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Evaluating the hematological impact of ethion and silver nanoparticles on avian embryos: Protective effects and toxicity at varying doses

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Abstract
This experiment investigated the effects of ethion, silver nanoparticles (AgNPs), and their combination on avian embryo hematological parameters. Exposure to ethion, an organophosphate insecticide, led to a dose-dependent decrease in hemoglobin, RBC count, PCV, MCV, MCH, and MCHC due to oxidative stress and disruption of erythropoiesis, resulting in smaller, less hemoglobin-rich red blood cells. Additionally, ethion treatment caused increases in WBC and platelet counts, reflecting an inflammatory response to the induced toxicity. When AgNPs were co-administered with ethion, they initially exhibited a protective effect by improving hemoglobin, RBC count, and PCV, likely due to their antioxidant properties that counteracted ethion-induced damage. However, at high doses, AgNPs themselves became toxic, causing reductions in these hematological parameters and increases in WBC and platelet counts, indicative of an excessive inflammatory response. The combination of high-dose AgNPs with ethion further exacerbated these effects, demonstrating a complex interplay between the substances and their potential for combined toxicity. These findings highlight the dual nature of AgNPs as both protective agents and potential sources of toxicity, underscoring the need for careful dosage management to balance therapeutic benefits with adverse effects. Further research is essential to elucidate the mechanisms of these interactions and optimize the use of AgNPs in mitigating pesticide-induced toxicity.

Keywords: Ethion, silver nanoparticles, hematology, toxicity, inflammation

1. Introduction
Pesticides are chemicals used to manage pests that threaten crops, animals and human health (Sharma *et al*., 2020) [1]. They enhance agricultural productivity by controlling insects, weeds, fungi and other harmful organisms. Among the pesticide groups, organophosphates (OPs), organochlorines (OCs), carbamates and pyrethroids (Mediene *et al*., 2011) [2] are more rampantly used these days. Among which organophosphorus pesticides (OPs) are most commonly used due to their lower environmental persistence compared to organochlorine pesticides (Van Dyk *et al*., 2011) [3]. OPs, including ethion function by inhibiting acetylcholinesterase, an enzyme critical for nerve function which effectively controls pests but also poses significant health risks (Kwong, 2002) [4]. Exposure to OPs can lead to neurotoxicity, respiratory issues, cardiovascular problems and endocrine disruption with potential developmental effects if exposure occurs during pregnancy including cognitive and behavioral impairments (Eskenazi *et al*., 1999) [5].
Ethion, an organothiophosphate is extensively used to control pests on various crops such as citrus fruits, cotton, and vegetables. It inhibits acetylcholinesterase, leading to pest paralysis and death (Mazloomi *et al*., 2024) [6]. However, ethion can cause acute neurotoxicity, respiratory distress and gastrointestinal issues (Singh *et al*., 2023) [7]. Long-term exposure can trigger apoptosis or programmed cell death by overstimulating neural pathways and causing oxidative stress and inflammation particularly in the liver and nervous system (Bhatti *et al*., 2010) [8].
Silver nanoparticles (AgNPs), with sizes between 1 and 100 nanometers; possess unique properties due to their small size and large surface area (Zawadzka *et al*., 2014) [9]. They are widely used for their antimicrobial properties in medical devices, water purification and food

packaging. Silver nanoparticles exhibit beneficial activities including antimicrobial, anti-inflammatory and anti-apoptotic effects making them useful in reducing oxidative stress and cell damage. However, high doses of AgNPs can lead to toxicity including cytotoxicity, genotoxicity and apoptosis highlighting the importance of careful dosage management (Kim and Ryu, 2013) [11].

Hematological parameters are crucial in toxicity studies as they reveal the impact of toxicants on blood cell production, immune function and overall health (Petterino and Argentino-Storino, 2006) [12]. Changes in these parameters can indicate conditions such as anemia, inflammation or immune suppression resulting from toxic exposure (Bignucolo *et al*., 2012) [13]. Monitoring hematological parameters helps assess the systemic effects of toxicants and their impact on biological systems (Pang *et al*., 1987) [14].

Thus, the present study was designed to evaluate the effects of ethion, silver nanoparticles either alone or in combination on developing avian embryos at multiple doses, with a focus on hematological parameters.

2. Materials and Methods

2.1 Drugs and Chemicals: Ethion was procured from Cheminova Industries Pvt. Ltd. (Mumbai, India). All other chemicals used in the study were of technical grade and obtained from reputable suppliers. Silver nanoparticles (AgNPs) were synthesized according to the method described by Yerragopu *et al*. (2020) [15].

2.2 Handling and Preparation of Fertilized eggs: Fertile White Leghorn eggs were sourced from the Instructional Livestock Farm Complex (ILFC) at the College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala with study approval from the Institutional Animal Ethics Committee (IAEC/COVAS/PKD/21/01/2023 dated 12.07.2023). The eggs were disinfected with 70% alcohol, dried weighed to approximately 50±5 grams and labelled. They were candled to discard any defective eggs and mark the air cell position, then incubated at 37.8 °C with 55% humidity, and turned every four hours (Farag *et al*., 2021) [16].

2.3 Yolk Sac Inoculation: On the fifth day of incubation, 120 eggs were candled to observe the vitelline vessels. Subsequently, each egg was inoculated with a 100 µL dose containing ethion, silver nanoparticles (AgNPs), or their combinations via the yolk sac (Bhanja *et al*., 2004) [17]. The eggs were allocated into ten groups: vehicle control, silver nanoparticle group, ethion treatment groups, and their combinations. The specifics of the experimental design are outlined in the table below.

2.4 Experimental design and Grouping: In this study, 10 groups of 12 embryonated eggs each were used to evaluate the effects of silver nanoparticles and ethion, individually and in combination on avian embryo development. Group 1 served as the control, receiving 100 µL of olive oil. Groups 2, 3, and 4 received silver nanoparticles at doses of 25, 50, and 100 µg/egg respectively, while Groups 5, 6, and 7 were treated with ethion at doses of 73.5, 147, and 294 µg/egg, all in 100 µL of olive oil. Groups 8, 9, and 10 received combined treatments of AgNP-25 µg/egg + ETH-73.5 µg/egg, AgNP-50 µg/egg + ETH-147 µg/egg, and AgNP-100 µg/egg + ETH-294 µg/egg, respectively. Dosing was initiated on the 5th day of incubation and eggs were incubated until day 19, when they were opened for hematological analysis to assess the effects of the treatments as detailed in table 1 respectively.

2.5 Hematology parameters: On the 19th day of incubation, blood samples were collected to assess hematological parameters. Approximately 1 mL of blood was extracted from each embryo via jugular puncture into EDTA vials. The samples were analyzed for hematological parameters using the Urit 3000 VetPlus (China).

2.6 Statistical analysis: The data are presented as Mean ± SEM (n=12). Statistical significance was determined using one-way analysis of variance, followed by Tukey's multiple comparison test, conducted with SPSS Version 24.0.

Table 1: Treatment and dosing schedule for *In-ovo* experimentation with ethion, AgNPs, and their combinations in the developing avian embryo model

Groups	Treatment	Dose/ Route (*In-ovo*)	No. of eggs
1	Vehicle	Olive oil @ 100 µL/egg	12
2	Silver Nanoparticle (AgNP-25)	25 µg/egg in 100 µL of olive oil	12
3	Silver Nanoparticle (AgNP-50)	50 µg/egg in 100 µL of olive oil	12
4	Silver Nanoparticle (AgNP-100)	100 µg/egg in 100 µL of olive oil	12
5	Ethion (ETH-73.5)	73.5 µg/egg in 100 µL of olive oil	
6	Ethion (ETH-147)	147 µg/egg in 100 µL of olive oil	12
7	Ethion (ETH-294)	294 µg/egg in 100 µL of olive oil	
8	Silver Nanoparticle + (AgNP-25)Ethion (ETH-73.5)	Silver nanoparticle (25 µg/egg) in 100 µL of olive oil+ Ethion (73.5 µg/egg) in 100 µL of olive oil	12
9	Silver Nanoparticle (AgNP-50) +Ethion (ETH-147)	Silver nanoparticle (50 µg/egg) in 100 µL of olive oil +Ethion (147 µg/egg) in 100 µL of olive oil	12
10	Silver Nanoparticle (AgNP-100) + Ethion (ETH-294)	Silver nanoparticle (100 µg/egg) in 100 µL of olive oil + Ethion (294 µg/egg) in 100 µL of olive oil	12

3. Results and Discussion

Table 2: Effect of *In-ovo* administration of Ethion (ETH), Silver nanoparticles (AgNp) and their combination on hematological parameters on the 19th day of avian embryo development

Treatment	Hb (g/dL)	PCV (%)	RBC (x10^6/µL)	WBC (x10^3/µL)	PLT (x10^3/µL)
Vehicle	12.69±0.98^d	28.39±1.39^d	5.21±0.18^d	9.86±0.69^a	49.23±1.89^a
AgNP-25 (A1)	12.58±0.86^d	28.45±1.28^d	5.28±0.16^d	9.78±0.59^a	48.69±1.84^a
AgNP-50 (A2)	12.86±1.08^d	29.28±1.26^d	5.30±0.16^d	10.02±0.63^a	49.21±1.72^a
AgNP-100 (A3)	11.09±1.06^c	25.69±1.24^{cd}	4.89±0.17^{cd}	10.98±0.71^b	50.28±1.96^a
ETH-73.5 (E1)	11.89±1.04^d	29.56±1.30^d	5.27±0.21^d	9.56±0.63^a	48.63±1.57^a
ETH-147 (E2)	9.85±1.03^b	21.85±1.27^b	3.86±0.22^b	14.39±0.71^c	56.39±1.86^b
ETH-294 (E3)	8.69±1.04^a	18.63±1.29^a	3.10±0.20^a	18.63±0.68^d	64.20±1.95^c
AgNP-25 +ETH-73.5 (AE1)	12.68±1.09^d	29.64±1.30^d	5.29±0.16^d	9.68±0.69^a	47.96±1.75^a
AgNP-50 +ETH-147 (AE2)	12.76±1.08^d	28.63±1.34^d	5.68±0.21^d	9.52±0.70^a	48.38±1.69^a
AgNP-100+ ETH-294 (AE3)	8.68±1.07^a	19.63±1.38^a	3.21±0.20^a	14.69±0.68^c	65.39±1.75^c

Units are expressed as grams (g), dL (decilitre), µL (microlitre), hemoglobin (Hb), packed cell volume (PCV), red blood cell (RBC), white blood cell (WBC), and platelets (PLT). Values are expressed as Mean ± SEM (n=12). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test. comparison was made between each groups having different superscripts denoting the level of significance indicates $p<0.05$ respectively.

Hematological parameters, including hemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), and platelets (PLT), were evaluated in avian embryos on the 19th day of development. These embryos were treated with ethion, silver nanoparticles, or combinations thereof. Results revealed a significant ($p < 0.05$) decrease in Hb, PCV, and RBC values in groups A3, E2, E3, and AE3 compared to the control. Conversely, groups AE1 and AE2 demonstrated a significant ($p < 0.05$) improvement in these parameters, approaching levels comparable to the control group.

Table 3: Effect of *In-ovo* administration of Ethion (ETH), Silver nanoparticles (AgNp) and their combination on MCV, MCH and MCHC parameters on the 19th day of avian embryo development

Treatment	MCV (fL)	MCH (pg)	MCHC (g/L)
Vehicle	105.69±2.36^d	39.68±1.86^d	35.25±1.21^d
AgNP-25 (A1)	108.63±2.37^d	38.54±1.75^d	34.63±1.18^d
AgNP-50 (A2)	107.57±2.52^d	38.69±1.68^d	34.58±1.16^d
AgNP-100 (A3)	101.53±2.68b^c	34.75±1.85b^c	30.42±1.20b^c
ETH-73.5 (E1)	108.36±2.42^d	39.63±1.63^d	35.68±1.14^d
ETH-147 (E2)	95.62±2.49^b	30.25±1.35^b	28.63±1.20^b
ETH-294 (E3)	90.26±2.38^a	26.96±1.71^a	25.86±1.16^a
AgNP-25 +ETH-73.5 (AE1)	106.95±2.85^d	39.36±1.39^d	34.75±1.21^d
AgNP-50 +ETH-147 (AE2)	108.67±2.69^d	38.54±1.74^d	35.69±1.15^d
AgNP-100+ ETH-294 (AE3)	91.38±2.75^a	31.27±1.79^a	28.74±1.16^a

The administration of ethion, silver nanoparticles, or their combinations to avian embryos on the 19th day of development significantly decreased mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Groups A3, E3, and AE3 exhibited a significant ($p<0.05$) decrease in these parameters compared to the control, while group AE2 demonstrated a significant ($p<0.05$) amelioration of ethion toxicity, approaching control levels.

4. Discussion

Globally, pesticide use is integral to agriculture, with around 4.1 million tonnes used annually as of 2021, led by countries like China, Brazil and the U.S. India, the fourth largest pesticide producers consumed 62,000 tonnes in 2021-2022, primarily in states like Maharashtra, Uttar Pradesh, and Punjab (Reddy *et al*., 2024) [18]. Organophosphates (OPs) like ethion are commonly used due to their effectiveness and lower bioaccumulation compared to organochlorines, though their overuse raises environmental and health concerns (Desouky *et al*., 2013) [19]. Ethion, widely applied to crops like cotton, citrus and vegetables, inhibits acetylcholinesterase in pests but poses risks to non-target organisms and can contaminate water sources (Foster *et al*., 2004) [20]. To mitigate such toxicity, silver nanoparticles (AgNPs) offer promise due to their antimicrobial, anti-inflammatory, antioxidant and anticancer properties (Sofi *et al*., 2022) [21]. AgNPs can promote wound healing, induce cancer cell apoptosis and enhance drug delivery by improving bioavailability, making them valuable in both agriculture and medicine (Shaikh *et al*., 2021) [22].

Exposure of the avian embryo to ethion led to a dose-dependent decrease in hemoglobin, RBC and PCV levels. This decline can be attributed to the toxic effects of ethion, an organophosphate which disrupts normal erythropoiesis (red blood cell production) by inhibiting acetylcholinesterase and inducing oxidative stress (Lu, 1995) [23]. Organophosphates are known to cause oxidative damage to cell membranes, including red blood cells leading to hemolysis and a reduction in their count (Nafisah *et al*., 2023) [24]. Hemoglobin, being a component of RBCs, decreases proportionally as RBCs are destroyed (Ozkul *et al*., 2021) [25]. PCV (packed cell volume), which is the percentage of blood volume occupied by RBCs also drops as RBC numbers decline (Shukla *et al*., 2022) [26]. The correlation between these parameters lies in the fact that RBC count directly affects both hemoglobin concentration and PCV; a reduction in RBCs lowers hemoglobin and consequently the PCV decreases (Ranjith *et al*., 2022) [27].

When silver nanoparticles (AgNPs) were co-administered with ethion, an increase in hemoglobin, RBC and PCV levels was observed suggesting a protective effect (Imani *et al*., 2015) [29]. AgNPs have antioxidant properties, which likely counteracted the oxidative stress induced by ethion, reducing RBC damage and preserving their numbers (Bhanja *et al*., 2022) [28]. By limiting oxidative damage, AgNPs helped to maintain normal erythropoiesis and prevent hemolysis leading to an improvement in hemoglobin and PCV levels (Kulak *et al*., 2018) [30].

However, at high doses silver nanoparticles themselves showed toxicity leading to a decrease in these parameters (Song *et al*., 2017) [31]. This toxicity could be due to excessive ROS (reactive oxygen species) generation at high concentrations of AgNPs, overwhelming the antioxidant defenses of the cells and resulting in oxidative damage similar to ethion (Ognik *et al*., 2016) [32]. Excessive oxidative stress can impair red blood cell production, increase hemolysis and disrupt cellular processes leading to a reduction in RBC count, hemoglobin and PCV as seen in the high-dose AgNP group (Flores-López *et al*., 2019) [33].

Thus, while AgNPs provide protection at lower doses by mitigating oxidative stress, higher doses can exert toxic effects which are likely due to excessive ROS production leading to similar hematological changes as those caused by ethion alone (Katarzyńska-Banasik *et al*., 2021) [34].

MCV (Mean Corpuscular Volume) measures the average size of red blood cells. Ethion exposure likely impairs red blood cell production and leads to oxidative stress-induced damage causing the formation of smaller RBCs (microcytosis), thus lowering MCV (Utreja *et al*., 2018) [35].

MCH (Mean Corpuscular Hemoglobin) reflects the average amount of hemoglobin per RBC. Ethion-induced oxidative stress can damage RBCs, decreasing their hemoglobin content. This oxidative damage may impair hemoglobin synthesis or lead to hemolysis of mature RBCs reducing the average hemoglobin content (MCH) (Shukla *et al*., 2022) [26].

MCHC (Mean Corpuscular Hemoglobin Concentration) measures the concentration of hemoglobin within a given volume of RBCs. A reduction in RBC size (microcytosis) and hemoglobin content leads to a proportional decrease in MCHC, indicating that the remaining RBCs are not only smaller but also carry less hemoglobin relative to their size (Ozkul *et al*., 2021) [25].

A reduction in RBC size (MCV) directly affects the amount of hemoglobin (MCH) each RBC can hold, and the concentration of hemoglobin in relation to RBC volume (MCHC) will also drop as a consequence of these reductions. The cumulative effect of ethion-induced oxidative stress likely disrupts normal erythropoiesis and RBC morphology contributing to decreases in these parameters (Elbialy *et al*., 2015) [36].

Co-administration of silver nanoparticles (AgNPs) with ethion led to an increase in MCV, MCH and MCHC levels, suggesting a protective role of AgNPs. AgNPs are known for their antioxidant properties which help neutralize the reactive oxygen species (ROS) (Bhanja *et al*., 2022) [28] generated by ethion by reducing oxidative damage to RBCs. This allows for normal red blood cell development and maturation, restoring RBC size (MCV) and hemoglobin content (MCH) and leading to normalization of MCHC. By preventing oxidative stress, AgNPs likely facilitate healthy erythropoiesis and prevent RBC shrinkage and hemolysis (Kulak *et al*., 2018) [30].

At high doses, silver nanoparticles showed a decrease in MCV, MCH and MCHC indicating toxicity (Rezaei *et al*., 2018) [37]. While AgNPs have protective effects at lower concentrations, excessive doses can lead to increased ROS production overwhelming the antioxidant defense mechanisms (Gallocchio *et al*., 2017) [39]. This excessive oxidative stress at high doses can impair red blood cell production, damage existing RBCs and disrupt hemoglobin synthesis (Ahmadi and Branch, 2012) [40]. As a result, RBCs become smaller and less efficient at carrying hemoglobin causing reductions in MCV, MCH and MCHC mimicking the toxic effects observed with ethion treatment alone (Ognik *et al*., 2016) [32].

WBC (White Blood Cells): Ethion, being an organophosphate insecticide, induces oxidative stress and systemic toxicity (Ozkul *et al*., 2021) [25]. The increase in WBC count is a common physiological response to toxic stress, reflecting an inflammatory or immune response (Nafisah *et al*., 2023) [24]. Ethion's toxicity can trigger an immune response to combat potential damage and inflammation leading to leukocytosis (an increase in WBC count). This response is typically a protective mechanism to handle and mitigate the effects of the toxin (Cortés-Iza *et al*., 2017) [41].

Elevated platelet counts (thrombocytosis) can also result from systemic inflammation or stress. Ethion-induced toxicity can stimulate the production of inflammatory cytokines and other mediators that prompt the bone marrow to produce more platelets (Dündar *et al*., 2015) [42]. Increased platelet production is often a response to tissue damage or inflammation aiming to enhance clotting and repair processes (Ranjith *et al*., 2022) [27].

When silver nanoparticles (AgNPs) were co-administered with ethion, WBC and platelet counts decreased (Wong *et al*., 2009) [43]. This indicates that AgNPs, at lower doses might counteract some of the inflammatory and stress responses induced by ethion. AgNPs have known anti-inflammatory properties which could reduce the inflammatory and immune responses triggered by ethion (Gholami-Ahangaran and Zia-Jahromi, 2014) [38]. Consequently, this leads to lower WBC and platelet counts reflecting reduced systemic inflammation and stress (Khafaga *et al*., 2022) [44].

At high doses, silver nanoparticles themselves can be toxic. High doses of AgNPs may generate excessive oxidative stress or inflammation, overwhelming the body's ability to manage the oxidative load (Ahmadi and Kurdestany, 2010) [45]. This can lead to leukocytosis and thrombocytosis as the body reacts to the excessive stress and inflammation caused by the high concentration of AgNPs (Hashem *et al*., 2023) [46]. The elevated WBC and platelet counts in this scenario indicate that high-dose AgNPs are inducing a similar inflammatory response as seen with ethion, potentially exacerbating the stress and inflammatory response rather than alleviation.

When high doses of AgNPs are combined with ethion, the combined toxicity can significantly elevate the inflammatory and stress responses (Song *et al*., 2017) [31]. The synergistic effect of both substances may lead to a heightened immune response, reflected in increased WBC and platelet counts (Glinski *et al*., 2016) [48]. The excessive oxidative stress and inflammation caused by both high doses of AgNPs and ethion contribute to this response demonstrating the complexity of their interactions and the potential for combined toxicity (Prasannaraj *et al*., 2017) [49].

In this experiment, avian embryos were exposed to ethion, silver nanoparticles (AgNPs), and their combination to assess the impact on hematological parameters (Rezaei *et al*., 2018;

Shukla *et al*., 2022) [37, 26]. Ethion treatment led to a dose-dependent decrease in hemoglobin, RBC count, PCV, MCV, MCH and MCHC attributing to oxidative stress and disruption of erythropoiesis resulting in smaller and less hemoglobin-rich red blood cells (Shukla *et al*., 2021) [50]. Simultaneously, ethion caused increases in WBC and platelet counts due to an inflammatory and stress response (Verma and Sethi, 2020) [47].

When AgNPs were co-administered with ethion, there was an improvement in hemoglobin, RBC count and PCV suggesting a protective effect of AgNPs against ethion-induced oxidative stress (Gholami-Ahangaran and Zia-Jahromi, 2014) [38]. AgNPs helped to mitigate the damage to RBCs, leading to restored levels of these parameters (Wong *et al*., 2009) [43]. However, at high doses AgNPs themselves exhibited toxicity causing a decrease in hemoglobin, RBC count and PCV similar to the effects seen with ethion alone (Song *et al*., 2017) [31]. Additionally, high doses of AgNPs increased WBC and platelet counts, reflecting a heightened inflammatory response (Glinski *et al*., 2016) [48]. The combination of high-dose AgNPs with ethion exacerbated this response demonstrating a complex interplay between the substances and their combined toxic effects (Prasannaraj *et al*., 2017) [49].

Overall, while AgNPs showed protective benefits at lower doses, their toxicity at higher concentrations, combined with ethion resulted in increased inflammatory responses and adverse effects on hematological parameters

5. Conclusion

This experiment investigated the effects of ethion, silver nanoparticles (AgNPs) and their combination on avian embryo hematological parameters. Exposure to ethion, an organophosphate insecticide led to a dose-dependent decrease in hemoglobin, RBC count, PCV, MCV, MCH and MCHC due to oxidative stress and disruption of erythropoiesis resulting in smaller, less hemoglobin-rich red blood cells. Additionally, ethion treatment caused increase in WBC and platelet count, reflecting an inflammatory response to the induced toxicity. When AgNPs were co-administered with ethion, they initially exhibited a protective effect by improving hemoglobin, RBC count and PCV, likely due to their antioxidant properties that counteracted ethion-induced damage. However, at high doses AgNPs themselves became toxic causing reductions in these hematological parameters and increases in WBC and platelet counts indicative of an excessive inflammatory response. The combination of high-dose AgNPs with ethion further exacerbated these effects, demonstrating a complex interplay between the substances and their potential for combined toxicity. These findings highlight the dual nature of AgNPs as both protective agents and potential sources of toxicity, underscoring the need for careful dosage management to balance therapeutic benefits with adverse effects. Further research is essential to elucidate the mechanisms of these interactions and optimize the use of AgNPs in mitigating pesticide-induced toxicity.

Conflict of Interest
Not available

Financial Support
Not available

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