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Research Article

Synergistic Developmental Toxicity of Cisplatin and Carboplatin in the *Gallus gallus domesticus* Embryonic Model

Atharva Milan Mulye *, Pinakin Shrikant Wagh, Vivaan Sameer Kelkar

Operon Research and Learning, Kothrud, Pune, MH 411038, India

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*For Correspondence:

Atharva Milan Mulye, Operon Research and Learning, Kothrud, Pune, MH 411038, India

Abstract

Cisplatin and carboplatin, two frontline platinum-based chemotherapeutics, are limited by toxicity and the emergence of resistance during monotherapy. Combination regimens are increasingly considered, yet their developmental safety profiles remain unclear. Here, we assess the individual and combined developmental effects of these drugs using the *Gallus gallus domesticus* embryonic model, a sensitive system for teratogenic evaluation. A multiparametric strategy was applied, including morphological assessment, Yolk Sac Membrane (YSM) vasculature analysis, and biochemical profiling of total protein, acetylcholinesterase (AChE), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP).

Both drugs individually impaired development, but their combination produced a striking synergistic toxicity. Embryos displayed severe craniofacial malformations, somite loss, and neural tube closure failure. YSM analysis revealed marked degeneration in vessel density, network length, branching, and segment number. Biochemically, combination treatment yielded maximal protein accumulation, sharp suppression of AChE and ALP, and a drastic reduction in LDH activity.

These findings demonstrate that cisplatin–carboplatin co-exposure disrupts embryonic viability, angiogenesis, differentiation, and neurodevelopment more severely than either drug alone. The results highlight a paradox: while combination therapy may overcome resistance in tumors, it substantially amplifies developmental toxicity. This underscores the need to balance therapeutic gain with embryotoxic risk when designing platinum-based drug regimens.

Keywords: Cisplatin, Carboplatin, Synergistic effects, Angiogenesis, Chicken Embryo, Developmental Toxicity

1. INTRODUCTION

Cisplatin is a widely used antineoplastic drug belonging to the first generation of platinum-based DNA-alkylating agents [1]. Its mechanism of action involves binding to nucleophilic sites in DNA, leading to the formation of intrastrand and interstrand cross links that inhibit replication and ultimately induce apoptosis. Clinically, cisplatin serves as the first line of treatment against wide spectrum of tumors, including ovarian, lung, bladder, head and neck, and cervical cancers, as well as pediatric tumors such as osteosarcoma and neuroblastoma. Despite its efficacy, the clinical use of cisplatin is limited by severe side effects, particularly nephrotoxicity, neurotoxicity, and ototoxicity, which restrict long-term administration [2 3 4]

Carboplatin, a second-generation platinum analogue, exerts cytotoxic effects through a similar mechanism of DNA cross-linking [5] but differs chemically by possessing a bidentate dicarboxylate group in place of cisplatin's chloride ligands [6]. This modification confers improved stability and a more favourable toxicity profile, making carboplatin preferable in certain treatment regimens [7]. It is commonly used in many of the same cancers as cisplatin [8, 9] and is also applied in conditioning protocols for bone marrow transplantation. Nevertheless, carboplatin is not devoid of adverse effects, with dose-limiting myelosuppression representing a major clinical challenge [10]. Comparative studies have highlighted subtle pharmacological differences between the two agents, including distinct aquation kinetics and DNA damage response profiles, which contribute to differences in both efficacy and toxicity.

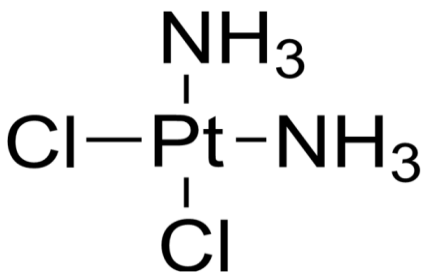


Figure 1 Molecular structure of Cisplatin

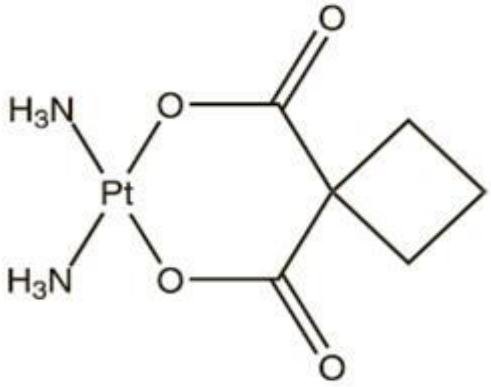


Figure 2 Molecular structure of Carboplatin

Although cisplatin and carboplatin are frequently evaluated as alternatives in monotherapy, their combined use has received less systematic attention but now needs to be taken into consideration due to the rise of monotherapy resistance against antineoplastics. Both drugs strongly activate components of the DNA damage response; however, phosphorylation of Chk1, H2AX and RPA2 is induced earlier by cisplatin than by carboplatin [11] Combination strategies have the potential to exploit synergistic antitumor effects while balancing toxicity, yet they may also produce unanticipated interactions in non-tumor tissues. Developing embryos, with their rapid cell division and sensitivity to DNA-damaging agents, represent a particularly vulnerable system for assessing such effects. Evaluating platinum drug combinations in embryonic models can thus provide critical insights into their developmental toxicity and teratogenic potential.

Gallus gallus domesticus is a scientific model organism that plays a vital role in the field of developmental biology, toxicology, and reproductive biology. *G.domesticus* has a well distinguished body plan and it is also amenable to genetic and physiological modifications. It is sensitive to manipulations and has rapid response to treatment of xenobiotics and toxicants. Fertilized chicken eggs are affordable to purchase in bulk and are easy to culture in a laboratory environment [12]. There is about 60% similarity between the corresponding genes of *G.domesticus* and humans. This makes thema dependable organism to study and draw relevant conclusions [13].

Its Yolk Sac Membrane (YSM)is a well-established platform to study angiogenesis and tumor growth, while the embryo itself serves as a sensitive system for assessing developmental and systemic toxicity [14 15]. YSM

To evaluate the embryotoxic and teratogenic potential of cisplatin, carboplatin, and their combination, we employed a multiparametric approach. The YSM assay was used to assess angiogenic disruption, a critical process in embryonic survival and tumour biology [16].

Lactate dehydrogenase (LDH) is an important enzyme of the anaerobic metabolic pathway with its function being to catalyze the reversible conversion of of lactate to pyruvate with the reduction of NAD+ to NADH and vice versa. Its release was measured as a marker of cytotoxicity and membrane integrity [17].

Alkaline phosphatase (ALP) are isoenzymes that catalyse the hydrolysis of extracellular organic phosphate esters. They are primarily found in liver and bone tissues but also found throughout the body. Activity was quantified to monitor developmental and differentiation-associated changes [18].

Acetylcholinesterase (AChE) activity was assessed as an indicator of neurotoxicity and neuromodulation [19] given the high vulnerability of the embryonic nervous system to DNA-damaging agents. Finally, total protein quantification provided an overall measure of metabolic and biosynthetic activity under drug exposure.

Through these complementary assays, this study aims to provide a comprehensive assessment of the developmental toxicity induced by cisplatin, carboplatin, and their combination in chicken embryos. The findings are expected to clarify whether these agents interact synergistically or antagonistically in modulating embryonic viability, angiogenesis, differentiation, and neurodevelopment, thereby offering new insights into the broader safety and mechanistic profiles of platinum-based drug regimens.

2. MATERIALS AND METHODS

Fertilized and pre-incubated eggs of 72hrs (HH 20-21) [20] *of Gallus gallus domesticus* (White-Leghorn strain) were procured from Venkateshwara Hatcheries Pvt Ltd Pune. Eggs were cleaned with 70% ethanol, labelled and incubated in BOD incubator (REMI®) at 37.5 °C at 70-80% relative humidity (Rh). Working solution of Cisplatin 100ppm (333.3 µM) and carboplatin 100ppm (269.3 µM) were prepared using the stock solution and filter sterilized (0.22micron pore size, 25mm diameter) before treatment.

Teratogenesis: Chick embryos (HH20-21) were treated with 100ppm Cisplatin, 100ppm Carboplatin and their combination (100ppm cisplatin +100ppm carboplatin) by air-sac route (in ovo) window technique. Eggs were sealed with Parafilm M and were incubated at 37.5 °C for 24 hours at 70-80% relative humidity. The embryos were harvested 24 hours later and transferred into sterile, chilled 1X PBS (pH 7.4) and were analysed for drug induced deformities.

YSM analysis: photographs of the YSM vasculature of the control and the treated embryos were taken after opening the eggshell and were analysed using WimCam(Wimasis™) software [21].

Biochemical studies: Control and treated embryos were harvested and homogenized (Potter-Elvehjem PTFE mortar & pestle) in sterile chilled 1X protein extraction buffer (PEB). Protein quantification of treated and control embryos was performed using Bradford method (Bradford MM, 1972). Optical density (OD) was measured using (Systronics µC colorimeter 115) at absorbance 595 nm for control and treated embryos. Enzyme assays for Alkaline Phosphatase (ALP) and Acetylcholinesterase (AChE) were performed using Meril Diagnostics AutoQuant 100 Amara- Alkaline phosphatase kit and AChE assay was performed using Delta Cholinesterase, Delta lab. Both the assays were analysed using HORIBA Yumizen, CA60 semi-automatic analyser.

3. RESULTS

3.1. Teratogenesis and embryonic malformations

Our research showed that when Cisplatin, Carboplatin and their combination is introduced into 3-4 day old (HH stage 20-25) *Gallus gallus domesticus* embryos, it induces teratogenic defects in for the selected doses. The embryos exhibited developmental anomalies, including neural tube defects (NTDs) alterations in neuromere morphogenesis, craniofacial abnormalities, haematoma in the heart, compression of antero-posterior axis, abnormal torsion and flexure throughout the embryonic axis, ocular defects like absence of pigmentation, deformed lens and optic cup.

3.2. Degeneration of YSM Vasculature

Cisplatin, carboplatin and its combination treatment resulted in severe degeneration in the vessel density (Figure 13), total vessel network length (Figure 14), total branching points (Figure 15) and total segments (Figure 16) of the blood vessels on the YSM of 3–4-day old chick embryos as revealed by analysis using WimCAM software

Figure 3: control embryo

Figure 4: control embryo

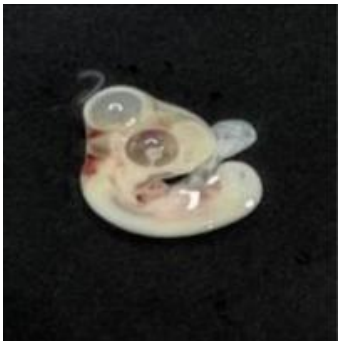
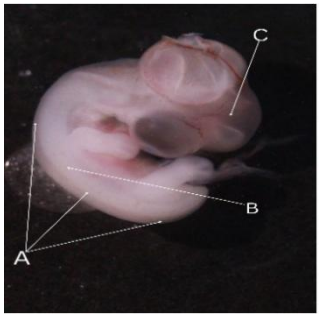


Figure 5: Cisplatin 100ppm HH 20-21



A: Cranial tube

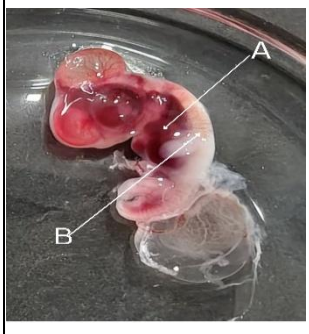
Dysmorphogenesis with

Pronounced ventral flexion.

B: Absence of somites.

C: Absence of forebrain structures.

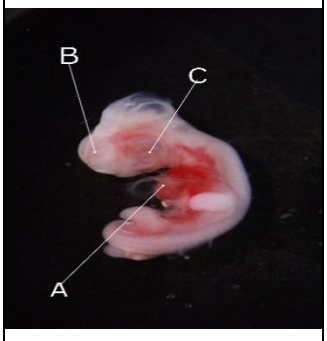
Figure 6: Carboplatin 100ppm HH 20-21



A: Severe hematoma in the heart and cervical region extending upto optic socket.

B: Reduced somite differentiation and asymmetric curvature of neural tube.

Figure 7: Combination 100ppm HH 20-21

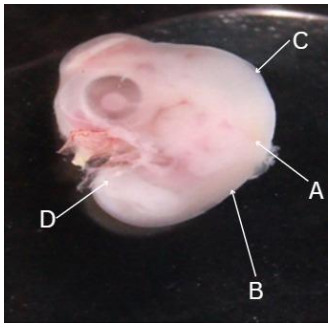


A: Severe hematoma in the thoracic region.

B: Abnormal enlargement of brain vesicles and cranial deformities.

C: Optic cup deformed.

Figure 8: Combination 100ppm HH 20-21

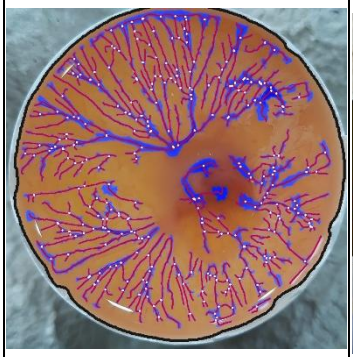
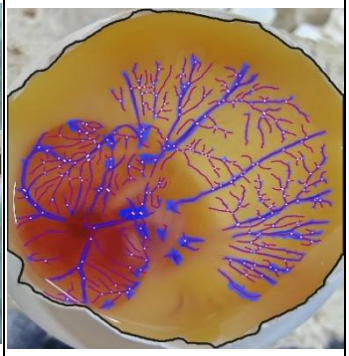
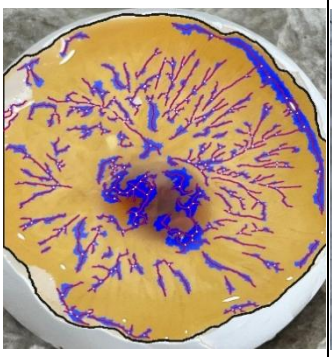
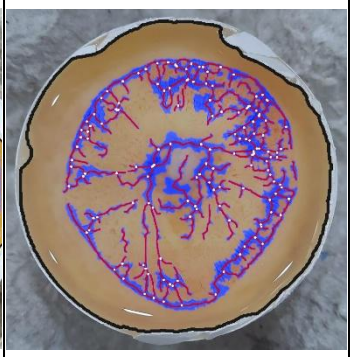


A: Severe torsion of embryonic axis.

B: Somite differentiation absent.

C: Highly deformed neural vesicles.

D: Absence of limb buds.

Figure 9: Control YSM vasculature HH 20-21	**Figure 10**: Cisplatin 100ppm YSM vasculature HH 20-21	**Figure 11**: Carboplatin 100ppm YSM vasculature HH 20-21	**Figure 12**: Combination 100ppm YSM vasculature HH20-21
			
Vessel density= 26.7% Total vessel network length=19102 px Total branching points=263 Total segments=512	Vessel density= 19.2% Total vessel network length=52939 px Total branching points=212 Total segments=438	Vessel density= 21.7% Total vessel network length=16156px Total branching points=158 Total segments =365	Vessel density=23.8 % Total vessel network length=8102 px Total branching points=133 Total segments=247

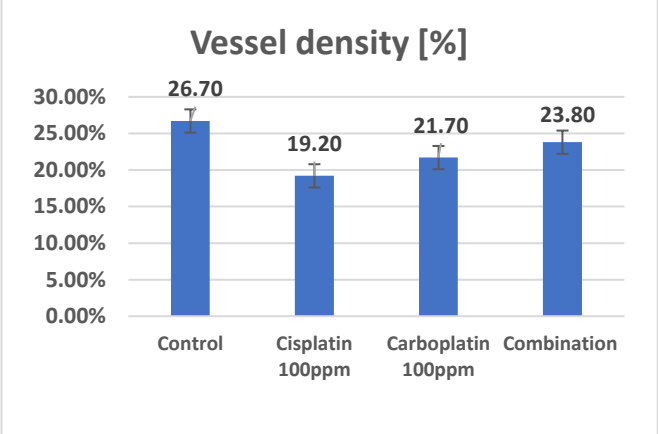


Figure 13: Vessel density of YSM in HH 20-21 treated chicken embryos

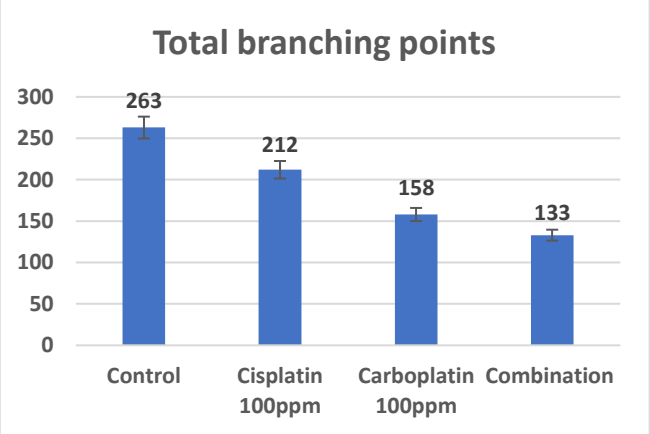


Figure 15: Total branching points of YSM of control and treated HH 20-21 chicken embryos

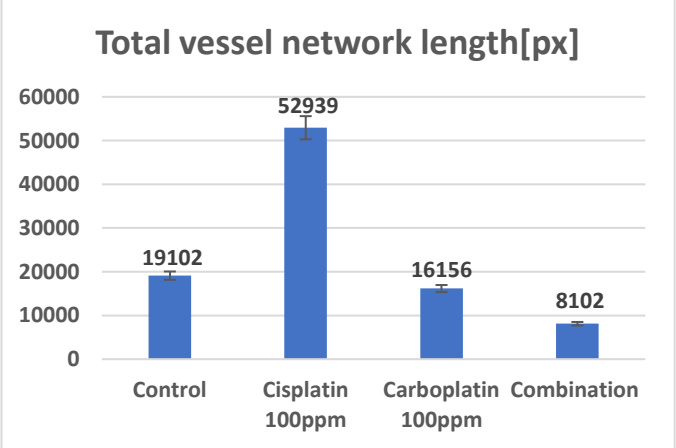


Figure 14: Total vessel network length of YSM of control and treated HH20-21 chicken embryos

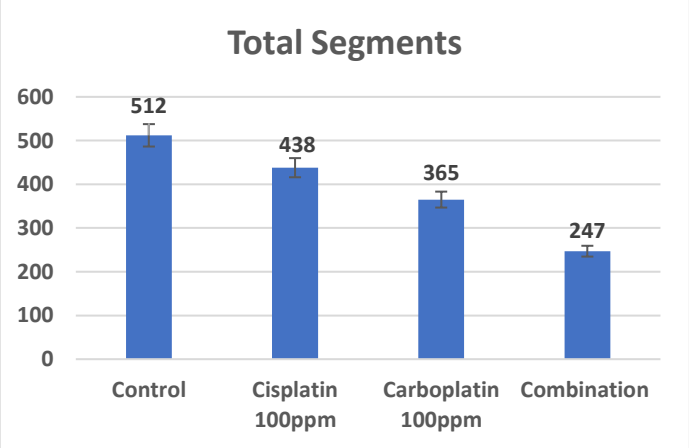


Figure 16: Total segments of YSM of control and treated HH20-21 chicken embryos

3.3. Biochemical studies

Biochemical studies revealed that treatment with cisplatin, carboplatin, and their combination altered the protein, AChE, LDH, and ALP levels of treated embryos compared to controls. Total protein content(Figure 17) was found to increase in treated groups, with cisplatin (3.88 µg/ml) and carboplatin (5.50 µg/ml) showing higher levels than the control (2.84 µg/ml), while the combination group exhibited the maximum increase (6.76 µg/ml). Acetylcholinesterase activity(Figure 18) was elevated in cisplatin (1187 IU/L) and carboplatin (989 IU/L) treated embryos compared to control (942 IU/L), but the level was markedly reduced in the combination group (767 IU/L). LDH activity(Figure 19), which was 2837 IU/L in the control, showed a slight reduction in cisplatin (2619 IU/L) and a mild increase in carboplatin (2948 IU/L), whereas the combination treatment caused a drastic reduction (944 IU/L). Similarly, alkaline phosphatase(Figure 20) activity was reduced from 216.7 IU/L in the control to 156.07 IU/L and 148 IU/L in cisplatin and carboplatin groups, respectively, with the combination group showing the lowest activity (52.68 IU/L).

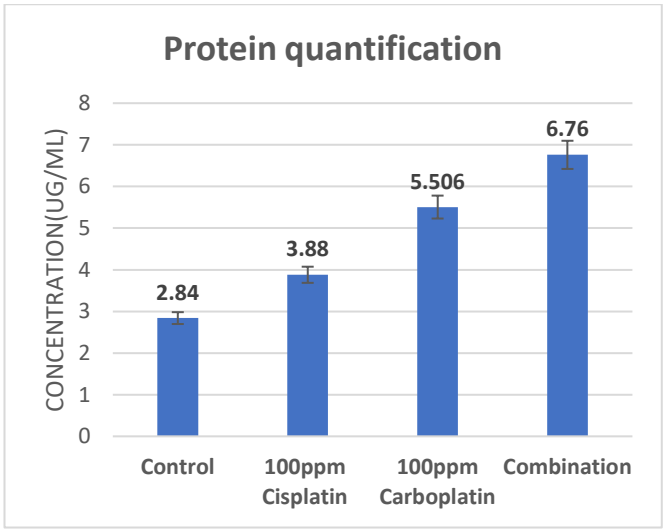


Figure 17: Protein quantification of control and treated HH20-21 chicken embryos

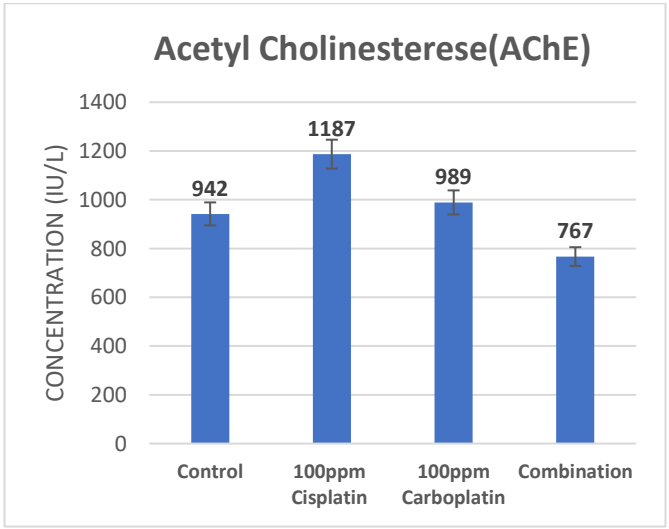


Figure 18: Acetyl Cholinesterase levels of control and treated HH20-21 chicken embryos

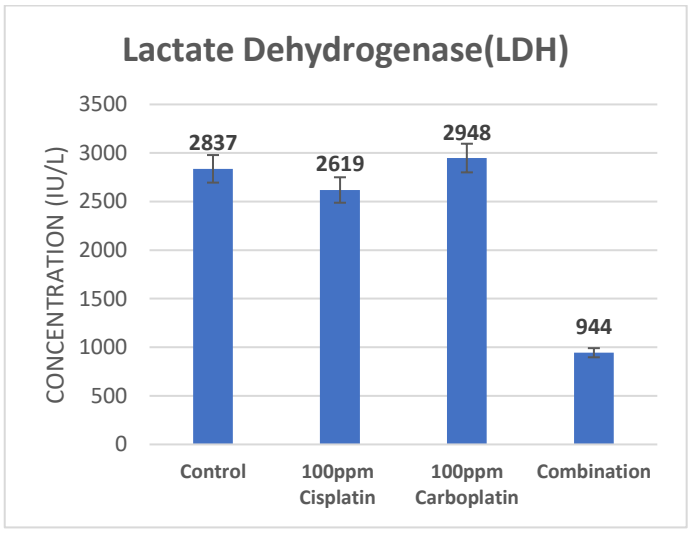


Figure 19: Lactate dehydrogenase levels of control and treated HH20-21 chicken embryos

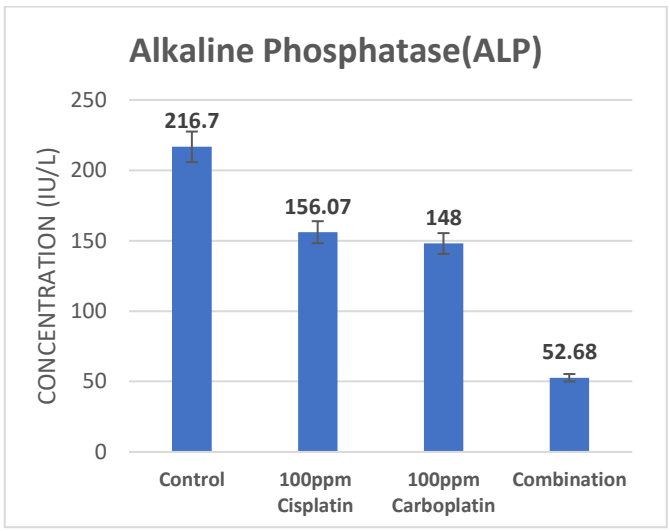


Figure 20: Alkaline phosphatase levels of control and treated HH20-21 chicken embryos

4. DISCUSSION

This study shows that when Cisplatin and Carboplatin individually retard embryonic development, but their combination (Cisplatin Carboplatin) produces a pronounced synergistic effect. Protein content in treated groups increases relative to control (2.84 µg/ml), rising in Cisplatin (3.88 µg/ml), Carboplatin (5.50 µg/ml) and highest protein level in combination (Cisplatin + Carboplatin) group (6.76 µg/ml), indicating amplified stress protein synthesis due to combined exposure

Enzyme activity assays revealed distinct signatures of toxicity. Acetylcholinesterase (AChE) activity rose with single-drug treatments—for Cisplatin group (1187 IU/L) and for Carboplatin group (989 IU/L)—relative to the control group (942 IU/L), reflecting neurotoxic hyperactivation of cholinergic signalling. However, in the combination group, AChE activity dropped sharply to 767 IU/L, suggesting that extensive cytotoxicity impaired normal cholinergic regulation. Lactate dehydrogenase

(LDH) levels followed a similar non-linear trend, while control group (2837 IU/L), cisplatin (2619 IU/L), and carboplatin (2948 IU/L) embryos retain measurable activity, the combination group showed a dramatic decline (944 IU/L). Rather than indicating reduced damage but rather reflects massive loss of viable cells that can release the enzyme. Alkaline phosphatase (ALP) activity, a marker of growth and differentiation, also declined progressively across treatments, falling from 216.7 IU/L in controls to 156.07 IU/L (cisplatin), 148 IU/L (carboplatin), and 52.68 IU/L in the combination group. This steep decline under dual treatment highlights profound retardation of developmental processes, and ossification [22].

These biochemical disruptions align closely with YSM and teratogenicity findings, where cisplatin and carboplatin together caused severe impairment of vascular development and embryonic morphology. Phenotypically, treated embryos exhibit severe cranial dysmorphogenesis, absent somites, and neural tube closure failure were consistent with the mechanism of platinum compound toxicity, which centres on DNA crosslinking, replication blockade, and apoptosis in highly proliferative embryonic tissues [23,24]. Differential effects were also evident, cisplatin induced more severe morphological malformations than carboplatin, while carboplatin produced hematomas, likely due to their distinct plasma protein binding kinetics—cisplatin binding irreversibly and carboplatin reversibly, influencing bioavailability and tissue distribution [25].

Yolk sac membrane (YSM) data analysis further confirmed profound vascular disruption. Reductions in vessel density, network length, and branching indicate anti-angiogenic activity, consistent with suppression of VEGF signaling [23]. Hematomas in carboplatin-treated embryos further suggest endothelial fragility [26].

Platinum-based compounds have been linked to hepatotoxicity, with reported alterations in ALP [27]. While some studies report increased ALP after cisplatin [28], our findings of decreased ALP in all groups suggest metabolic dysfunction and impaired nutrient exchange across the compromised yolk sac vasculature [29]. Likewise, the observed decline in LDH and AChE under combination treatment parallels literature noting synergistic Platinum effects on glycolytic metabolism [30] and cholinergic disruption [31,32]. The sharp reduction in AChE activity directly correlates with neural tube defects, pointing to disrupted cholinergic system maturation [33].

Collectively, the above study reveals that cisplatin and carboplatin act synergistically to hijack embryonic developmental mechanisms. Dual exposure triggers aberrant protein synthesis, neurodevelopment disturbances and angiogenesis inhibition, culminating in catastrophic morphological outcomes.

5. CONCLUSION

This study demonstrates that while cisplatin and carboplatin individually impair embryonic development, their combined administration produces a markedly synergistic toxicity in *Gallus gallus domesticus* model. Co exposure exacerbated morphological malformations, vascular degeneration and biochemical effects beyond the effects of either agent alone, highlighting profound interference with angiogenesis. These findings underscore a critical paradox: although cisplatin–carboplatin combinations are pursued clinically to counteract resistance, their embryotoxic potential is significantly magnified. The work emphasizes the importance of evaluating developmental toxicity when designing platinum-based regimens and supports the use of chick embryo models as sensitive platforms for mechanistic and safety profiling of chemotherapeutic drug combinations.

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