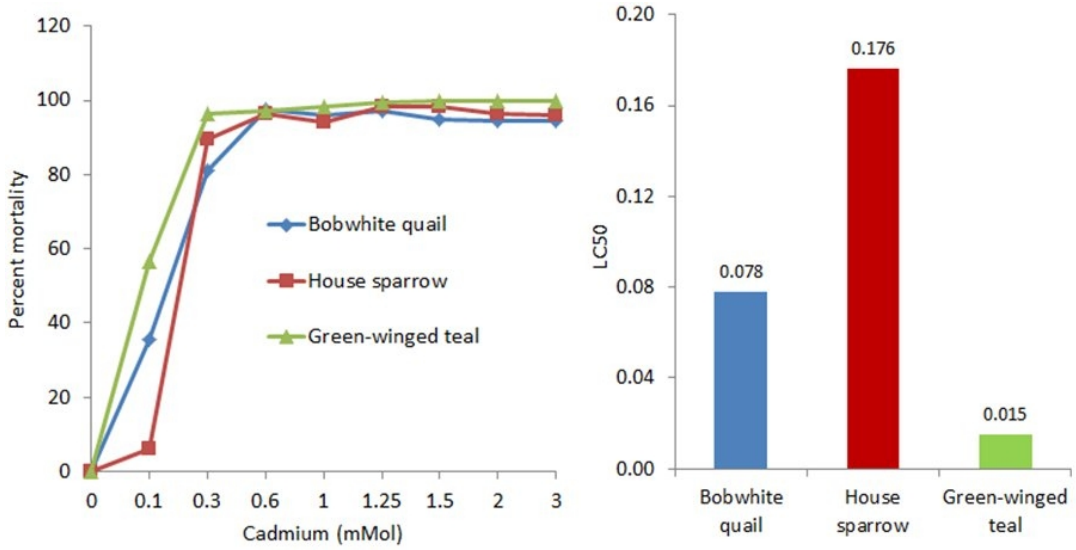

Fig. 2. Percent mortality of skin fibroblasts from three bird species exposed to different concentrations of cadmium (left panel). Right panel shows the LC_{50} of cadmium for skin fibroblasts from different bird species

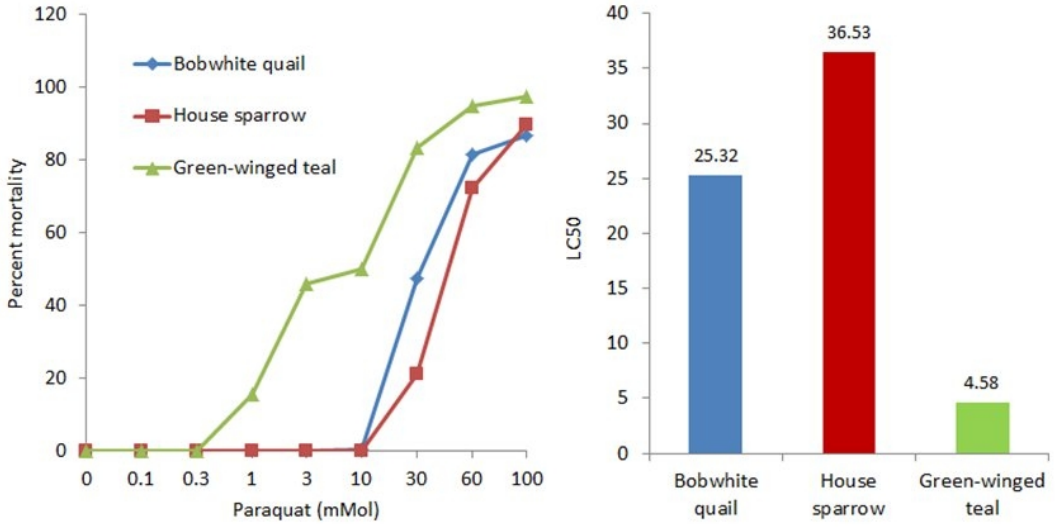

Fig. 3. Percent mortality of skin fibroblasts from three bird species exposed to different concentrations of paraquat (left panel). Right panel shows the LC_{50} of paraquat for skin fibroblasts from different bird species

A comparative view of cytotoxicities of Cd and PQ in fibroblasts from different bird species (this study) with those observed in skin fibroblasts from different mammalian species including mouse, rat and bat [32] is given in Table 1.

Table 1. Comparison of cytotoxicity of cadmium and paraquat between birds (this study) and mammals [32]

Species	Body mass (g)	Lifespan (y)	LC_{50} (mM)	
			Cadmium	Paraquat
Bobwhite quail	150	5	0.0780	25.32
House sparrow	30	15	0.1760	36.53
Green teal	300	25	0.0150	4.58
House mouse	40	2.7	0.0057	2.00
Wild mouse	20	3.4	0.0016	3.40
Rat	200	5	0.0093	2.70
Bat	10	34	0.0680	1.24

4. DISCUSSION

Our results showed that skin fibroblasts from green-winged teal were highly susceptible to Cd (LC_{50}=0.015 mM) whereas house sparrows, an invasive species in North America, showed appreciable resistance to Cd toxicity (LC_{50}=0.176 mM) (Fig. 2). The LC_{50} of Cd was found to be significantly higher in skin fibroblasts from long-lived dwarf mice (0.0126 mM) as compared with normal mice (0.0045 mM) [37]. In the human hepatocellular carcinoma (hepg2) cell line, the LC_{50} values of Cd were 0.0062 mM for naive cells (no previous Cd exposure) and 0.0107 mM for pre-exposed cells indicating that adaptive tolerance or increased resistance had occurred in the cells that had a previous exposure to the same

toxicant [38]. Olabarrieta et al. [39] assessed the effects of Cd on two different in vitro cell models, a cell line derived from proximal tubule renal cells (LLC-PK1) and blood cells of mussels. Their findings showed that 24 h LC_{50} was 0.05 mM in LLC-PK1 cells and 0.4 mM in haemocytes, suggesting that mussel haemocytes were more resistant to Cd exposure than LLC-PK1 cells [39].Tan et al. [40] used lactate dehydrogenase (LDH) leakage as an indicator of cellular viability and cytotoxicity for studying the time- and dose-dependent effects of Cd in cultured primary rat hepatocytes. They observed that a lower dosage of Cd (1.25 µM) did not affect the LDH release whereas a higher dose (2.0 µM) caused a significant leakage of LDH at 12and 24 h after Cd exposure [40]. Thus, skin fibroblasts from birds were comparatively more resistant to Cd toxicity than the cells from other taxonomic class, such as mammals (Table 1). It is interesting to note that birds tend to live longer than mammals of the same body size, despite the fact that they have a higher rate of metabolism. One might suspect that the higher the rate of metabolism, the more free-radical production, however, the generation of free radicals in mitochondria is a complex process influenced by several factors [41-43]. Although birds have higher metabolic rates and longer lifespans, they have lower rates of ROS generation than similarly sized mammals [43,44]. Thus, birds provide a unique study system to address how ROS production affects aging and cellular stress tolerance processes [45].

Hinkle and Osborne [46] studied the role of calcium channels in the cytotoxicity of Cd using two rat pheochromocytoma cell lines including PC12 cells, which express voltage-sensitive calcium channels, and PC18 cells, which do not. The LC_{50} of Cd for PC12 and PC18 cells were 0.012 mM and 0.015 mM respectively. The Cd toxicity in the former cells was significantly inhibited by calcium antagonist whereas the latter cells were not protected suggesting a potential role of calcium in Cd transport across the cells [46]. The findings of a time course study on the effect of Cd exposure, with or without glutathione (GSH)-depleting agent, to A549-T27human tumor cells have shown that GSH plays an important role in early cellular protective responses to Cd [47]. Son et al. [48] have shown that Cd induces apoptosis through the caspase-independent mitochondria-mediated pathway in mouse skin epidermal cell lines, where the decrease of Bcl-2/Bcl-xL, the upregulation of GADD45a, and the nuclear translocation of apoptosis-inducing factor (AIF) are the critical events for apoptosis induction. Cadmium exposure has been associated with abnormal denaturation of cellular proteins while certain cell stress response genes such as heat shock proteins (HSPs) are stimulated [49,50]. HSPs are known to act as molecular chaperones to help denatured proteins refold to protect the cells against stress-induced damage. Tan et al. [40] have observed increased expression of Hsp8, Hspb1, Hspa1a and Hspca as a response to the cadmium toxicity in primary rat hepatocytes. To counteract the toxic effects of Cd, cell protection machinery respond by activating a variety of genes involved in numerous processes and pathways such as chelating the metal to nullify its activity, scavenging ROS, repairing membrane lipid and DNA damage and degradation of unfolded proteins [51,52].

The pattern of PQ toxicity was similar to Cd toxicity as the skin fibroblasts from green-winged teal showed a low tolerance (LC_{50}=4.58 mM) while the cells from house sparrow exhibited higher tolerance (LC50= 36.53 Mm) to PQ exposure (Fig. 3). Murakami et al. [37] have observed that LC_{50} of PQ is significantly higher in skin fibroblasts from long-lived dwarf mice (2.08 mM) as compared to normal mice (1.28 mM). The respective LC_{50} of PQ for undifferentiated and differentiated PC12 cells were found to be 42.5mM and 26.5 mM [53]. The enhanced susceptibility of the differentiated cells were attributed to decreased activity of glutathione peroxidase and the concentration of its substrate, GSH; however, the preincubation of PC12 cells with alpha-tocopherol (antioxidant) or L-buthionine-(R,S)-sulfoximine (GSH depleting agent) lowered or enhanced their cytotoxicities, respectively

[53]. Machaalani et al. [54] compared the cytotoxicity of PQ in primary culture of baboon proximal tubule cells (bPTC) with a positive reference using LLC-PK1 cells of proximal tubule origin and a negative reference using MDCK cells of distal tubule origin. The LC_{50} of PQ after 24 h were 0.076, 0.061 and 0.455 mM for the bPTC, LLC-PK1 and MDCK cells respectively indicating that proximal tubule cells are more susceptible to PQ toxicity compared to distal tubule cells [54]. The mitochondria are thought to be essential targets of PQ and there is evidence that PQ disrupts the mitochondrial electron transfer chain resulting in an impairment of metabolic function leading to cellular damage [23]. It is well established that PQ induces toxicity mainly through its metabolism and subsequent generation of ROS by redox cycling [55]. Kim et al. [56] have found that 0.10 mM PQ can sufficiently induce intracellular ROS level in human alveolar epithelial A549 cells. Paraquat-induced oxidative stress in keratinocytes is accompanied by increased expression of antioxidant genes in order to protect the skin from PQ-mediated cytotoxicity [57].

The inter-specific variations in stress tolerance, as observed in this study, may be attributed to species-specific differences in basal metabolic rate (BMR) and/or in the extent of cellular antioxidant defenses. Certain factors such as the absence of tidally-induced food restrictions, low salinity, and less windy microclimates associated with inland freshwater habitats may reduce the levels of energy expenditure and hence BMR [58].Variations in BMR are related to the composition of cell membrane fatty acids which are targeted by excessive generation of oxygen-derived free radicals causing lipid peroxidation [59]. Different bird species possess a different composition of membrane lipids [60,61], which differ in their susceptibility to peroxidation [59] and may account for variation in the stress tolerance among different bird species. Galvan et al. [62] have reported that antioxidants and life-histories coevolve while the accumulation of liver antioxidants is a positive fitness indicator and the avian species with higher antioxidant levels live longer. However, antioxidant-life history associations differed between tropical and temperate species and varied with respect to taxonomic sampling [63].Williams et al. [35] have also observed that birds that live longer invest more in self-defense such as maintaining the intracellular antioxidant levels.

5. CONCLUSION

The skin fibroblasts from three different bird species showed differential vulnerability to Cd and PQ. The magnitude of LC_{50} of Cd and PQ in avian fibroblasts was found to be higher (less toxicity) than the previously reported LC_{50} of these toxicants for different mammalian cells. The exact mechanism behind the inter-specific and inter-class variations in the cytotoxicity of environmental toxins is not clearly understood and requires further studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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