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REPVAS
RESEARCH & PRACTICE in VETERINARY & ANIMAL SCIENCE

Volume: 1 Issue: 2 Year: 2024 Research Article ISSN: 3023-6681

Determination of the Effect of Melatonin and Vitamin E on Cadmium Chloride-Induced Pathologies in the Stomach and Small Intestine of Lohmann Chicken Embryos

Erhan ŞENSOY[1*] Eda GÜNEŞ[2]

[1*]Faculty of Health Sciences, Karamanoglu Mehmetbey University, Karaman, Türkiye

[2] Necmettin Erbakan University, Faculty of Tourism, Konya, Türkiye

Article Info

Received: 29.04.2024
Accepted: 08.06.2024
Published:31.07.2024

Keywords:
Cadmium,
Melatonin,
Vitamin-E,
Histopathological effect,
Stomach,
Small intestine.

ABSTRACT

Cadmium (Cd), one of the most important heavy metals contributing to environmental pollution, represents a significant risk factor for public health. The human body is exposed to cadmium through the ingestion of food and respiration. Cadmium accumulates in tissues such as the stomach and small intestine, where it can cause pathological changes in the organs. Melatonin is an antioxidant hormone secreted by the pineal gland, whereas vitamin E is a powerful antioxidant with therapeutic properties. The aim of this study was to determine the toxicity of Cadmium chloride ($CdCl_2$) in the stomach and small intestine of Lohmann breed chicken embryos and to treat them with melatonin and vitamin E.A total of 30 fertile eggs of the Lohmann breed were utilized in this study, and five experimental groups were established (n=6): distilled water (DW), DW+ ($CdCl_2$), DW+Melatonin, DW+Vitamin E, DW+$CdCl_2$+Melatonin+Vitamin E. After a single dose of $CdCl_2$ (0.430 mM), Melatonin (5 mg) and vitamin E (3 mg) were administered to each egg via in-ovo injection, the hole was closed with liquid paraffin. The fertile eggs were transferred to the incubator and the study was completed before the chicks hatched (day 21). The stomach and small intestine tissue of the chicks were excised, fixed in 10% formaldehyde, and processed using an alcohol and xylene series. Following the completion of routine histological procedures, the samples were stained with Hematoxylin-Eosin and examined under a light microscope. A decrease in average body and organ weight was observed in the $CdCl_2$ group, while the mixture of melatonin and vitamin E prevented this decrease. The administration of $CdCl_2$resulted in the necrosis and hemorrhage of gastric epithelial cells, as well as the deformation of small intestine crypts, the rupture and fracture of villi, and hemorrhage. The administration of $CdCl_2$ induced pathologies in the gastrointestinal system, and the mixture of Melatonin and Vitamin E may have a limited effect in treating the damage. Further studies are required to determine the protective properties of melatonin and Vitamin E against $CdCl_2$ in different experimental animals.

To cite this article:

Şensoy, E., & Güneş, E. (2024). Determination of the effect of melatonin and vitamin e on cadmium chloride-induced pathologies in the stomach and small intestine of lohmann chicken embryos. *Research and Practice in Veterinary and Animal Science (REPVAS),1(2),* 73-85. https://doi.org/10.69990/repvas.2024.1.2.3

***Corresponding Author:** Erhan Şensoy, *erhansensoy@kmu.edu.tr*

INTRODUCTION

Nowadays, increasing industrial activities and the release of heavy metals into the environment due to natural phenomena represent significant challenges that pose a threat to public health. Heavy metals exhibit a high degree of resistance to adverse environmental conditions, which enables them to persist in nature and in the human body for extended periods (Şavran and Küçük, 2022). Cadmium (Cd) is a heavy metal with which humans, at the top of the food pyramid, are in constant contact (Blasiak, 2001). Cd is a white element with an atomic number of 48, a density of 8.64 g ml, an atomic weight of 112.4, and a valency of "+2" (IPCS, 2005; IPCS, 2007). In its natural state, Cd is found in a variety of forms, including Cd oxide, chloride, sulfide, and sulfate. It can be ingested orally through food or inhaled in powder or aerosol form. It has been reported that Cd, which is known to have toxic effects in both forms (Yıldızgören et al., 2014), causes pathology by accumulating especially in the stomach and small intestine, as well as in the lungs, liver, kidneys, pancreas, and prostate (Demirkıran et al., 2020). The half-life of Cd is approximately 30 years (Coşan et al., 2017), it reduces vitamin D levels, and it negatively affects bone mineralization (James and Meliker, 2013). Advanced pathologies have been documented in individuals engaged in occupational activities with high exposure to Cd oxide compounds (Ateş, 2008).

Sources of Cd, such as foods and contaminated drinking water, as well as industrial activities like battery production, textiles, electronics, and refineries (Coşan et al., 2017; Rani et al., 2014), may also negatively affect the endocrine system. Cd causes an increase in oxidative stress by disrupting the functioning of natural enzyme groups such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), which are known as antioxidants and are involved in reducing oxidative stress (Cuypers et al., 2010; Güneş and Şensoy, 2022). The World Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have established the acceptable value of Cd for humans (Acceptable Daily Intake; ADI) for nutrients at 5 ppm/kg/day and for drinking water at 0.005 mg L day (Kara et al., 2016). Some therapeutic agents may play an active role in the treatment of pathologies caused by Cd toxicity and in the reduction of oxidative stress. Melatonin, a neurohormone secreted from the pineal gland, small intestine, skin, and leukocytes, is a natural antioxidant with therapeutic properties (Baltacı 2001; Baran et al., 2020; Karaca et al., 2014; Öner et al., 2003). Melatonin plays an active role in ensuring sleep patterns and coordinating the immune and reproductive systems (Candan et al., 2017). Furthermore, it plays an active role in preventing oxidative damage (Forsling, 2001) because of its ability to trap free oxygen radicals, prevent lipid peroxidation, and activate SOD, GSH-Px, and CAT enzymes (Erdem 2010; Özmete 2009; Uluocak et al., 2010). Vitamin E is a powerful antioxidant with therapeutic properties that can suppress free oxygen radicals (Pranay Kumar et al., 2019).

The easiest and cheapest method to determine the Cd toxicity process is the use of live animals. This method not only permits the active ingredient to be tested in animal tissues but also offers a significant advantage in terms of time. In studies on the therapeutic aspects of melatonin and vitamin E, a variety of experimental animals have been used (El-Sokkary et al., 2010; Gültekin et al., 2001; Kim et al., 1998; Karaca et al., 2014). Poultry holds an important place in histopathological studies due to its short incubation period, high reproductive capacity, ease of breeding, low rearing costs, and genetic diversity. Chicken embryos were preferred to determine the effects of melatonin and vitamin E against Cd damage because they have physiological systems that are similar to those of humans and their histological structures are largely similar (Shabalout et al 2023). In the literature, studies investigating the efficacy of natural therapeutic agents in the treatment of $CdCl_2$ toxicity in fertile eggs. The results of these studies are often contradictory. The aim of this study was to treat pathologies induced by $CdCl_2$ toxicity in the stomach and small intestine of Lohmann breed chicken embryos with melatonin and vitamin E.

MATERIAL METHOD

Ethics Committee Permission

The ethics committee permission was obtained from the Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (16.02.2021, 2021/01-18).

Material

Lohmann breed fertile eggs were used (n: 30). The eggs were examined under a light box to ensure normal porosity and disinfected with a mixture of 21 g potassium permanganate and 42 ml formaldehyde/m^3 in a steam environment for 15 min, after which they were placed in the incubator. The weights of the eggs were recorded before and after incubation.

Experimental Groups

Five groups were created (n: 6): Group I: Distilled water (DW), Group II: DW+cadmium chloride ($CdCl_2$), Group III: DW+melatonin, Group IV: DW+vitamin E, and Group V: DW+$CdCl_2$+Melatonin+Vitamin-E (Table1).

Incubation Process

The fertile eggs were maintained at 37.5±0.5°C and 65-70% humidity for 2 days to prevent heat stress before being transferred to the incubator. Throughout the incubation process, the eggs were rotated once every hour and provided with a suitable growth and living environment (maintaining a temperature of 37.8°C and relative humidity of 65%).

Table 1

Groups and Applied Procedures (n:6)

Experimental Groups	Chemical Substance	Dose
Group I	DW	20µl
Group II	DW	20µl
	$CdCl_2$	0.430 mM
Group III	DW	20µl
	Mel	5µg
Group IV	DW	20µl
	Vit-E	3µg
Group V	DW	20µl
	$CdCl_2$	0.430 mM
	Mel	5µg
	Vit-E	3µg

Applications

The level of $CdCl_2$ was determined on the basis of the human physiological dose (30 mg kg) and applied as 0.430 mM, according to literature knowledge (Nordberg et al., 2007; Venter et al., 2015). Melatonin's levels (5 µg) and vitamin E (3 µg) were also measured. Before injection, the eggs were disinfected using 70% ethanol. All applications were administered as a single dose on the eighth day of the incubation process, which is a time of intensive metabolic activation. Following the

injections, the holes were closed by applying liquid paraffin (Özparlak 2015; Wolf and Leupke 1997).

Tissue Samples

On the 21st day of the incubation process, the eggs were hatched from the blunt end. The embryos were then placed in moistened cotton jars. Once their inactivity was confirmed, the sacrifice process began. The stomach and small intestine tissues were removed, weighed, and transferred to a 10% phosphate-buffered formal-saline solution (0.1M PBS, pH: 7.4).

Histopathological Examinations

Following fixation, standard follow-up procedures were conducted. Six-micrometer (µm) sections were obtained from the paraffin-embedded tissues. Subsequently, the preparations were stained with Hematoxylin-Eosin (HE) and examined under a light microscope, with important regions being recorded (Şensoy and Öznurlu 2019).

Statistical analyzes

Experiments were conducted in 4 repetitions to give mean and standard errors. Data transferred to the SPSS package program (version 27.0, IBM Corporation, Armonk, NY) were analyzed using ANOVA and LSD tests. Analysis of variance was applied to mean body weight, mean stomach weight, and mean small intestine weight followed by LSD test for the parameters were performed with one-way ANOVA. The significance level was determined as $p<0.05$ in all statistical tests in the study.

RESULTS

Average body and organ weights

The mean body weight decreased in Group II; moreover, it did not change in Groups III and IV. We observed a decrease in Group V compared with the control, but it increased significantly compared with Group II ($p<0.05$). The mean stomach and small intestine weights decreased in Groups II and V compared with the control group ($p<0.05$), (Table 2).

Table 2

Average Body and Organ Weights of the Embryos (Mean ± SE)

Group	Mean Body Weight (g)	Mean Stomach Weight (g)	Mean Small Intestine Weight (g)
Group I	38.25±2.03[a]	1.09±0.01[a]	4.13±0.03[a]
Group II	32.12±3.44[b]	0.78±0.02[b]	3.66±0.02[b]
Group III	39.89±1.23[c]	1.05±0.01[c]	4.51±0.01[c]
Group IV	38.71±2.99[c]	1.03±0.02[c]	4.08±0.01[c]
Group V	34.63±1.37[d]	0.89±0.04[d]	3.69±0.03[d]
Sig	0.01	0.03	0.02

SE: Represents Standard Error. Values Shown With Different Letters Differ From Each Other At The P<0.05 Level.

Histopathological evaluation

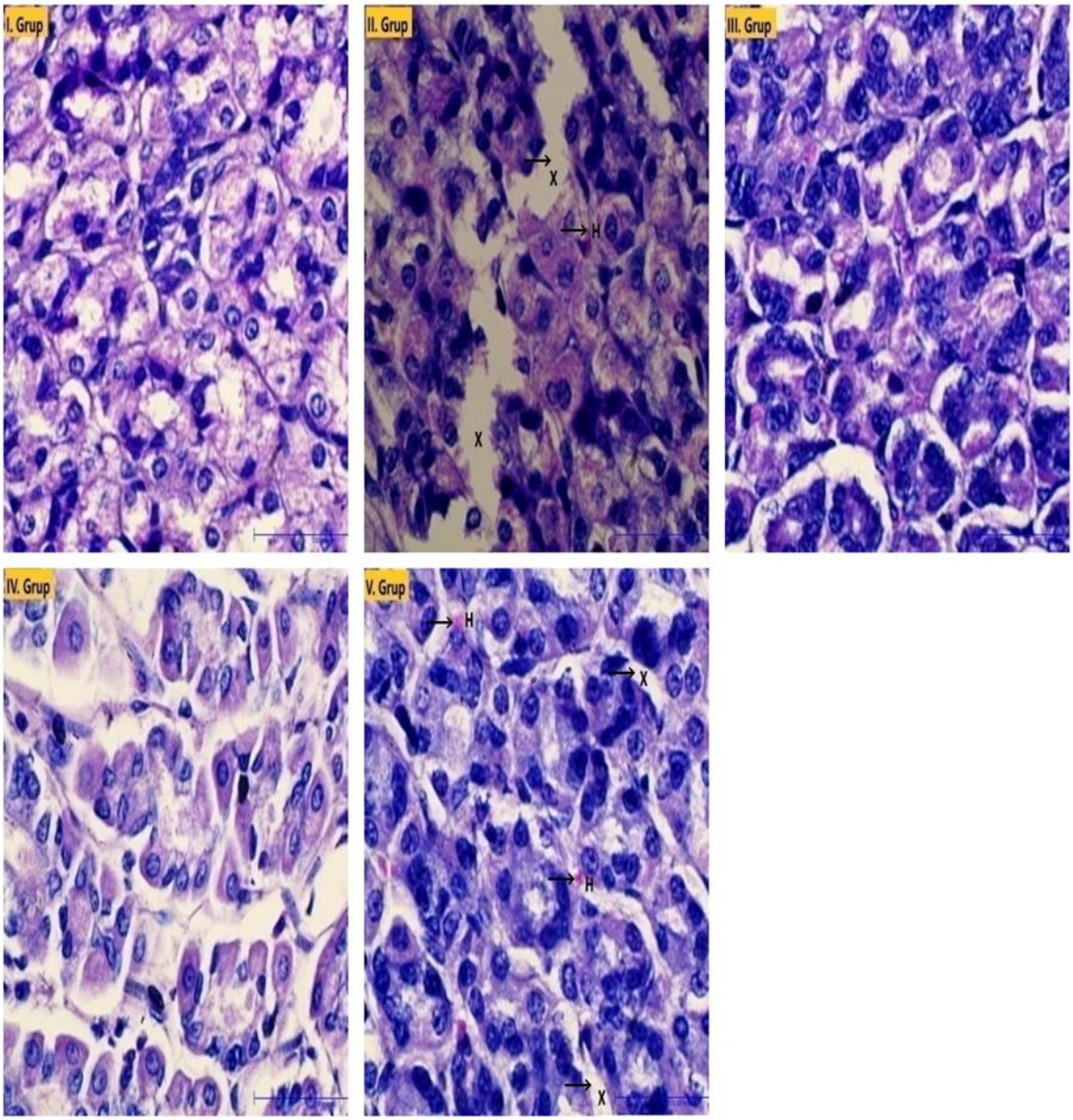
Stomach tissue

The histopathological appearance of the stomach tissue was normal in the control group,

Group III, and Group IV. In these groups, the stomach contours were regular, and the cytoplasm and nucleus were not stained darkly. Additionally, there were no gaps or hemorrhages between the epithelial cells. In Group II, the nucleus and cytoplasm were stained dark, and gaps and hemorrhage were observed between the epithelial cells. In Group V, the nucleus and cytoplasm stained normally, but there were partial gaps and hemorrhage between the epithelial cells (Figure 1).

Figure 1

Light Microscopy Images of Stomach Tissue (HE Staining, Magnification:100 µm)
Spaces Between Epithelial Cells: X, Hemorrhage: H).

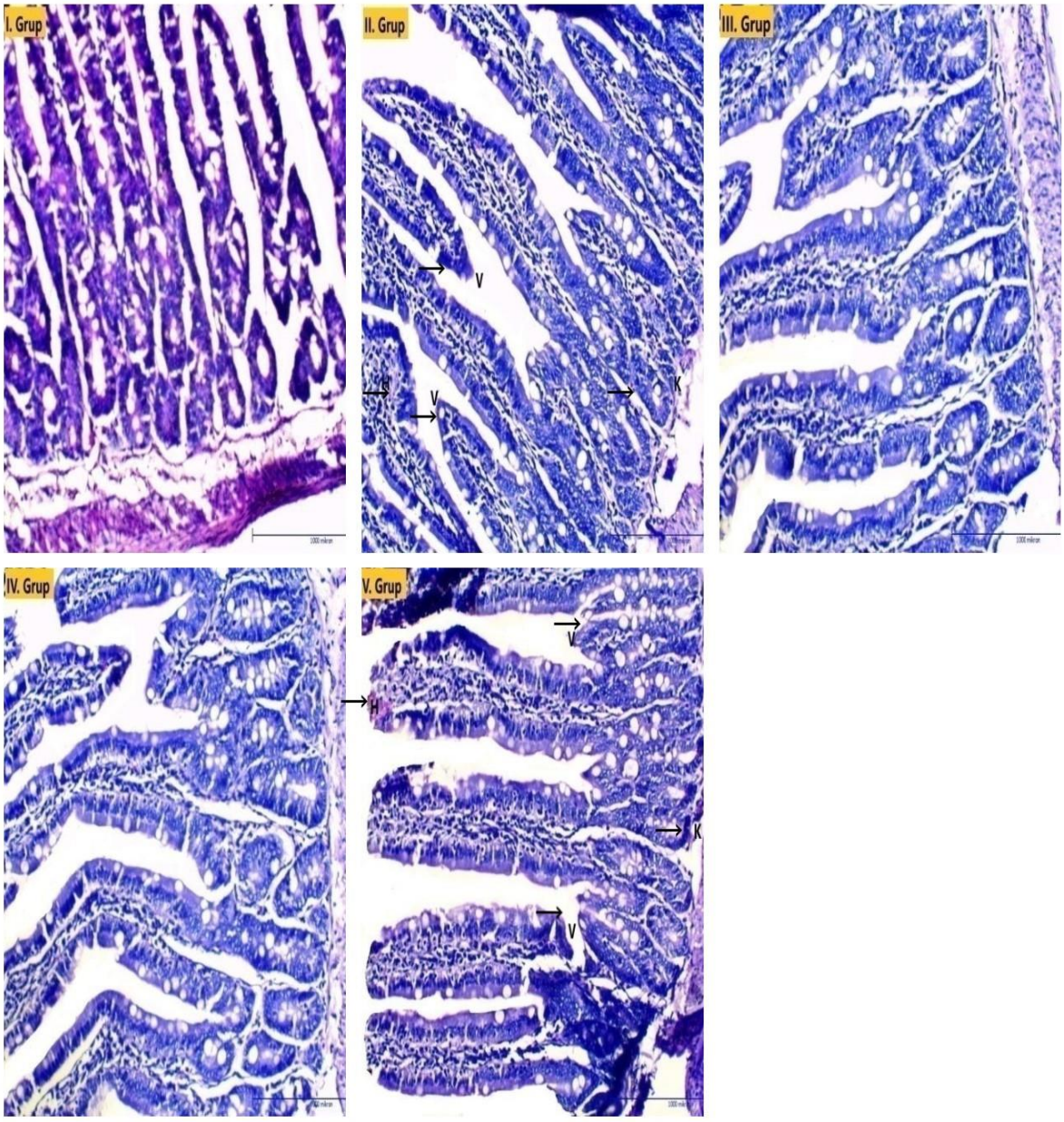


Small intestine tissue

The histological structure of the small intestine tissue in the control group, Group III, and Group IV was normal, and the contours of the mucosal layer were smooth. In these groups, no deformations in the crypts, ruptures, fractures, necrotic areas, or hemorrhage were observed. In Group II, deformations in the crypts, ruptures, fractures, and extensive hemorrhage were detected in the villi. In Group V, deformations of the crypts, rupture of the villi, and hemorrhage were detected (Figure 2).

Figure 2

Light Microscopy Images of Small Intestine Tissue (HE Staining, Magnification: 100 µm, Deformation In The Crypts: K, Ruptures And Fractures In The Villi: V, Hemorrhage: H).



DISCUSSION

The accumulation of heavy metals in the environment is a growing concern, as it has the potential to negatively impact public health. Heavy metals, which have very long half-lives, accumulate in tissues and organs, causing pathologies. Heavy metals such as Cd cause developmental disorders in organs and a reduction in average body and organ weights, which can be attributed to alterations in the protein, carbohydrate, and fat balance of cells (Şensoy 2023). Because the embryonic period is when growth and development are the fastest, Cd may cause retardation in organ development (Çağlar and Saral 2014). The impact of Cd during the embryonic period has been investigated by numerous researchers on different experimental animals, and contradictory results. For instance, in one study, a single dose of $CdCl_2$ (5 µg) and $Pb(NO_3)_2$ (50 µg) was administered to fertile chicken eggs by in-ovo injection, resulting in a reduction in average body weight (Kril et al., 2011).

Another study found that fertile Lohmann breed eggs administered three doses of Cd (50.2 mg kg) by in-ovo injection led to increased relative intestinal weights and decreased body weights (Berzina et al., 2007). In contrast, a different study reported a decrease in body weight in 100-day-old Ross breed chickens fed $CdCl_2$ (50 and 100 ppm) in their diets (Teshfam et al., 2006). Similarly, a study reported that six-week-old chickens fed Cd (10 mg day) for four weeks experienced a decrease in body weight (Ali et al., 2016). In our study, the average body, stomach, and small intestine weights were affected by $CdCl_2$, with our results indicating a decrease in weight that is consistent with similar studies. It is thought that the observed decrease in average weight values is due to alterations in the carbohydrate, protein, and fat composition of tissues and organs resulting from $CdCl_2$ exposure.

Cd toxicity can directly or indirectly affect all systems. The gastrointestinal system is one of the first systems to be affected, as Cd is mostly ingested through food (Anetor 2012). Short-term Cd exposure affects the liver, while long-term exposure affects the intestines, stomach, kidneys and heart (Valko et al., 2005). Cd is absorbed from the small intestine, where it accumulates in the intestinal mucosa with little diffusion into the organism (Bishak et al., 2015). Cd accumulation in the proximal region of the human intestine causes degeneration and severe gastrointestinal disorders (Şimşek and Alabay 1999). Cd toxicity leads to increased lipopolysaccharide production and changes in metabolic activity, triggering cellular damage in the intestinal wall (Tinkov et al., 2018). In a study, 100-day-old Ross breed chickens fed $CdCl_2$ (5, 50, and 100 ppm) for 49 days showed decreased villus length, width and crypt depth (Teshfam et al., 2006). Berzina et al. (2007) reported that Lohmann breed brown roosters orally fed Cd (50.2 mg kg) for 3 days showed decreased villus length. In addition, it was Cd decreased the rate of Ca^{+2} absorption in the intestine of chicks (Fulmer et al., 1980). In a similar study, Cd showed radioactivity in various parts of the small intestine of newborn rats and was retained in the intestine for a long time (Sasser and Jarboe, 1977). Cd also increases metallothionein expression (Danielson et al., 1982) and causes damage and inflammation in the small intestine of mice (Ninkov et al., 2015). In addition, Cd-induced intestinal damage causes irregularities in the microbiota, which leads to a decrease in body resistance (Velayatzadeh 2023). However, some studies have reported intestinal resistance to heavy metals, with no signs of atrophy, necrosis or hyperplasia in the small intestinal mucosa of Cd-fed rats (Nai et al., 2013).In various models of Cd toxicity, it has been shown to cause nuclear degeneration (Younis et al., 2016), inflammation (Liu et al., 2019), villi erosion, necrosis, vacuole formation, and irregularities in the lamina propria of the intestinal mucosa (Duan et al., 2023; Li et al., 2017; Xie et al., 2019; Yu et al., 2021).

Because the gastric mucosa is very sensitive to Cd, in cases of toxicity, the protective structures of the gastric mucosa weaken and become more susceptible to stress (Öner et al., 1994). Rats orally administered $CdCl_2$ (15 ppm/day) for 30 days experienced decreased stomach acid and mucin values, dilated mucosal vessels, decreased parietal cell numbers, and tubule vesicular membrane ruptures (Asar et al., 2002). In addition, cellular degeneration, necrosis, and vesicle formation in gastric tissue were observed. Similarly, mild dysplasia and interstitial inflammation have been detected in the gastric mucosa of rats fed $CdCl_2$ via water (Nai et al., 2013). Our results, which indicate that Cd causes necrosis and hemorrhage among epithelial cells in stomach tissue and deformation of the crypts, ruptures and fractures, and hemorrhage in the villi of the small intestine, are consistent with similar studies.

There is no effective treatment for cadmium toxicity. Studies in the literature have used agents with therapeutic effects in various models (El-Sokkary et al., 2010; Karaca et al., 2014; Kim et al., 1998). It has been reported that melatonin (single dose 10 mg kg), a hormone with therapeutic effects, reduces Cd-induced inflammation, regulates cytokine expression and accelerates proliferation in fertile chicken eggs (Li et al., 2018). Similarly, melatonin was reported to increase enzyme levels in rats whose antioxidant enzyme levels were decreased by CdCl2 toxicity (Aydoğdu et al., 2007). Vitamin

E, a therapeutic and strong antioxidant, reduces reactive oxygen molecules generated by aerobic respiration and increases antioxidant defensive capacity (Pranay Kumar et al., 2019). It has been reported that fertile eggs administered vitamin E (30 mg) by novo injection showed reduced mortality and increased immunity levels (Salary et al., 2014). According to Goel et al. (2013), vitamin E increases IgM levels in chicks. There are no studies in the literature combining melatonin and vitamin E for the treatment of cadmium toxicity. The results of our study, which are expected to shed light on future studies in this area, indicate that the combination of melatonin and vitamin E has a limited therapeutic effect in treating $CdCl_2$ toxicity. The combination of melatonin and vitamin E, which prevents the decrease in average body and organ weights due to $CdCl_2$, has a limited effect in the treatment of gastric and small intestinal pathologies.

CONCLUSION

Natural therapeutic agents may indeed hold promise in mitigating the effects of CdCl2 toxicity, which affects organ development and induces pathologies. The identification of pathologies and negative developmental effects in fertile eggs exposed to $CdCl_2$highlights the importance of investigating interventions such as melatonin and vitamin E. Although these treatments showed partial efficacy, their potential warrants further investigation. Raising public awareness of Cd toxicity and advocating for measures to prevent heavy metal exposure, especially during critical developmental periods, are critical steps in protecting public health. By promoting awareness and advocating for preventive measures, we can minimize the adverse effects of heavy metal exposure on human health and development.

Ethics Committee Approval

16/02/2021 dated and 2021/01-18 numbered was given by Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by EŞ and EG. The first draft of the manuscript was written by EŞ and EG, with input and revisions from other authors. All authors have read and approved the final manuscript.

Funding

There is no funding or supported.

Conflict of Interest

The author declares that there are no conflicts of interest.

Sustainable Development Goals (SDG): 3 Good Health and Well-Being

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