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Detection of plastics particles in equine blood by Scanning Electron Microscopy

Detección de partículas plásticas en sangre de equinos mediante Microscopía Electrónica de Barrido

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ABSTRACT

The study was conducted in the province of Guayas, located in the coastal region of Ecuador. The researchers analysed blood samples from 30 horses of different breeds (purebred, pony and mixed breeds) to detect the presence of micro- and nanoplastics (MPs and NPs). Blood smear and scanning electron microscopy (SEM) techniques were used to identify and quantify plastic particles in randomly selected animals aged between 2 and 12 years, with a body weight (BW) between 100 and 380 kg and a body condition score (BCS) between 5 and 6 (on a scale of 1 to 9), fed on natural grass and balanced supplements. The results did not show the presence of MPs, but NPs were identified in the blood smear of all animals, with an average of 51 particles per field of 1700 square microns (µm²) at a depth of 5 micrometres (µm) and an average size of 426.33 nanometres (nm). No significant difference was found in the number or size of NP particles between the sexes (females and males) (P=0.288); a greater presence of NPs was observed in younger horses (P<0.040). The pure-blood breed had a larger size of plastic particles (P < 0.020) and the crossbreeds had a greater amount of NP particles (P < 0.010) compared to other breeds. The research concludes that NPs are present in equine blood, highlighting the ability of these contaminants to enter the body and potentially cause adverse health effects. In particular, younger animals showed a higher presence of NPs in blood, suggesting that the effects of exposure may be more severe in the early stages of life.

Key words: Plastic particles; young animals; scanning electron microscopy

RESUMEN

El estudio se llevó a cabo en la provincia del Guayas, ubicado en la región Costa del Ecuador, donde se analizaron muestras de sangre de 30 equinos de diversas razas (Pura Sangre, Poni y Mestizos) para detectar la presencia de micro y nanoplásticos (MPs y NPs), con animales de entre 2 y 12 años de edad, un peso corporal (PC) de entre 100 y 380 Kg, y una condición corporal (CC) de 5 y 6 (en escala del 1 al 9), seleccionados aleatoriamente, alimentados con pastizales naturales y suplementos balanceados, se utilizó técnicas de frotis sanguíneo y microscopía electrónica de barrido (MEB) para identificar y cuantificar las partículas plásticas, los resultados no mostraron la presencia de MPs, pero se identificaron NPs en el extendido de sangre de todos los animales, con un promedio de 51 partículas por campo de 1700 micrómetros cuadrados (µm²) a una profundidad de 5 micrómetros (µm), y un tamaño promedio de 426,33 nanómetros (nm), no se evidenció significancia en la cantidad o tamaño de las NPs entre los géneros (hembras y machos) (P=0,288), se observó una mayor presencia de NPs en equinos de menor edad (P<0,040), en la Raza pura sangre, las partículas plásticas encontradas fueron de mayor tamaño (P<0,020), y los Mestizos presentaron mayor cantidad de NPs (P<0,010), en comparación con otras razas, la investigación concluye que las NPs están presentes en la sangre de los equinos, lo que deja en evidencia la capacidad de estos contaminantes para ingresar al organismo y potencialmente causar efectos adversos en la salud, en particular, los animales más jóvenes mostraron mayor presencia de NPs en sangre, lo que sugiere que los efectos de la exposición podrían ser más severos en las primeras etapas de vida.

Palabras clave: Partículas plásticas; animales jóvenes; microscopía electrónica de barrido

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INTRODUCTION

Growing concern about plastic particles has led to an increase in research into their presence, distribution and effects on ecosystems and living organisms. Microplastics are plastic particles less than 5 millimetres in size, while nanoplastics are those with a diameter of less than 100 nanometres. These particles originate both from the fragmentation of larger plastics through processes such as ultraviolet radiation, mechanical degradation and biological weathering [1], and from industrial products that contain these materials in particulate form, such as cosmetics, personal care products and synthetic textiles [2].

The presence of plastic particles in aquatic and terrestrial ecosystems has been widely documented, with detrimental effects on both fauna and flora. These pollutants not only affect organisms that accidentally ingest them, but can also be absorbed by biological tissues, causing toxic effects at the cellular level. It has been observed that micro- and nanoplastics can induce a number of adverse physiological responses, such as oxidative stress, alterations in the immune system, DNA damage, and dysfunction of the reproductive and metabolic systems [3 ,4].

A key part of the rural and agricultural ecosystem, it is estimated that 39 million donkeys (*Equus asinus*), 40.5 million horses (*Equus caballus*), and 12.3 million mules ((*Equus asinus* X *Equus Caballus*), live in developing countries, making up over 85% of the world's equids. In these countries, they are primarily used as labour, pack animals, often performing tasks in harsh and impoverished conditions for long hours of the day [5]. In addition, exposure in animals consuming natural roadside pastures, a common resource in rural Ecuador, as plastics are found in many environments, equines may be exposed through their diet, so it is crucial to study how these contaminants affect various species in nature [6]. The presence of microplastics and nanoplastics provides insight into the dispersion of these contaminants in various species, not just those that directly enter the human food chain [7]. Exploring the existence in terms of accumulation and effects of micro- and nanoplastics can reveal more about the biological susceptibility of different animal groups [8]. The results may alert us to the long-term effects of plastic exposure on the health of animals and, consequently, on the ecosystems in which they live [9].

The effects on living organisms, especially large terrestrial mammals, have emerged as a serious challenge due to their wide distribution and complex effects on ecosystems [10]. Plastics are a major global pollutant, with large quantities being released into the oceans every day due to mass production, overuse of this resilient material and poor environmental management [11].

The effects of plastic particles on human and animal health have caused global concern, which calls for a sound toxicological approach using appropriate methods to further investigate and understand the health problems caused by these pollutants [12]. This problem also affects developed countries, where large quantities of plastic waste are generated [13]. Therefore, understanding the magnitude of the situation and the importance of disseminating information about plastics in the body is crucial to raise awareness and prioritise public health [14]. The aim of this work was to detected the presence of micro- and nanoplastics in a selected area of equine (*Equus caballus*) blood smears using scanning electron microscopy.

MATERIALS AND METHODS

Bioethical aspects

The criteria for scientific research with animals established by the National Commission for Scientific and Technological Research (Fondecyt-Conicyt, Chile) [15], were applied in the development of this study.

Location of the study

The present study was carried out in the coastal region of Ecuador, in the province of Guayas, located in the south-east of the country. Animals from the EQUIMAS research centre, located on the coast with the following coordinates: latitude: -2.272346 and longitude: -80.144874 [16].

Experimental design

The coastal region, province of Guayas, has 16,138 horses [17], for this study 30 horses from the EQUIMAS Equestrian Centre were used, of the following breeds: Thoroughbred, Poni and Mestizos, with an age between 2 and 12 years, a body weight (BW) between 100 and 380 kg, and a body condition (BW) between 5 and 6 (on a scale of 1 to 9) [18]. Horses were randomly selected, fed with balanced supplements and natural grasses based on Brachiaria decumbens (17,585 kg DM/ha/year and PC 7-12%), Brachiaria brizantha (26,970 kg DM/ha/year and PC 8-14%) [19].

Sample collection

The animals were placed in a suitable area and muzzled with a rope to immobilise them. Biosafety standards were followed [15]. Blood collection equipment was carefully prepared to preserve the condition of the samples. In addition, the use of materials such as plastic syringes was excluded, Vacutainer tubes were used for blood collection with minimal vacuum necessary to reduce the contact of the plastic-coated needle with the blood, which helps to reduce the release of microplastics and transfer of contaminants [20]. The lateral region of the neck, where the jugular vein is located, was palpated and the site was disinfected with cotton wool and alcohol. A vacutainer (Medlab, EDTA Glass-Tube, Ecuador) was used, the jugular vein was punctured at a 45-degree angle to the skin, the tube containing ethylenediaminetetraacetic acid (EDTA) (Medlab, EDTA Glass-Tube 5mL, Ecuador) was placed and 5 mL of blood was withdrawn. The needle was withdrawn and pressure was applied to the puncture site to stop the bleeding. The blood was homogenised with gentle agitation. Samples were identified and stored at 4°C (Medicalpro, Transport refrigerator for blood samples, Ecuador) from the time of collection to the Public Health Research Institute (INSPI) laboratory in Guayaquil [21].

Blood smear

The samples collected were processed in the laboratory of the Public Health Research Institute (INSPI) in Guayaquil. A drop of blood was taken with a glass capillary (Medical Supplies, Haematology Glass Capillaries, Ecuador), the drop of blood was placed in the centre of a clean glass slide (Medical Supplies, Glass Slides, Ecuador), with another slide (in a gentle and controlled manner), the drop of blood was spread, forming a thin and uniform layer, taking care that the smear was homogeneous,

avoiding the cells to break or agglutinate. The aim was to obtain an even and thin smear, thus achieving the desired characteristics. In addition, the corresponding code of 5 (five) smears/sample was placed on the slides and stored in special boxes (MedLab, Glass Petri dish, Ecuador) that had previously been subjected to heat drying [22].

Sample drying

The samples were kept for four days (d) in a controlled environment chamber, free of contamination, at a temperature of 24 to 25°C (room temperature) (BINDER, ED115, Germany), after which the slides were placed (Medical Supplies, Glass Slides, Ecuador), in an oven for 24 h at a temperature of 40°C, 24 h at a temperature of 60°C and 24 h at a temperature of 80°C. From day 4 to d 6 they were kept at a temperature of 100°C. The plates were then subjected to 150°C for 3 h to complete the Critical Point Dryer (CPD) phase without bubble formation or deformation, which is essential to preserve the structural integrity of the particles [23].

Metallization of samples for SEM

The coating process was carried out by sputtering with gold for 20 s using the (JEOL, JPC1200 Fine Counter, Japan), which consists of pumping metal onto the surface of the sample using an electric current. This produced a very thin layer (5 to 10 nm thick), which allowed observation without electrostatic charging of the sample and also increased the contrast of the images to obtain high quality microphotographs [24].

Implementation of Positive and Negative Controls in the Detection of Microplastics

The positive control consisted of the inclusion of samples containing known plastic particles, such as polyethylene or polypropylene microplastics in ultrapurified water, to verify the ability of analytical techniques, such as scanning electron microscopy (SEM), to detect these particles. This control allowed validation of the efficacy and sensitivity of the detection process, ensuring that the methods used were appropriate for identifying plastic contaminants [25 , 26].

On the other hand, the negative control involved the use of samples without plastic particles, such as contaminant-free ultrapure water, to detect any cross-contamination during the analysis process. The negative control samples were observed under SEM to confirm that the plastic particles found in the experimental samples were not the result of handling or collection equipment [27].

Sample mounting and scanning electron microscope (SEM) observation

The plates were placed in the SEM sample chamber, which is maintained at a high vacuum. The (SEM) (JEOL, JSM-7001F, Japan), emits a beam of electrons into the vacuum chamber, which is focused on the surface of the sample, producing electrical signals as secondary electrons. For imaging, the resulting electrical signal was amplified, then processed and digitised by the microscope software to produce a three-dimensional grey-scale image of the sample based on the surface topography and particle size present in the samples [28].

Analysis by SEM confirmed the presence of plastic particles in the blood samples, using the JSM IT500 version 1.300 software integrated with the SEM, counting the particles on the erythrocytes at a depth of 5 µm and measuring the plastic particles in a field of 1700 µm² of the blood smear, excluding possible contamination by scanning the upper parts of the smear and the outer areas of the smear. The results were recorded by INSPI-certified technicians.

Variables

- Presence of nanoplastics / field of 1700 square microns (µm²).
- Size of nanoplastics in the nanometre (nm) range.
- Presence of nanoplastics / Field of 1700 square micrometres (µm²) according to sex, age and race.

Statistical analysis

The data obtained for each study variable were first tested for normality and then subjected to parametric or non-parametric tests as appropriate [29]. The data were analysed using generalised linear mixed models with particle size and quantity as continuous variables and sex, age and race as discrete variables [30].

RESULTS AND DISCUSSION

No microplastics were found, but the presence of nanoplastics was detected in all animals, with a result of 1530 NPs, with an average of 51 NPs/field of 1700µm². TABLE I and FIG. 1 show the results of the presence and quantity of nanoplastic particles in a field of 1700 (µm²).

TABLE I. Amount of nanoplastic particles present in equine blood spread in a 1700µm2 field

Particles/Nanoplastics	Sample	Average	TOTAL
Size	30	426.33nm	12790.16nm
Quantity	30	51un	1530um
Nanometers (nm) and Unites (um)			

Ingestion of nanoplastics is a common route of exposure for many animals. Marine and terrestrial animals have been shown to accidentally ingest plastics by ingesting contaminated food or drinking water containing plastic particles. In the case of horses, nanoplastics are likely to be ingested via the diet, specifically by consuming grass or water contaminated with plastics present in the environment [31]. This may occur in rural and agricultural areas where plastic contamination is more prevalent due to the use of plastic packaging and other polluting products.

Similarly, to Prata *et al*. 2022 [32] presented results from a study in which 18 cats (*Felis catus*) and 17 dogs (*Canis lupus familiaris*) from urban areas were analysed and microplastics were found in kidney, lung, liver and blood clot samples, which was attributed to high levels of urban pollution. As well as the presence of nanoplastics in sheep (*Ovis aries*) liver tissue [33].

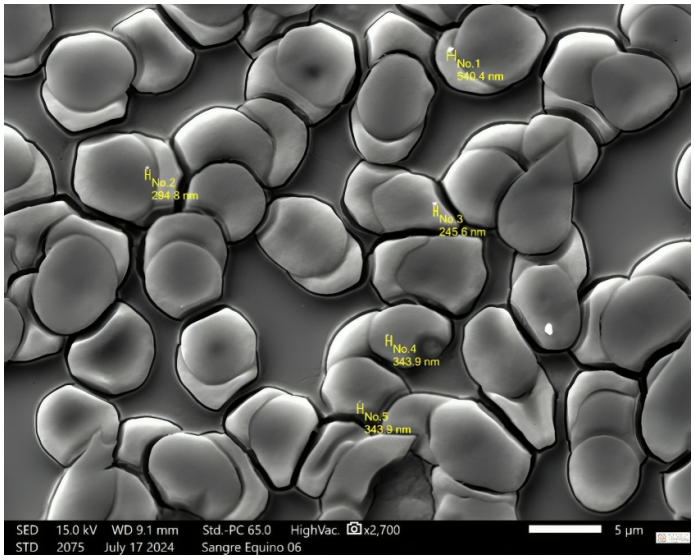

FIGURE 1. Size measurement of five plastic nanoparticles present in erythrocytes of equine, with an average of 426.33 nm, by SEM. erythrocytes, with an average of 426.33 nm

Shows the results of the particle size of nanoplastics found in equine blood, where the number of particles was counted in a field of 1700 µm² with a depth of 5 µm. In addition, a representative number of particles (five particles) were measured and marked with their respective size in nanometres (nm) (TABLE ll).

TABLE II. Particle size of nanoplastics in equine blood identified by SEM over an area of 1700 µm²

Number of particle	Sample	Size/nm	TOTAL /nm	P
1	30	395.8[a]	11872.8	0.288
2	30	425.0[a]	12750.8	0.288
3	30	416.7[a]	12500.0	0.288
4	30	455.2[a]	13656.2	0.288
5	30	439.0[a]	13171.0	0.288

Similar letters do not show significant differences, (P=0.288).

The average particle size of nanoplastics was 426.33 nm. It should be noted that microplastics are fragments smaller than 5 mm and nanoplastics are those smaller than 100 nm [34]. Two main sources of origin for the impact of microplastics and nanoplastics on the environment were identified: primary origin, which are intentionally produced for direct use (cosmetics, etc.), and secondary origin, which are plastics that degrade over the years and break down into micro and nanoparticles of the same size [35].

Bilal *et al.* [36] observed the presence of nanoplastics in a group of poultry (*Gallus gallus domesticus*), with the possible sources of contamination being the feed supplied and the farm environment. Oxidative stress was associated with exposure to nanoplastics and higher rates of inflammatory bowel disease were found compared to healthy animals in the absence of nanoplastics.

The gender variable has been used as a reference, where the result between females and males did not differ significantly in terms of average size and presence of nanoplastic particles [37]. In a study that detected the presence of polyethylene, polyvinyl chloride and polypropylene in breast milk samples analysed, it was suggested that exposure and absorption of residues not only reach different parts of the body, but can even be transmitted through breastfeeding (TABLE lll) [37].

TABLE III. Nanoplastics in equine blood by gender (female and male), over an area of 1700 µm²

Variables	Female (8/30)	Male (22/30)	t	Prob.	Sign.
Average size/nm	425.09	426.79	-0.06	0.48	ns
Nanoplastics in the field at 1700 µm².	59.00	48.09	0.54	0.30	ns

Nanometres (nm), Square microns (µm²), Probability (Prob.), Significance (sign), No significant difference (ns)

In addition, microplastics have been identified in the placentas of mammals, in which at least several types of plastic waste have been found, with polypropylene predominating in the chorioamniotic membranes, highlighting the importance and urgency of assessing the risks that microplastics may pose during pregnancy and the effects this may have on the foetus and the mother [38].

Plastic particles such as bisphenol A, phthalates and polychlorinated biphenyls have been linked to infertility as endocrine disrupting chemicals and acute exposure can cause low fertility and reproductive problems in farm animals [39].

One of the characteristics of certain plastic particles is lipophilicity, which means an affinity for lipid-rich tissues such as the epididymis and testes, which could facilitate transfer to semen, which is associated with semen quality in terms of volume, sperm count, motility and morphology [40].

According to TABLE IV, the presence of a higher amount of plastic particles is evident in the younger animals. Since, there is a significant difference in inverse relation between the age of the horse and the amount of NPs, respectively.

TABLE IV. Nanoplastics in equine blood by age (2 - 12 years), in a 1700 µm² field

Variables	Intersect (a)	Regression (b)	Correlation (r)	Determination (r^2)	Prob.
Average size/nm	386.70	6.23	0.24	0.06	0.21
Nanoplastics in the field at 1700 µm².	93.68	-6.70	0.38	0.142	0.040

Nanometres (nm), Square microns (µm²), Probability (Prob), Significant difference (P<0.040)

The presence of plastic particles observed at early ages suggests that most of the effects are not exerted by the plastic particles but by their metabolites, which originate in the liver [41]. In rats, it has been found that higher doses than those to which humans are normally exposed can cause severe disruption to the developing male reproductive system. In addition, the presence of these particles and other additives may cause toxicity, carcinogenicity and mutagenicity, as these compounds have been found in high concentrations in urine and breast milk, which is the first food of all mammals in the early stages of life [42].

The problem with plastic nanoparticles is that they cause injury after ingestion of plastic fragments, affecting the digestive system, leading to starvation and even physiological damage ranging from oxidative stress to carcinogenesis [43]. In addition, the accumulation of microplastic particles in the body has a prolonged period of storage, particularly in liver tissue, causing liver disease and metabolic problems [44].

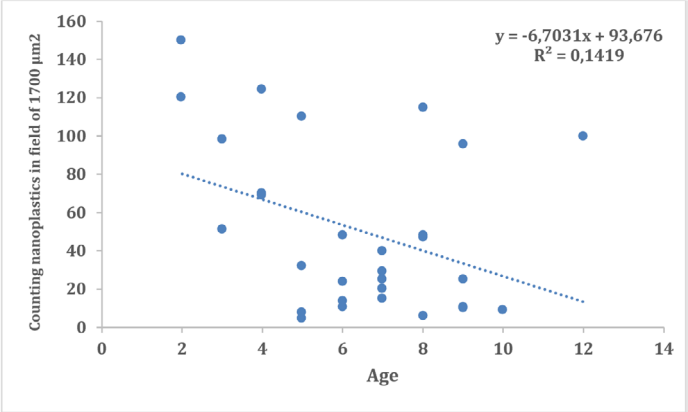

FIGURE 2. Relationship between equine age and the presence of nanoplastics in blood

In resource-poor urban and rural areas, living conditions and the environment are more exposed to pollution, including that from the degradation of plastic products and their fragmentation into smaller particle sizes, such as nanoplastics (EPA, 2023). In these cases, although exposure to nanoplastics cannot be directly related to breed, this can be argued according to the results shown in TABLE V, Thoroughbred horses have a larger size of plastic particles and half-bloods have a larger number of plastic particles, as they are more exposed to these pollutants due to the conditions of their environment [45].

TABLE V. Nanoplastics in equine blood by breed, observed over an area of 1700 µm²

Variables	Pure blood	Mestizo	Ponny	Prob.
Average/nm	471.74[a]	404.81[b]	393.06[b]	0.02
Nanoplastics in the field at 1700 µm².	23.60[b]	69.89[a]	18.00[b]	0.01

Nanometres (nm), Square microns (µm²), Probability (Prob), Different letters present significant difference (P<0.020), (P<0.010)

CONCLUSION

The results obtained show the presence of nanoplastics in the blood of equines tested by blood smears at a depth of 5 µm, with an average of 51 particles per 1700 µm² field. These findings suggest that nanoplastics have the ability to penetrate the body of animals, raising concerns about their potential to cause long-term adverse health effects.

In particular, younger animals had higher levels of nanoplastics in their blood, suggesting that the effects of exposure may be more pronounced at earlier stages of development.

As no differences in the presence or size of plastic particles were observed between females and males, the results indicate that nanoplastic contamination affects equines across the board, regardless of sex.

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Conflict of interests

The authors declare no conflict of interest regarding the publication of this manuscript.

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