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Blood from Horses and Cows In Vitro Exposed to Quaternium-15 and Thiacloprid: Haematology and Erythrocyte Osmotic Fragility Alterations

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Abstract: Blood cells are constantly exposed to several pollutants, including xenobiotics, and they can be considered a useful target for pollution exposition of the animal. The aim of this study was to investigate the effects of two xenobiotics (Quaternium-15, a preservative used in personal care products, and Thiacloprid, a neonicotinoid pesticide) on the haematological profile and erythrocyte osmotic fragility (EOF) of equine and bovine blood samples. Ten blood samples from horses and cows were exposed for 24 h to Quaternium-15 at a concentration of 0.1 and 1 mg/L and to Thiacloprid at a concentration of 30 and 60 µg/mL. A decrease in the values of the red blood cells, white blood cells, haematocrit, haemoglobin, mean corpuscular volume, and platelets, and an increase of EOF were found in blood samples exposed to xenobiotics compared to the control. According to the results gathered in the current study, the two xenobiotic compounds herein tested negatively affect the haematological indices causing haemolysis both in cattle and horse blood. This study, despite being preliminary, emphasizes the concept that blood cells are an excellent target for evaluating the effects of xenobiotics.

Keywords: erythrocytes; haematological profile; xenobiotics; osmotic fragility; equine; bovine

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1. Introduction

The haematological profile is a collection of certain parameters obtained from the analysis of whole blood [1]. The evaluation of haematological indices allows us to understand the health status of the animal and evaluate the presence of pathological states [2–5]. Among the haematological parameters, the most used are haematocrit, haemoglobin, circulating erythrocyte, and leukocyte counts. However, the interpretation of haematological parameters in animals can be complex, and the benchmarks are very broad; indeed, haematological indices are influenced by various factors, including breed, age, reproductive status, season, and environmental factors [6–8]. When analysing these values, it must be understood that they can also be affected by sample collection and preparation, transport time, and the instrument used for the analysis [9–11]. Noteworthy, the complete haematological profile also makes it possible to assess environmental pollution. For this reason, there are numerous model organisms, including plants, amphibians, and fish, that are used for analysing the haematological profile for different purposes [12–15]. Moreover, animals, particularly grazers, including large and small ruminants and horses, are considered good indicators of environmental pollution. As a matter of fact, these animals inhabit the same space as humans and are exposed to the same pollutants through the ingestion of polluted vegetation, small amounts of soil, and drinking water [16]. Given the significance of environmental pollution, the evaluation of the presence of compounds in the environment is of paramount

importance, and, in this regard, the investigation of compounds' presence in certain biological indicators represents a valuable tool to assess animal exposition to pollutants. Among pollutants, xenobiotics, divided into natural and synthetic, are chemical compounds which are considered foreign to living beings exposure to these substances can be through the skin, the respiratory and digestive systems [17]. Blood cells are constantly exposed to various xenobiotics and metabolites, and thus, these cells can be considered a useful target for pollution exposition. It has been previously demonstrated that these compounds cause a breakdown in the integrity and metabolism of the entire blood cell population [18,19]. The effects of xenobiotics on red blood cells are many; for example, they cause the programmed death of these cells, known as eryptosis [20]. Eryptosis is often linked to an increase in osmotic fragility, and precisely because of this, the major signals are cell contraction and loss of membrane symmetry. This is important because osmotic fragility can be used as a biomarker of oxidative damage following exposure to xenobiotics [21]. Additionally, it will be possible to observe the translocation on the erythrocyte surface of certain membrane proteins, including phosphatidylserine. Furthermore, eryptosis is indicated by increased cytosolic calcium, ROS activity, oxidative stress, and energy depletion [22–24]. It has been demonstrated that xenobiotics can induce substantial direct or indirect damage to the cytoskeleton and alter cell metabolism [25]. Horses and cattle, which are grazers like sheep and goats, were identified for this study. The interest in studying the exposition of horses and cattle instead of sheep and goats is because they are exposed to significant amounts of substances and different pollutants by ingesting vegetation, soil, and water. For this reason, they can be considered detectors of environmental pollution. Blood samples from these two species were exposed to two compounds, Quaternium-15 and Thiacloprid, selected based on their concentrations in the environment and their effects on organisms. Specifically, Quaternium-15 is a preservative used for cosmetics such as shampoos, creams and lotions, disinfectants, and laundry soaps. According to the current literature, this compound could release formaldehyde into the environment, which is considered carcinogenic. Moreover, *Mytilus galloprovincialis* showed alteration of haemolymphatic and oxidative parameters following exposure to Quaternium-15 [26]. This compound is also under the magnifying glass because of its ability to resist metabolic degradation; in fact, its main characteristic is that it is lipophilic and is essential to be absorbed by target organisms [27]. Thiacloprid is a neonicotinoid pesticide and insecticide that have cytotoxic and genotoxic effects on human peripheral blood lymphocytes by reducing mitotic, proliferation, and nuclear division indexes. This class of pesticide has a function similar to that of nicotinic acetylcholine receptors and binds to GABA receptors located on the postsynaptic membrane, interrupting neuronal transmission [28–31]. In amphibians, fish, birds, and bats, even low exposure to neonicotinoids can cause immunosuppression. Neonicotinoids also bind to nicotinic (nACHr) and muscarinic receptors present on the surface of lymphocytes, which can cause a range of functional and biochemical effects. In addition, these pesticides are considered endocrine disruptors, causing an increase in thyroid hormones [32]. Given the above considerations, the aim of the present study was to investigate the effects of Quaternium-15 and Thiacloprid at different concentrations on the haematological profile together with erythrocyte osmotic fragility in two livestock species, horse and cattle, through in vitro whole blood exposure.

2. Materials and Methods

From 10 horses (Sella Italiana breed, aged 4–5 years) and 10 cows (Simmental breed, aged 3–4 years) living in the same area of Sicily, Italy (38°00′49″ N 15°25′18″ E, 80 m above sea level), blood samples were collected using 5 mL vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) upon the owner's informed consent. Before starting the study, horses and cows were subjected to clinical examination, routine haematology, and biochemistry analyses to assess their health status. Whole blood samples collected from horses and cattle were divided into 5 aliquots of 1mL each (one aliquot represents the control blood sample which was not exposed to xenobiotics, the other aliquots were exposed

to Quaternium-15 and Thiacloprid at two different concentrations for each xenobiotic). Thiacloprid (purity 99.9%) was obtained from Sigma-Aldrich, Česko, Czech Republic, and was dissolved in distilled water. The whole blood collected from horses and cattle was exposed for 24 h at 37 °C to Thiacloprid at a concentration of 30 µg/mL and 60 µg/mL, according to a previous study carried out on mammalian species [31]. The Quaternium-15 (hexamethylenetetramine chloroallyl chloride) concentrations to which whole blood was exposed were 0.1 mg/L and 1 mg/L, according to its presence in the environment as previously demonstrated [27]. The Quaternium-15 was obtained from Alfa Aesar GmbH & Co. KG A Johnson Matthey Company, Karlsruhe, Germany. The haematological profile was investigated using an automated hematology analyzer (HeCo Vet C; SEAC, Florence, Italy). Specifically, the values of red blood cell (RBC), haematocrit (Hct), haemoglobin (Hgb), mean corpuscular volume (MCV), white blood cell (WBC), and platelets (PLT) were assessed. Moreover, a manual analysis was performed on all whole blood samples to evaluate cell morphology. Specifically, two peripheral blood smears were performed for each sample, and after air drying, the obtained slides were stained through Dif-Stain kit (Titolchimica srl, Rome, Italy). The same laboratory professional later performed the microscopic analysis of blood films by using an optical microscope (Nikon Eclipse e200; Nikon Instruments Europe BV, Amsterdam, The Netherlands). For the determination of the erythrocyte osmotic fragility (EOF) test, a sodium chloride (NaCl) solution was prepared as previously described [33,34]. Briefly, 10 plastic tubes without anticoagulant or other additives (Nunc, Thermo Scientific, Wyman Street, Waltham, MA, USA) were prepared with 3 mL of serially increasing concentrations of NaCl solution (0.0% up to 0.9%) at pH 7.4, checked by a pH meter (HI 3220, Hanna Instruments, Milan, Italy). An aliquot of 0.05 mL of whole EDTA blood was transferred to each tube. After gentle mixing, the tubes were maintained at room temperature (26–27 °C) for 30 min, mixed once more, and centrifuged at 327 g for 15 min. The supernatant was transferred to a glass cuvette, and the concentration of haemoglobin in the supernatant solution was assessed by employing an automated analyzer UV Spectrophotometer (Slim SEAC, Florence, Italy) at 540 nm. For each tube, haemolysis was expressed as a percentage, with a 100% maximum after haemolysis in straight distilled water (0.0% NaCl concentration). The percentage of haemolysis was calculated according to the formula [33]:

$$\text{Haemolysis (\%): } = (\text{OD of test}/\text{OD of distilled water}) \times 100$$

where OD is optical density.

The EOF curve was obtained by plotting the percentage of haemolysis at each saline concentration.

Statistical Analysis

One-way repeated measures analysis of variance (ANOVA) was applied to determine the statistical effect of Quaternium-15 and Thiacloprid on the values of RBC, Hct, Hgb, MCV, WBC, PLT, and EOF obtained from blood samples collected from horses and cattle. When statistical significances were found, the Bonferroni post hoc comparison test was applied. p values < 0.05 were considered statistically significant. The statistical analysis was performed using the software Prism v. 9.00 (Graphpad Software Ltd., San Diego, CA, USA, 2020).

3. Results

3.1. Haematological Analyses

As shown in Figure 1, one-way ANOVA showed a significant effect of Quaternium-15 and Thiacloprid on the values of RBC, WBC, Hgb, Hct, MCV, and PLT measured in blood samples collected from horses and cows ($p < 0.05$). In particular, decreased values of RBC were observed in horse and bovine blood samples after exposure to Quaternium-15 at 0.1 mg/L and 1 mg/L and Thiacloprid at 30 µg/mL and 60 µg/mL compared to untreated blood samples. The WBC of both species showed decreased values after the exposition

to thiacloprid and Quaternium-15. The Hgb concentration measured in the equine and bovine blood samples treated with the two xenobiotics showed a statistically significant decrease than untreated blood samples. The tested xenobiotic compounds led to decreased Hct, MCV, and PLT values of equine and bovine blood than the untreated samples. The observation in RBC morphology on blood smears utilizing a light microscope highlighted the loss of normal morphology of RBC from bovine (Figure 2) and equine (Figure 3) blood samples after exposure to Quaternium-15 at 0.1 mg/L and 1 mg/L and Thiacloprid at 30 µg/mL and 60 µg/mL.

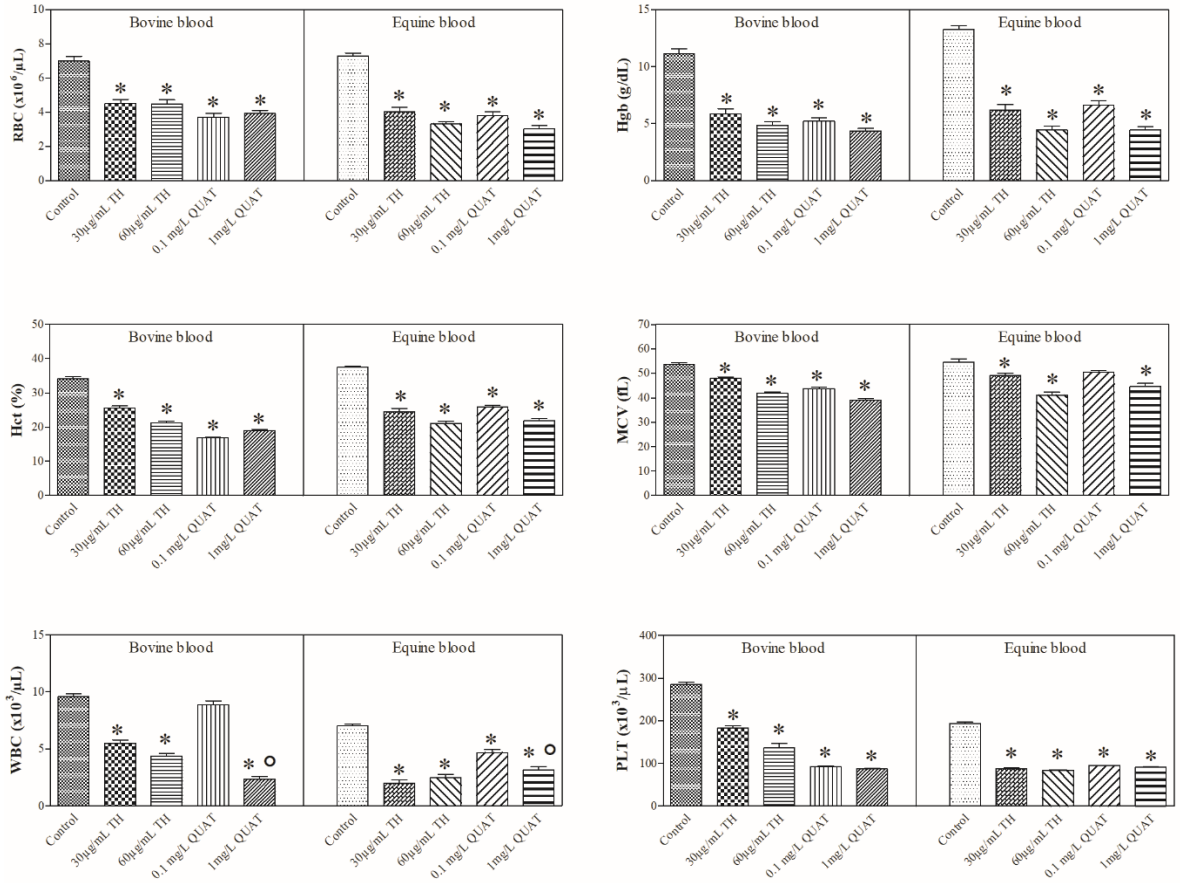

Figure 1. Mean values ± standard error of the mean (SEM) of red blood cell (RBC), haematocrit (Hct), haemoglobin (Hgb), mean corpuscular volume (MCV), white blood cell (WBC), and platelets (PLT) obtained from equine and bovine blood samples not exposed to xenobiotics (control) and blood samples exposed to Quaternium-15 at 0.1 mg/L (0.1 mg/L QUAT), Quaternium-15 at 1 mg/L (1 mg/L QUAT), Thiacloprid at 30 µg/mL (30 µg/mL TH), and to Thiacloprid at 60 µg/mL (60 µg/mL TH).

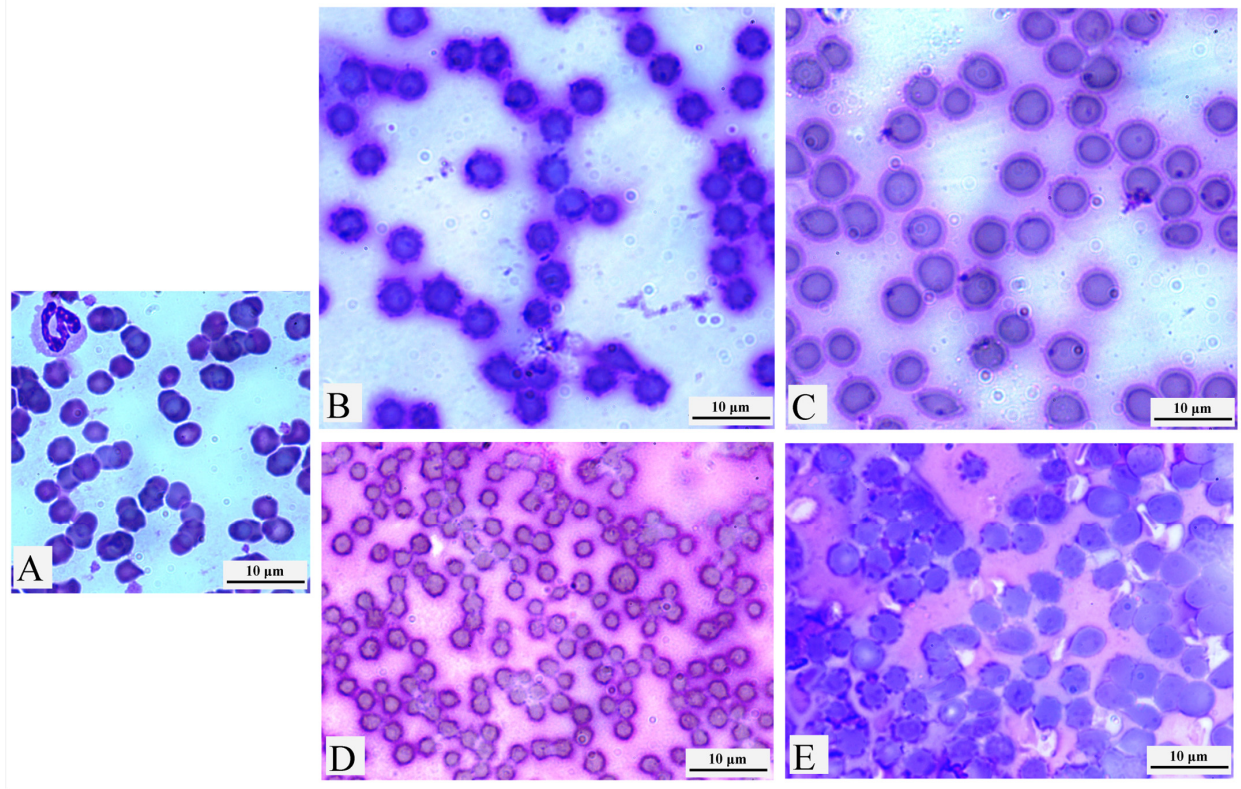

Figure 2. Erythrocytes appearance at light microscope (100× objective) obtained from bovine blood samples not exposed to xenobiotics (**A**) and blood samples exposed to Thiacloprid at 30 µg/mL (**B**), Thiacloprid at 60 µg/mL (**C**), Quaternium-15 at 0.1 mg/L (**D**), and Quaternium-15 at 1 mg/L (**E**).

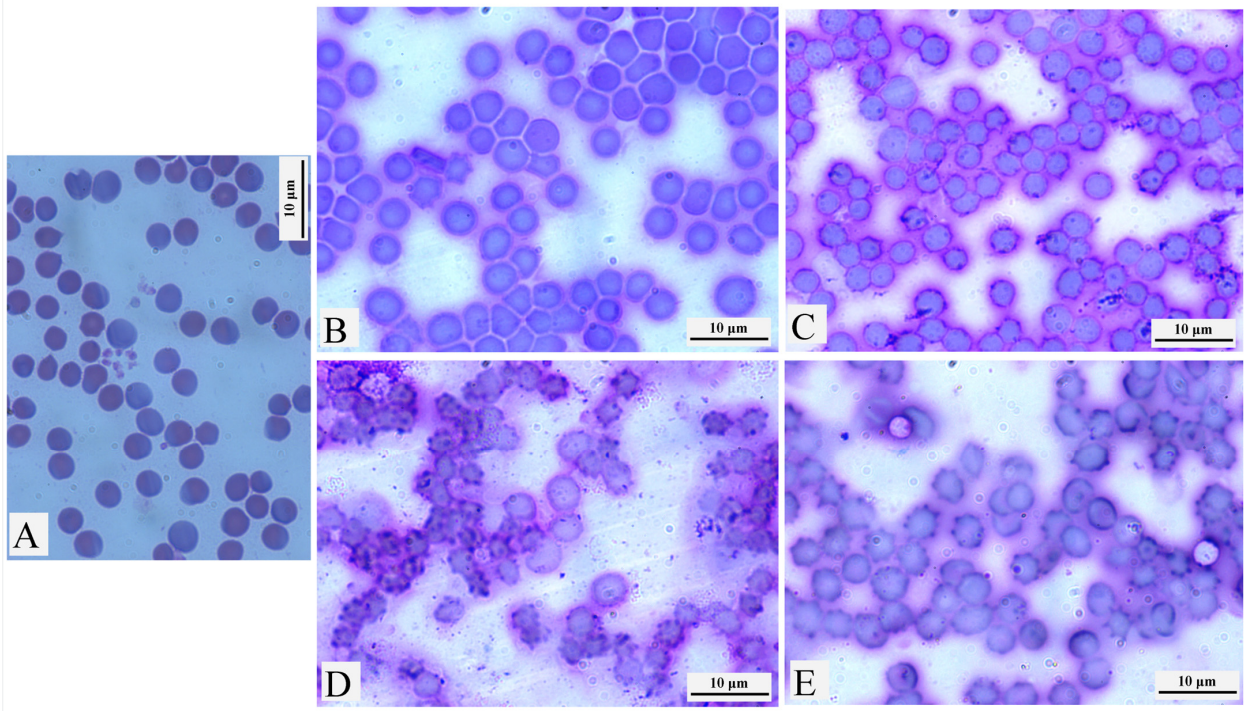

Figure 3. Erythrocytes appearance at light microscope (100× objective) obtained from equine blood samples not exposed to xenobiotics (**A**) and blood samples exposed to Thiacloprid at 30 µg/mL (**B**), Thiacloprid at 60 µg/mL (**C**), Quaternium-15 at 0.1 mg/L (**D**), and Quaternium-15 at 1 mg/L (**E**).

3.2. Erythrocyte Osmotic Fragility Analysis

The one-way ANOVA showed significant differences ($p < 0.05$) in the haemolysis trend of the analyzed horse and bovine blood samples at all concentrations of both xenobiotics tested (Figure 4). A statistically significant increase in EOF values at 0.9%, 0–8%, 0.7%, and 0.6% NaCl was observed in blood samples collected from cows treated with Quaternium-15 and Thiaclopird at all tested concentrations. A statistically significant increase in EOF values was observed in blood samples collected from horses treated with Thiacloprid at the concentration of 60 µg/mL and with Quaternium-15 at the concentration of 1 mg/L at 0.9%, 0.8%, and 0.7% NaCl, whereas, the EOF values were higher in blood samples treated with Quaternium at a concentration of 1mg/L compared to the control at 0.6% NaCl.

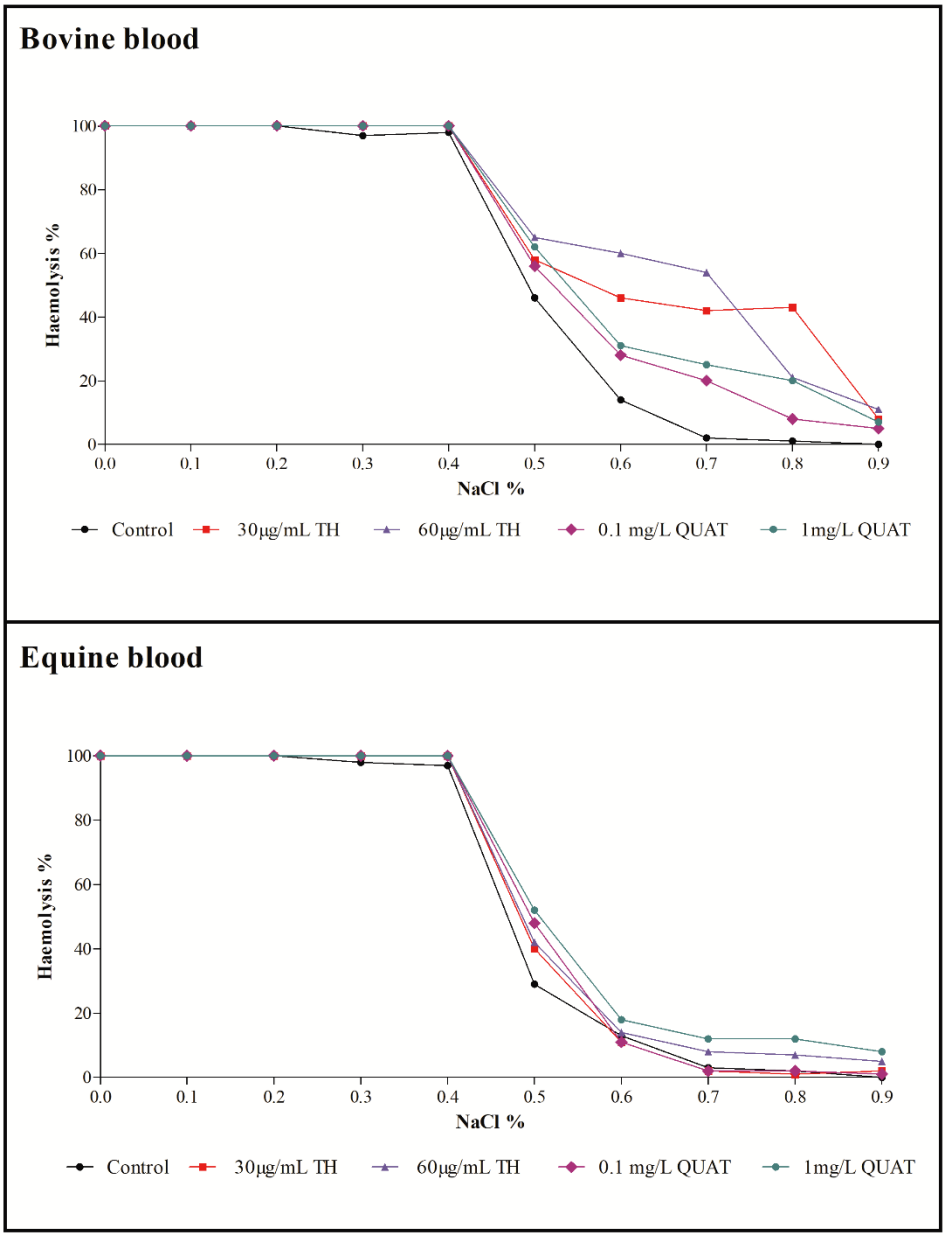

Figure 4. Erythrocyte osmotic fragility (hemolysis) curves obtained from bovine and equine blood samples exposed to Thiacloprid at 30 µg/mL, Thiacloprid at 60 µg/mL, Quaternium-15 at 0.1 mg/L, and Quaternium-15 at 1 mg/L.

4. Discussion

Industrialization, urbanization, and agricultural production represent important sources of extraneous chemicals, including xenobiotic compounds, for living organisms.

Xenobiotics, in particular pesticides and their careless use, can negatively affect the environment and animal and human health [34]. To the authors' knowledge, this is the first study assessing the effects of Quaternium-15 and Thiacloprid on erythrocytes parameters, including EOF in horse and cattle blood, through in vitro whole blood exposure. Despite being preliminary, the current study emphasizes the concept that all blood cells are an excellent target for evaluating the effects of xenobiotics.

The investigation of tissue, including blood, in animal species, represents a valuable tool to assess their health status as well as to investigate the relationship between blood properties and habitat, as well as the species' ability to adapt to the environment. The evaluation of haematologic variables is one of the most common investigations performed in clinical practice. Within the haematologic profile, erythrocyte variables represent a valuable tool for the assessment of the severity of many pathologic conditions. According to the results gathered in the current study, a decrease in RBC, Hct, Hgb, and MCV was observed both in blood samples from horses and cows exposed to Quaternium-15 and Thiacloprid. According to previous studies, the decrease of RBC and related erythrocyte parameters (i.e., Hgb, Hct, and MCV) is mainly caused by the ability of these xenobiotics to interfere with the biosynthesis of haemoglobin in the bone marrow, thus causing a reduction in the life length of circulating erythrocytes [35,36]. The decrease in RBC could also be due to the lipid peroxidation of their cell membrane and to the destruction of these cells already in the bone marrow [37]. There is also evidence that exposure to xenobiotics can affect their ability to incorporate iron and its destruction within erythrocytes [38]. In particular, decreases in Hgb, Hct, and MCV also appear to be induced by oxidative stress in erythrocytes, resulting in an increased production of ROS that can oxidise certain macromolecules such as phospholipids, DNA, and proteins [39]. Additionally, the flexibility of the erythrocyte membrane exposed to xenobiotic compounds could be compromised. Therefore, tests of the integrity and elasticity of the RBC membrane have value for the assessment of the proper function of RBC. One method used to determine the membrane elasticity of RBC is the EOF test. The degree of resistance of RBC to lysis, as a result of a decrease in the NaCl concentration of their environment, is the basis of the EOF test [21]. Osmotic fragility is widely used both in the human and veterinary field to elucidate mechanisms of the influence of different factors on the osmotic properties of RBC membranes, such as shear stress and mechanical hemolysis, drugs [40], temperature [41], ultrasound effects and irradiation [42], physical exercise [43], peripartum and neonatal period [44]. As animals enrolled in the study lived in the same area of Sicily, an influence of pasture diet can be excluded. Additionally, although no environmental pollution problems have been documented in the area where the animals live, we cannot exclude the presence of other pollutants in that area. The EOF is important for assessing cell permeability and is considered a valuable biomarker for oxidative membrane damage caused by xenobiotics [45]. As the concentration of NaCl increased, an increase in haemolysis was noted in horse and bovine blood samples exposed to higher levels of Quaternium and Thiacloprid. In this regard, the identified osmotic fragility is linked to decreased cell deformability, membrane fluidity, and structural damage to RBC membranes [35]. To corroborate this, a change in the morphology of erythrocytes can be visualised in images acquired by light microscopy. Indeed, these show more aggregates in the experimental blood smears than in the control ones in both bovine and horse blood samples at all concentrations tested. In agreement with the current study, [46] report an increase in the osmotic fragility of rat erythrocytes after exposure to certain insecticides. According to [47], on the other hand, the use of certain detergents induced osmotic fragility in erythrocytes caused by the formation of pores in the lipid region of the membrane, resulting in the release of red blood cell vesicles. Moreover, it has been shown that osmotic fragility in erythrocytes can be induced by the elevation of erythrocyte calcium caused by impairment of the ATP-Ca2+ pump [21].

The results obtained in the present study clearly showed that also WBC and PLT from blood samples of horses and cows suffered the exposition to both xenobiotic compounds. As a matter of fact, these cell populations showed significantly decreased values following

Quaternium and Thiacloprid exposure. Concerning PLT, it has been previously shown that the production of free radicals in the bone marrow as a consequence of xenobiotic exposition causes a decrease in PLT, altering thrombopoiesis [37]. The decreased values of PLT as well as of the WBC found in the blood samples from horses and cows exposed to Quaternium and Thiacloprid, compared to the blood samples not exposed to these xenobiotics, may be related to the toxic effects of these compounds on the cells in the blood circulation. Indeed, being this study an in vitro investigation on peripheral blood samples, it is not possible to know the outcomes of Quaternium and Thiacloprid at the hematopoiesis level.

5. Conclusions

According to the results found in the present study, the two xenobiotic compounds herein investigated (Quaternium-15 and Thiacloprid) significantly affect the blood cell physiology in bovine and equine species. Specifically, after 24 h exposure, Quaternium-15 and Thiacloprid caused a decrease in analyzed haematological indices and an increase of EOF as confirmed by high haemolysis observed in blood samples exposed to xenobiotics. Despite being a preliminary investigation, the current study emphasizes the concept that blood is an excellent target for the evaluation of the effects of xenobiotics and/or other environmental pollutants on animals. Further studies are needed to better understand the mechanisms of cellular toxicity of Quaternium and Thiacloprid in horses and cattle, as well as in other grazers species (i.e., goat and sheep) considered good indicators of environmental pollution. Moreover, the assessment of the possible effects of these xenobiotics on the animal's production performance, including milk yield in dairy cows and/or horse's reproductive physiology or athletic performance, would be worthy of investigation.

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Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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