

# Introduction to the International Journal of Equine Science

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Dr. Carlos Alberto Hussni is an Editorial Board Member of the International Journal of Equine Science. Dr. Hussni received his bachelor's degree in veterinary medicine from Universidade Estadual Paulista Jlio de Mesquita Filho in 1984, a master's degree in veterinary medicine from Universidade Estadual Paulista Jlio de Mesquita Filho in 1991, and a doctorate in functional anatomy of equines from Tierärztliche Hochschule Hannover in 1994. He has veterinary medicine experience, with a specialty in animal surgical clinics, equine, and large animal surgery.

Studies, knowledge, and science involving equine species go back centuries due to humanity's need to improve the performance, production, and health of these animals that have always participated in people's lives and thus contributed to the development of humanity. This involved veterinary medicine and its specialties, which began to continually seek and improve solutions to different problems. Notably, it was possible to observe that based on the historical needs of the use of horses in society, the field of equine medicine has evolved tremendously in terms of research and study. Horses were active participants in the creation and development of nations, in military actions and conquests, as an invaluable animal labor force, in the production of goods, and in the transportation of people and cargo. The use of horsepower has undergone changes, with the intensification of its use at work, in sports, in different modalities, and in leisure. It currently reaches the therapeutic dimension by having a key role in hippotherapy, which is widely used in different world regions. This variety of jobs in different activities makes horses a prominent species and recognized as beneficial to people and societies. Currently, horses represent a source of income for people, generate work and resources, and have an impact on the pleasure and health of the populations that have horses as part of their lifestyle. Because of how important

they are in people's lives, horses have become prominent in the field of scientific research seeking more knowledge about the development of the species and its possibilities of use. Initially, the research and efforts were aimed at improving animal health via equine veterinary medicine. As a result of this endeavor, new equine-related disciplines of research have emerged and are constantly expanding. Other themes, thoughts, and understandings have evolved, as well as an ever-increasing necessity to promote equine science-related subjects. These developments have resulted in a large growth in the areas of research with an ever-innovative and essential deepening in the face of each challenge encountered in the scientific exploration of the species in question. Equine science is distinguished by deepening knowledge in the various fields of research related to horses, updating and expanding information and knowledge, and directly benefiting animals and people who relate to this species, touching animal health and public health which is in line with the "One Health" approach. This field of studies related to horses tends to grow and expand, responding to existing questions and seeking other benefits with scientific and technological development. The incessant search for animal health, for human and social well-being, and development, allied to ethical and animal

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well-being issues make the expansion of studies, research, and knowledge happen in a consistent and accessible way.

On this occasion, we will have the first edition of the International Journal of Equine Science (IJES), a new journal associated with the different areas of equine science. IJES intends to contribute to the advancement in technical and scientific dissemination of Equine Science in its numerous domains; equine health, physiology, reproduction, nutrition, pharmacology, and equine sports medicine. The journal's editorial board consists of well-known scholars with extensive experience in the field of equine science. IJES is a peer-reviewed open-access publication. Everyone is welcome to read, disseminate, and build upon its content.. This new action

for knowledge becomes a strong ally, seeking collaboration in a consistent way with this vision and this concept of this branch of animal health and science, in the expectation of development, collaboration, and participation in this relevant scientific specialty.

### **Conflicts of Interest**

The author declares that there is no conflicts of interest.

#### **How to Cite**

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# Haptoglobin, Magnesium, Adiponectin, and Leptin as Biomarkers for Obesity and Insulin Dysregulation in Horses

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## Abstract

The aim of this project was to identify blood analytes which are associated with obesity and/or insulin dysregulation (ID) in horses. For this study, 57 horses were selected. At the start, body condition score (BCS) was established, and an oral sugar test (OST) was performed. Furthermore, the following blood analytes were determined: basal glucose, insulin, albumin, high molecular weight (HMW) adiponectin, leptin, haptoglobin, total protein, calcium, magnesium, phosphate, non-esterified fatty acids, and also glucose and insulin 75 min after an OST. Horses were classified according to their body condition as 'lean' (BCS 4/9-6/9) or 'obese' (BCS > 6/9). Horses were classified as insulin dysregulated (ID-positive) when insulin was 40 mU/L or higher than 75 min after OST. Multivariable logistic regression analysis showed a correlation between obesity and HMW adiponectin, leptin, total protein, and magnesium and between ID, haptoglobin, and leptin. Receiver operating characteristic plots (ROC) of obesity with leptin, adiponectin, total protein, and magnesium, which had an area under the curve (AUC) of 0.932, showed that the combination of these four blood analytes has a high association with obesity. The AUC of the ROC analysis of ID with haptoglobin and leptin was 0.805 implicating that this combination has a high association with ID. Although obesity can be observed (eyes and hands), the health risk of obesity can be monitored with a combination of leptin, adiponectin, total protein, and magnesium, while leptin and haptoglobin are good markers for ID (a health risk of obesity).

## Keywords

Horse; obesity; adiponectin; leptin; haptoglobin; insulin dysregulation

## 1. Introduction

Approximately 400,000 horses live in The Netherlands [1]. About 50,000 of these are active competitive athletes, while the remaining 350,000 horses are kept for recreational purposes. It has been estimated that approximately 55% of horses kept for recreational purposes are 'overweight' [2]. Excessive weight has been associated with various health issues such as poor performance, abnormal thermoregulation, reduced fertility, mesenteric lipomata, insulin dysregulation (ID), and laminitis [3,4]. It is obvious that not every health risk is

easy to determine. A body condition score (BCS) above 6.8/9 indicates that the total body fat is greater than 20% and thus a risk of adipose-related adverse health effects [5].

Basal insulin concentration is traditionally often used to identify horses suffering from ID and thus risks of disorders in horses due to prolonged or excessive hyperinsulinemia as part of EMS [6,7]. However, other biomarkers measured in blood are also described that could give a better indication of whether adverse health consequences of adiposity are present [6,8]. When obesity or any associated health consequences

are indeed established, an attempt should be made to reduce body weight by dietary measures and/or exercise [9]. In the recent literature, adiponectin [10–14], leptin [6,15], NEFA [16], haptoglobin and IL-6 [17,18], SAA [11], insulin [19–21], and RBP4 (retinol binding protein 4 [22] are all mentioned as potential markers for obesity and associated with health consequences. The aim of this project was to identify blood analytes, which are associated with obesity and/or ID in horses for monitoring health risk.

## 2. Materials and Methods

The study protocol was ethically weighed and approved by the Animal Welfare Body (IVD) committee of GD Animal Health Service Deventer.

### 2.1. Animals

In total, 57 horses were selected as described in the study by van den Wollenberg *et al.* [23]. The breeds eventually included were the following: Belgian Warmblood (6), Belgian Sport Horse (2), Dutch Warmblood (8), Arabian (6), Friesian (2), and mixed breeds of these (9). Also included were Tinkers (2), Haflingers (6), Lusitanos (4), Welsh Ponies (7), New Forest ponies (3), and Fjord horses (2). The mean weight of the horses was 498 kg (SD: 105 kg, min: 199, max: 693 kg). All horses were in good health.

### 2.2. Study Design

Horses were visited at their home premises, and BCS was determined as described in the research by van den Wollenberg *et al.* [23]. The BCS of the horse was assessed as described by Henneke [24,25], ranging from BCS 1 (very poor) to BCS 9 (extremely fat). A horse with a score of 4/9 to 6/9 was considered 'lean'. A horse with a BCS higher than 6/9 was considered to be 'obese'.

### 2.3. Blood Samples

Blood samples were taken by two experienced veterinarians and sent to the laboratory (chilled) within 24 hours after sampling. Upon arrival, the blood samples were centrifuged (10 min/3000 g) and stored at -20°C until further analysis was performed. Testing was performed at the laboratory of the GD Animal Health (Deventer, The Netherlands), except for HMW adiponectin and leptin. HMW adiponectin was analyzed at the Liphook Equine Hospital (UK), and leptin was analyzed at the laboratory of the KU-Leuven (Belgium).

#### 2.3.1. Blood Parameters

At T = 0, the following analytes were determined in the blood sample: albumin, haptoglobin, total protein, calcium, magnesium, phosphate, and non-esterified fatty acids (NEFA). Tests were carried out using standard operating procedures of the laboratory on a Synchron DxC 600 analyzer. All tests were validated for equine samples: haptoglobin: Tridelta kit; calcium: arsenazo method; magnesium: xylidyl blue method; phosphate: ammonium-molybdate method; albumin: bromocresol green method; total protein: biuret method; NEFA: oxidase/peroxidase method.

High Molecular Weight (HMW) adiponectin was measured (Liphook Equine Hospital, UK) using an ELISA (MyBiosource Inc., P.O. Box 153308, San Diego, CA 92195-3308, USA): 'Horse High Molecular Weight Adiponectin ELISA Kit', Catalog # MBS028647. The adiponectin assay has a detection limit of 0.1 mg/L and a range of 0.25–8 mg/L. The intra-assay

and inter-assay variabilities are 5.1% and 12.9%, respectively, with 93.7% recovery from mixed horse samples of varying concentrations.

Leptin was analyzed with RIA (Merck XL-85K) at the laboratory at the KU-Leuven (Belgium) (Dr. Daniel Vermeulen).

#### 2.3.2. Insulin Dysregulation (ID)

All horses were examined for ID using an oral sugar test (OST) that was carried out as described in the study by van den Wollenberg *et al.* [23]. A horse was considered to have ID (ID-positive) when insulin concentration at T = 75 min was 40 mU/L or higher [26].

Insulin was measured in heparinized plasma with a Siemens Immulite 1000 using the insulin kit 6602443. Plasma was used instead of serum because during our validation of insulin in horse blood, we found insulin to be stable during 24 hours in plasma, while in serum insulin it appeared to be 15% lower after 24 hours ( $\approx$  the average time needed for field samples to arrive at the laboratory by post). Determination of glucose in NaF-blood was performed using the hexokinase method (Beckman) on a Synchron DxC 600.

### 2.4. Statistical Analysis

Stata (14.1, Stata Corp US) was used for statistical analysis. All figures were made with SigmaPlot (version 13, Systat Software). Regression analysis was performed, and all residuals of variables were tested for normality (Shapiro Wilk W test for normal data). Residuals of glucose, insulin, HMW adiponectin, leptin, NEFA, and haptoglobin were found to be not normally distributed. Median (P50) and interquartile ranges are reported for not-normally distributed biomarkers. Statistical differences were tested using Kruskal Wallis calculations. All blood analytes were tested for significant differences when using binomial classification of ID (yes or no) and obesity (yes or no). Blood analytes that were significantly different in outcome were combined in logistic regression. Logistic regression was performed for obesity (yes or no) or ID (yes or no) for blood analytes with a *p* value less than 0.25 combined in one regression. When regression yielded an outcome with a significance of 0.05, the blood analyte was used in a receiver operating characteristic plot, and sensitivity and specificity were calculated for each concentration. Youden's index analysis was used to calculate optimal cut-off values.

## 3. Results

The median age of the 57 horses used in this study was 10 years (interquartile range / minimum and maximum: Q1 = 7, Q3 = 13, min: 3, max: 20). There was a tendency ( $R^2 = 0.10$ ) that younger horses had a higher BCS compared to older horses.

The number of animals per combination (obese or lean and ID-positive or ID-negative) is shown in **Table 1**. In the 'obese' group, 50% of the horses were considered to be ID-positive, while in the 'lean' group, this was only the case for 17% of the horses.

Data were tested for normality. The outcome of the Shapiro-Wilk W test is shown in **Table 2**. Haptoglobin, NEFA, leptin, and adiponectin were not normally distributed ( $p > [z] <$

0.05), albumin, total protein, calcium, magnesium, and phosphate were normally distributed.

**Table 3** lists the mean results of blood analytes of obese horses (BCS > 6/9) and lean horses (BCS < 6/9). HMW adiponectin showed a tendency to be lower in obese horses, as expected. An unexpected finding was that magnesium was significantly lower in obese horses. Leptin and NEFA were significantly higher in obese horses.

**Table 1:** Number of horses classified according to BSC and ID.

		Obese	Lean	Total
Insulin	Positive	17	4	21
Dysregulation	Negative	17	19	36
Total		34	23	57

Obese horse: BCS > 6/9; lean horses: BCS < 6/9

ID positive: insulin after OST  $\geq$  40mU/L; ID negative: insulin after OST < 40mU/L

**Table 2:** Statistical results of Shapiro-Wilk W test for blood analytes.

Variable	Obs	W	Z	$p >  z $
Albumin	57	0.97430	0.630	0.264
Haptoglobin	57	0.92673	2.882	0.002
Total protein	57	0.98248	-0.193	0.577
NEFA	57	0.86407	4.210	0.001
Calcium	57	0.98288	-0.242	0.596
Magnesium	57	0.98772	-0.957	0.831
Phosphate	57	0.96542	1.268	0.102
Leptin	57	0.82630	4.737	0.001
Adiponectin	57	0.61412	6.452	0.001

**Table 3:** Results of blood analytes in obese horses compared to lean horses.

Analyte	Obese (n = 34)		Lean (n = 23)		$p$ value
	P50	IQR	P50	IQR	
HMW adiponectin (mg/L)	0.97	0.55	1.46	1.48	0.06
Glucose (resting) (mmol/L)	4.6	0.30	4.6	0.30	0.53
Insulin (resting) (mU/L)	5.4	4.9	3.4	6.5	0.58
Glucose after OST (mmol/L)	6.4	1.2	5.4	1.5	< 0.002
Insulin after OST (mU/L)	38.6	53.8	10.2	16.3	< 0.002
Leptin ( $\mu$ g/L)	14.2	16.4	2.5	4.8	< 0.001
Haptoglobin (g/L)	1.4	1.1	1.3	0.4	0.36
NEFA (mmol/L)	0.16	0.13	0.11	0.08	0.03
	Mean	SE	Mean	SE	$p$ value
Total protein (g/L)	68.5	0.76	66.3	1.13	0.12

Albumin (g/L)	35.0	0.35	34.8	0.44	0.49
Calcium (mmol/L)	2.89	0.017	2.92	0.022	0.47
Phosphate (mmol/L)	1.18	0.03	1.11	0.03	0.089
Magnesium (mmol/L)	0.75	0.013	0.81	0.019	< 0.005

Obese horse: BCS > 6/9; lean horses: BCS < 6/9

P50: median, IQR: interquartile range, SE: standard error

The results of analytes measured in the blood of ID-positive horses and ID-negative horses are shown in **Table 4**. Basal glucose and basal insulin were not different between ID-positive and ID-negative horses, as expected. Magnesium in the blood of ID-positive horses was not different from ID-negative horses, but haptoglobin and leptin were both statistically significantly higher in ID-positive horses compared to ID-negative horses.

Blood analytes that showed a difference ( $p < 0.25$ ) between lean and obese horses were used in a logistic regression. The same was done for blood analytes that showed a difference ( $p < 0.25$ ) between ID-positive and ID-negative horses. The outcome of the logistic regressions is shown in **Tables 5** and **6**. The results of these calculations show that HMW adiponectin, leptin, total protein, and magnesium were correlated with BCS when using multivariable logistic regression. The results of the calculations of the multivariable logistic regression of ID with blood analytes showed that leptin and haptoglobin were correlated with ID.

Blood analytes that were significantly different ( $p < 0.05$ ) in the multivariable logistic regression of obesity (HMW adiponectin, leptin, total protein, and magnesium) were used to calculate receiver operating characteristic plots, and sensitivity and specificity were calculated for each concentration. Corresponding plots are shown in **Figure 1** (BCS) and **Figure 2** (ID).

The cut-off values for both sets of plots and the AUC are listed in **Table 7**. The optimal cut-off values for BCS (obese or lean) were calculated using Youden's index: 8.9  $\mu$ g/L for leptin, 0.77 mmol/L for magnesium, 1.46 mg/L for HMW adiponectin, and 65.1 g/L for total protein. For ID (ID-positive or ID-negative), the optimal cut-off values were 5.66  $\mu$ g/L for leptin and 1.3 g/L for haptoglobin.

When the significant blood analytes are combined into one ROC-plot, the AUC of the plot for BCS is 0.932 and for ID it is 0.805 (see **Figure 3**).

#### 4. Discussion

The aim of this project was to identify blood analytes, which are associated with obesity and/or ID in horses for monitoring health risk. In our study group, 50% of the obese horses showed an insulin after OST of 40 mU/L or higher, while in the lean group only 17% were diagnosed to be ID-positive. This ratio (50/17 = 2.94) is comparable with that found in humans: in a study performed in 2015 [27], 17.3% of the human population (above 4 years of age) in the Netherlands which were classified as 'obese' were diagnosed with insulin dysregulation, while only 3.3% of people classified as lean were diagnosed with insulin dysregulation (ratio = 5.2) [27]. In the UK, 12.4% of people with obesity aged 18 years or older

are diagnosed with diabetes, five times as much as people having a healthy weight [28].

We found magnesium to be significantly different in lean versus obese horses (0.81 versus 0.75 mmol/L,  $p < 0.005$ ). This finding that magnesium is lower in obese horses is novel. In humans, a relation between obesity and lower magnesium has already been described [29]. Nadler et al. postulated that in humans a lower magnesium concentration is causally associated with ID [30]. Subclinical hypomagnesaemia is common in critically ill humans and animals [31], but obesity is not a critically ill situation. A possible explanation could be taken from the fact that that hypomagnesaemia is associated with increased cytokine production as in humans [32]. Reactive white adipose tissue in horses secretes cytokines [33] and therefore could result in magnesium being lower in obese horses.

We found that the differences between HMW adiponectin in the blood of obese horses and lean horses were less pronounced compared to previous studies in horses [9,11,33]. Menzies-Gow *et al.* had a similar finding in their study [34]. Adiponectin is inversely associated with the quantity of white adipose tissue (WAT) in horses [11,14] and is a protein hormone that regulates a number of metabolic processes in humans: glucose regulation and fatty acid oxidation [35]. Recently, Karikoski *et al.* also found that horses with ID had lower serum adiponectin concentrations [36], longer neck circumference, and larger height than horses in the non-ID group. Menzies-Gow *et al.* found total adiponectin to be significantly lower ( $p < 0.01$ ) in previously laminitic ponies (mean  $\pm$  S.D. 8.9  $\pm$  2.9 mg/L) compared to non-laminitic ponies (24.2  $\pm$  11.8 mg/L) [37]. An immunoturbidimetric method was used for measuring HMW adiponectin, and it was compared with ELISA methods. They found that none of the ELISA methods could be validated satisfactorily, and therefore, concluded that ELISA methods are not suitable for the measurement of equine adiponectin. The HMW adiponectin concentrations found in this study were comparable to those found in previous studies in horses [11,30] and humans [30,38]. However, leptin was an even better marker for obesity (median 2.5  $\mu$ g/L for lean horses versus 14.2  $\mu$ g/L for obese horses,  $p < 0.001$ ). Leptin values can differ markedly depending on the analytical method and specific antibody used, and one must be careful when comparing results from different methods mutually. Therefore, our results cannot be directly compared with older leptin results found in horses [9,14,38,39]. However, our results are comparable with newer publications [34,40]. Leptin results in 'lean' horses (median 2.5  $\mu$ g/L, interquartile range 4.8  $\mu$ g/L) in our study are comparable with the results of other studies: for example, Kedzierksi found a basal leptin concentration of 1.4 – 3.1  $\mu$ g/L for horses with a BCS of 5.0/9 or lower and 1.2 – 8.2 for horses with a BCS of 6.6/9 [40]. Pleasant *et al.* reported an average leptin of 3.5  $\mu$ g/L (CI: 3.2 – 3.7) in 300 mixed light breed horses in Southwest Virginia [41]. Leptin itself is not considered to be a good indicator for ID [26]; however, in our study, we found leptin to be significantly correlated with ID ( $p < 0.05$ ): higher leptin is

higher risk for ID (AUC of ROC-plot is 0.664) because obese horses have a higher chance of having ID.

**Table 4:** Results of blood analytes in ID-positive horses compared to ID-negative horses.

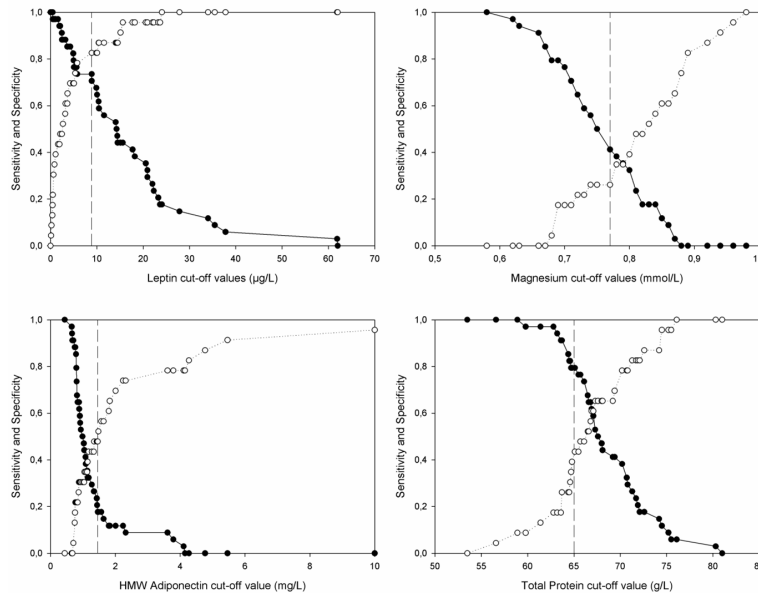
Analyte	ID positive (n = 21)		ID negative (n = 36)		p value
	P50	IQR	P50	IQR	
HMW adiponectin (mg/L)	0.99	0.47	1.17	1.11	0.25
Glucose (resting) (mmol/L)	4.6	0.4	4.6	0.3	0.27
Insulin (resting) (mU/L)	6.9	2.9	2.6	3.6	< 0.001
Glucose after OST (mmol/L)	6.6	1.2	6.0	1.2	< 0.02
Insulin after OST (mU/L)	70.0	57.0	12.2	1.5	< 0.001
Leptin ( $\mu$ g/L)	11.6	15.1	4.6	13.0	< 0.05
Haptoglobin (g/L)	1.8	0.8	1.2	0.7	< 0.002
NEFA (mmol/L)	0.18	0.15	0.14	0.08	0.14
	Mean	SE	Mean	SE	p value
Total protein (g/L)	68.7	0.88	66.9	0.89	0.26
Albumin (g/L)	35.2	0.40	34.8	0.35	0.22
Calcium (mmol/L)	2.91	0.018	2.90	0.020	0.61
Phosphate (mmol/L)	1.14	0.05	1.16	0.03	0.65
Magnesium (mmol/L)	0.77	0.015	0.77	0.017	0.88

ID positive: insulin after OST  $\geq$  40mU/L; ID negative: insulin after OST < 40mU/L  
P50: median, IQR: interquartile range, SE: standard error

**Table 5:** Results of multivariable logistic regression of obesity with blood analytes.

Variable	coef.	Z	p >  z	N = 57	
				LR (Chi <sup>2</sup> )	Prob > Chi <sup>2</sup>
BCS (lean or obese)				48.64	0.0001
				Pseudo R <sup>2</sup>	0.6326
Variable	coef.	Z	p >  z	95% CI	
HMW adiponectin	-1.824	1.96	0.050	-3.64	-0.01
Glucose after OST	0.124	0.01	0.988	-1.60	1.63
Insulin after OST	0.527	2.55	0.159	0.066	0.507
Leptin	0.287	2.55	0.011	0.066	0.507
NEFA	10.99	1.62	0.104	-2.27	24.26
Total protein	0.475	2.23	0.026	0.057	0.894
Phosphate	3.07	0.83	0.405	-4.16	10.31
Magnesium	-15.44	-1.98	0.048	-30.743	-0.141
Constant	-26.186	-1.78	0.076	-55.082	2.709

CI: confidence interval



**Figure 1(a):** ROC-plots showing sensitivity (solid line) and specificity (dotted line) of leptin at a range of cut-off values, with reference to BCS (lean or obese). The black long dashed line indicates the recommended cut-off value (8.90 µg/L). **(b):** ROC-plots showing sensitivity (solid line) and specificity (dotted line) of magnesium at a range of cut-off values, with reference to BCS (lean or obese). The black long dashed line indicates the recommended cut-off value (0.77 mmol/L). **(c):** ROC-plots showing sensitivity (solid line) and specificity (dotted line) of HMW adiponectin at a range of cut-off values, with reference to BCS (lean or obese). The black long dashed line indicates the recommended cut-off value (1.46 mg/L). **(d):** ROC-plots showing sensitivity (solid line) and specificity (dotted line) of total protein at a range of cut-off values, with reference to BCS (lean or obese). The black long dashed line indicates the recommended cut-off value (65.1 g/L).

We found ID-positive horses to have a higher haptoglobin concentration in blood (median 1.8 g/L) compared to ID-negative horses (median 1.2 g/L) ( $p < 0.002$ ). In obese versus lean horses, haptoglobin was not significantly different. In humans, higher haptoglobin serum levels seem to be a strong marker of hyperinsulinemia, independently of body mass index as reported by Pergola *et al.* [42]. Obesity is associated with inflammation of WAT resulting from chronic activation of the innate immune system, which can subsequently lead to insulin resistance, impaired glucose tolerance, and even diabetes mellitus in horses [9]. WAT depots secrete cytokines including IL-6 in horses [33,43] which has been shown to activate transcription of haptoglobin and other acute phase proteins [33]. Haptoglobin is also directly released from adipose tissue, but the amount is quite low in comparison to the concentrations of haptoglobin in the blood of humans [17]. Some previous researchers have also found acute phase proteins to be associated with obesity and ID in humans [17,18,43]. The source of most of these acute phase proteins (such as haptoglobin) is white adipose tissue which upon excessive weight gain becomes infiltrated with macrophages and lymphocytes and undergoes changes in gene expression: IL-6 is excreted when WAT becomes ‘reactive’ in human [13] and horses [22,44]. As IL-6 activates the production of haptoglobin in the liver, a higher haptoglobin could be expected in horses that are obese and thus at risk for ID. The disadvantage of haptoglobin is the sensitivity for hemolysis. Although in the haptoglobin kit from Tridelta mildly hemolyzed samples may be used, grossly hemolyzed samples

(hemoglobin > 2.5 g/l) should be avoided as results may not be reliable.

**Table 6:** Results of multivariable logistic regression of ID with blood analytes.

				N	57
				LR (Chi <sup>2</sup> )	20.2
				Prob > Chi <sup>2</sup>	0.0011
				Pseudo R <sup>2</sup>	0.2693
ID (positive or negative)					
Variable	coef.	Z	$p >  z $	95% CI	
HMW adiponectin	-0.356	-1.17	0.244	-0.954	0.242
Leptin	0.0724	2.35	0.019	0.012	0.133
Haptoglobin	2.194	2.81	0.005	0.666	3.722
NEFA	0.266	0.08	0.940	-6.613	7.145
Albumin	-0.0163	-0.1	0.922	-0.344	0.312
Constant	-3.671	-0.65	0.519	-14.819	7.477

CI: confidence interval

**Table 7:** AUC and cut-off values calculated with Youden’s index.

BCS / blood analyte	AUC	Cut-off value	Youden’s index
HMW adiponectin	0.351	1.46 mg/L	-0.35
Leptin	0.831	8.90 µg/L	0.56
Total protein	0.621	65.1 g/L	0.23

Magnesium	0.293	0.77 mmol/L	-0.35
ID / blood analyte	AUC	Cut-off value	Youden's index
Haptoglobin	0.751	1.30 g/L	0.44
Leptin	0.664	5.66 µg/L	0.42

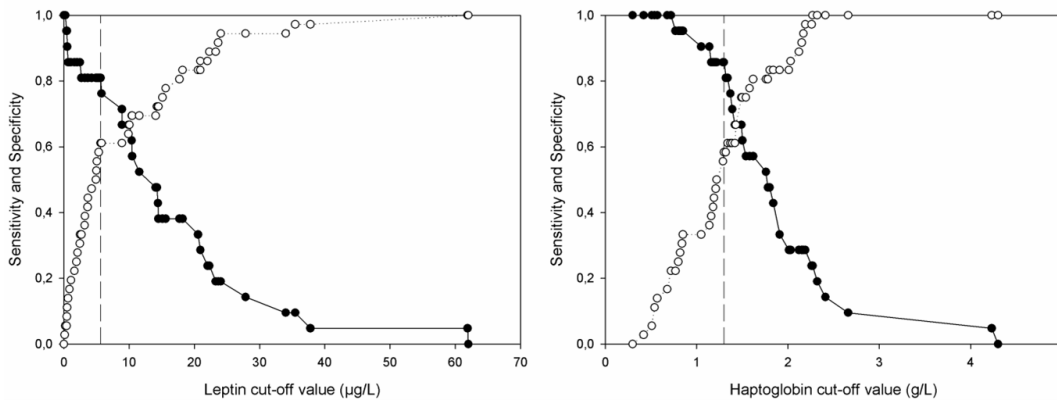
Because leptin and NEFAs were higher in obese horses compared to lean horses and HMW adiponectin and magnesium were lower in obese horses, we combined these findings in a multivariable logistic regression. This analysis showed a significant correlation between obesity and adiponectin, leptin, total protein, and magnesium with an AUC of 0.932 implicating that these four blood analytes have a very good association with obesity.

Furthermore, a significant correlation (multivariable logistic regression analysis) was found between ID and haptoglobin

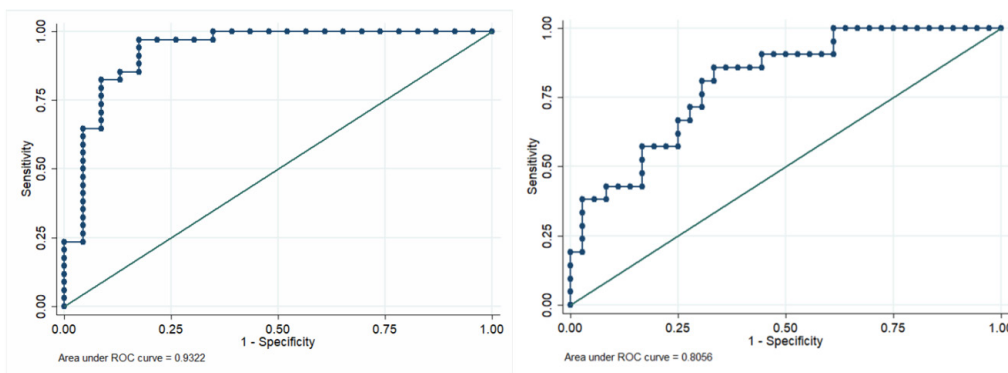
and leptin. The AUC of the combined ROC analysis of insulin dysregulation with haptoglobin and leptin is slightly lower: 0.805, but it still indicates a good association between ID and these two blood analytes.

### 5. Conclusion

A combination of leptin, adiponectin, total protein, and magnesium was found to have a good association with obesity, and the combination of leptin and haptoglobin had a good association with ID. Therefore, these biomarkers can be used as a monitoring tool for health risk associated with obesity and ID in horses.



**Figure 2(a):** ROC-plots showing sensitivity (solid line) and specificity (dotted line) of leptin at a range of cut-off values, with reference to ID (ID-positive or ID-negative). The black long dashed line indicates the recommended cut-off value (5.66 µg/L). **(b):** ROC-plots showing sensitivity (solid line) and specificity (dotted line) of haptoglobin at a range of cut-off values, with reference to ID (ID-positive or ID-negative). The black long dashed line indicates the recommended cut-off value (1.30 g/L).



**Figure 3(a):** Combined ROC-plot: BCS (obese or lean) with significant ( $p < 0.05$ ) blood analytes (leptin, magnesium, HMW adiponectin, and total protein). **(b):** Combined ROC-plot: ID (positive or negative) with significant ( $p < 0.05$ ) blood analytes (leptin and haptoglobin).



## Authors' Contributions

**G. H. M. Counotte** was responsible for laboratory results, statistical analysis, and preparation of the manuscript.

**A. Durham** was responsible for the adiponectin results (validation and laboratory results), reviewing of the manuscript, and discussing the results.

**V. Vandendriessche** was responsible for selection of the horses, blood sampling, and contact with horse owners.

**L. van den Wollenberg** was responsible for the background information, literature, and reviewing of the manuscript.

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## Data Availability Statement

The data supporting the findings of this study are openly available in <https://doi.org/10.17026/dans-2b3-f8wj>.

## Conflicts of Interest

The authors declare that there is no conflicts of interest.

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## Ethical Approval

The Animal Welfare Body of the Royal GD approved the experiments (according to 2010/63/EU).

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# Physical Assessment, Hematological, and Serum Amyloid A Levels Pre- and Post-Galloping Exercise in Arabian Horses in Maiduguri and Jere, Borno State, Nigeria

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## Abstract

Long-duration activities, such as endurance races, cause significant changes in hematological and biochemical components as a result of central and peripheral exhaustion. The aim of the present study was to assess the physical health status as well as hematological and serum amyloid A (SAA) levels pre- and post-galloping exercise in Arabian horses in Maiduguri and Jere, Borno State, Nigeria. Before and after the galloping exercise, the health status of 50 healthy Arabian horses was physically assessed and blood samples were collected for hematological and serum biochemistry analyses. Packed cell volume (PCV), red blood cell (RBC), haemoglobin (Hb) concentrations, white blood cell (WBC), and differential leucocytes such as neutrophils, eosinophils, basophils, monocytes, and lymphocytes were determined using ballottment methods and haematocrit cyanmethaemoglobin. SAA levels pre- and post-galloping were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit. The physical parameters such as temperature, heart, and respiratory rates values were significantly increased at  $p < 0.001$ ,  $0.001$ ,  $0.050$ , and  $0.001$ , respectively, after the galloping exercise as compared with the pre-galloping values. Haematological parameters such as PCV, RBC, and Hb were increased significantly at  $p < 0.001$ ,  $0.001$ , and  $0.015$ , respectively, as well as eosinophils and monocytes at  $p < 0.040$  and  $0.001$ , respectively, after galloping as compared with the pre-galloping values. The mean values of SAA increased significantly ( $p < 0.007$ ) post-galloping. Therefore, it was concluded that the galloping type of exercise affects some physical parameters such as temperature, respiration, and heart rates as well as various hematological parameters and biochemical parameters such as SAA, and an increase in SAA could be due to resistance provided by the sandy terrain, leading to exhaustion in exercising the muscles of horses in the study area.

## Keywords

Exercise; hematology; serum amyloid A; Arabian horses

## 1. Introduction

This is the first documented study conducted under sandy terrain about galloping exercise in Arabian horses in the studied area. Horses are useful in agriculture, transport, policing, games, sports, and others [1–3]. Humans, horses, camels, and greyhounds are the four primary athletic species

although horses are the quickest. As a result, horses drew the most interest in the study of athletic science, second only to humans [4]. A horse's competence is the consequence of a complex interplay of numerous factors. The horse's age, gender, breed, genetic potential, stride, nutrition, behaviour, strength, and neuromuscular coordination, as well as

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the ability for labor or fitness, are all factors to consider [5,6]. In Nigeria, there are approximately 240,000 horses predominantly of Arabian breed with a large population in Borno State [7]. Exercise is among the stressful events an animal may face during his life, and during elevated exercise, the metabolism of horses undergoes transitory homeostatic alterations [8,9]. Due to severe environmental conditions and central and peripheral exhaustion, protracted activities, such as endurance races, provide a significant stimulus to those components [10]. It has been reported that exercise influences hematological parameters [11]. In relation to its age, sex, breed, degree of fitness, health, and dietary sufficiency, a healthy horse retains a concentration of red and white blood cells, as well as other blood components, within the normal range [12–14]. After activity, deviations from the normal range in one or more blood components, either above or below the range's boundaries, might indicate a response to exhaustion by a working horse. In reaction to a number of stresses, acute phase proteins (APP) such as serum amyloid A (SAA) are released into the bloodstream [15,16]; another generally held belief is that APP is common in horses and is caused by glycogen depletion in exercising muscles, which leads to the release of IL-6 [17]. Pleiotropic effects are promoted by IL-6, which raises the synthesis of SAA in the liver [18]. Hepatocytes produce the majority of it as part of the APP [16]. The most significant metabolic alterations involve a huge family of proteins produced by the liver, known as APPs, being greatly increased or reduced [15,16,19]. The present study was aimed to evaluate the influence of galloping exercise on physical and hematological parameters as well as SAA levels pre- and post- galloping in Arabian horses located in Maiduguri and Jere local Government Areas of Borno State.

## 2. Materials and Methods

### 2.1. Study Area and Sample Population

This study was conducted in Maiduguri and Jere local Government areas, Borno State, which lies within the semi-arid zone of the Northeastern part of Nigeria, with an area of 69,436 sq km. Maiduguri lies between latitude 11°N and longitude 13°E. It has a mean day temperature of 38°C. The horses used in the present study are stallions of the Arabian breed which are most predominant in Borno State, Nigeria.

### 2.2. Sample Size Determination and Study Design

Cross-sectional study design and convenient sampling methods were used to select fifty stallion horses in Maiduguri and Jere. The galloping type of exercise was selected for fifty stallion horses of the Arabian breed weighing between 350 kg and 450 kg and grouped into two sessions of a twenty-five kilometer galloping that was covered in one hour, twenty-seven stallions were from Maiduguri and twenty-three from Jere, respectively. Physical conditions such as the body conformation and body condition score of the horses (the horses selected for the study have approximately the same body condition score; hence, this result was not presented

in the study) were determined as well as the vital parameters which include the temperature, respiration, and heart rates before and after exercise in the study area. The galloping exercise was conducted on a sandy terrain in the study area.

### 2.3. Collection of Blood Samples

Blood samples were collected from the left jugular vein of each sampled horses using the procedure described in [20]. The jugular vein area was cleansed with a swab moistened with 70% v/v alcohol, and it was allowed to dry. Using 21 gauge needles, approximately 5 ml of the blood sample were aseptically collected before the exercise and 5 ml after the galloping exercise from each of the horses into heparinized vacutainer tubes for biochemical analyses. SAA levels were determined using ELISA kit (Cusabio), and ethyl diaminetetraacetic acid (EDTA) vacutainer tubes for hematological tests such as PCV, RBC, Hb, WBC, and differential leucocytes were determined using Microhaematocrit, Cyanmethaemoglobin, and Battlement Methods as described by Coles [21]. The EDTA vacutainer tubes were rocked and rolled gently to allow for uniform mixing of the blood with the anticoagulant. The samples were labelled appropriately. Blood was drawn from horses that appeared healthy and at rest, and a thorough clinical examination was performed to determine their health state. The samplings were carried out with caution to ensure that the horses were not compelled by the process, which could have skewed off the horses' hematological and immunologic characteristics. Before the galloping exercise, the blood sample was taken 24 hours before the galloping starts and when the horses were at rest (between 8 and 10:30 a.m.) and tested in the event's on-site laboratory. The galloping exercise began at 4 p.m., and ended at 6:30 p.m. on the same day. The post-galloping blood sample was collected, and the clinical parameters of the horses were checked after a maximum of 20 to 30 minutes of the recovery period of the galloping events [22–26].

### 2.4. Data Analyses

Nonparametric test: Shapiro Wilk W test was used to compare the pre- and post-galloping values, and the data was examined using a student paired t-test. The changes on hematological and physical parameters during galloping were analyzed with JMP version 11 software (SAS Institute Inc, Cary, NC). Values were considered statistically significant at  $p < 0.05$ .

## 3. Results

The determined physical parameters revealed that temperature, respiration, and heart rates showed significant differences, as compared to the pre-exercise values as shown in **Table 1**. The revealed mean values of PCV, HB, and RBC pre- and post-galloping were significantly different. The mean values of differential leucocytes count, monocytes, and eosinophils pre- and post-galloping were significantly different as shown in **Table 2**. The mean values of SAA pre- and post-galloping revealed a significant difference as shown in **Table 3**.

**Table 1:** Changes in the physical parameters of Arabian horses in Maiduguri and Jere as a result of galloping events 20 to 30 minutes of the recovery period (n = 50).

Physical parameters	Pre-galloping	Post-galloping	P-values
Body temperature (°C)	36.52 <sup>b</sup> ± 0.19	38.82 <sup>a</sup> ± 0.14	0.001 <sup>*</sup>
Heart rate (Beat/mins)	64.46 <sup>b</sup> ± 0.81	85.46 <sup>b</sup> ± 0.96	0.050 <sup>*</sup>
Resp. rate (Breath/mins)	21.20 <sup>b</sup> ± 0.36	24.52 <sup>a</sup> ± 0.39	0.001 <sup>*</sup>

All values are expressed as mean ± SE; values with different superscript <sup>a, b</sup> within rows are significantly different at  $p < 0.05$ .

**Table 2:** Changes in the hematological parameters of Arabian horses in Maiduguri and Jere as a result of galloping events 20 to 30 minutes of the recovery period (n = 50).

Blood Parameters	Pre-galloping	Post-galloping	P-values
Packed Cell Volume (L/L)	29.76 <sup>b</sup> ± 0.69	31.72 <sup>a</sup> ± 0.71	0.001 <sup>*</sup>
Haemoglobin (mmol/L)	11.55 <sup>b</sup> ± 0.43	12.91 <sup>a</sup> ± 0.48	0.001 <sup>*</sup>
RBC (x 10 <sup>12</sup> /L)	6.27 <sup>b</sup> ± 0.30	7.03 <sup>a</sup> ± 0.34	0.015 <sup>*</sup>
WBC (x 10 <sup>9</sup> /L)	9.25 <sup>a</sup> ± 0.22	9.49 <sup>a</sup> ± 0.18	0.312
Neutrophils (x 10 <sup>9</sup> /L)	2.96 <sup>a</sup> ± 0.09	2.97 <sup>a</sup> ± 0.10	0.881
Lymphocytes (x 10 <sup>9</sup> /L)	5.21 <sup>a</sup> ± 0.16	5.27 <sup>a</sup> ± 0.13	0.603
Monocytes (x 10 <sup>9</sup> /L)	0.37 <sup>b</sup> ± 0.02	0.42 <sup>a</sup> ± 0.02	0.040 <sup>*</sup>
Eosinophils (x 10 <sup>9</sup> /L)	0.66 <sup>b</sup> ± 0.03	0.81 <sup>a</sup> ± 0.03	0.001 <sup>*</sup>
Basophils (x 10 <sup>9</sup> /L)	0.03 <sup>a</sup> ± 0.01	0.06 <sup>a</sup> ± 0.02	0.231

All values are expressed as mean ± SE; values with different superscript <sup>a, b</sup> within rows are significantly different at  $p < 0.05$ . RBC = red blood cells; WBC = white blood cells.

**Table 3:** Pre- and post-galloping serum amyloid A (SAA) of Arabian horses in Maiduguri and Jere after 20 to 30 minutes of the recovery period (n = 50).

Blood Parameters	Pre-galloping	Post-galloping	P-values
SAA (mg/mL)	7.07 <sup>b</sup> ± 0.64	10.43 <sup>a</sup> ± 0.95	0.007 <sup>*</sup>

All values are expressed as mean ± SE; values with different superscript <sup>a, b</sup> within rows are significantly different at  $p < 0.05$ .

#### 4. Discussion

This is the first reported galloping exercise in Arabian horses in the studied region, which was conducted on sandy terrain to test the physical health statuses, hematological, and serum amyloid A (SAA) levels in Arabian horses in Maiduguri and Jere, Borno State, Nigeria, before and after the galloping exercise. However, there are some variations with other studies, possibly as a result of the sandy terrain of the studied

area. It is possible that the variances are attributable to the sandy terrain's resistance to the horses' galloping efforts. In the present study, only stallions were used for the galloping type of exercise; this is done to avoid fluctuations in the final results which might create bias in the study as the presence of mares during such an event might cause distraction due to the mating behaviour. It was observed that if mares and stallions were allowed to perform together at the same time on the same terrain, they tend to sustain injuries because of the exhibited mating behaviour. The results revealed that the galloping exercise has caused some fluctuations in the physical parameters such as temperature, respiration, and heart rate in the horses. This could be due to the requirement of the muscles for more oxygen during the galloping exercise. The finding in the present study is consistent with the previous research reported in [11,14], that reported similar findings on endurance horses in Malaysia. This increase could be as a result of the conditioning of the horses to the galloping exercise because most of the horses used for this research were also event horses. The considerable rise in PCV, Hb, and RBC in the present study could be attributed to sympathetic stimulation-induced splenic mobilization of erythrocytes, which is intensity-dependent as reported in [12]. Ok-Deuk and Yong-Soo (2017) also reported a significant increase in PCV and Hb values after exercise due to the effects of environmental factors such as the shape of the track, topography, the type of the actual exercise, the breed of the horse, and the exercise career [27]. These elevations were equivalent in size to those seen in earlier investigations as reported in [28]. After the race, however, there were no modifications in the overall leukocyte counts or neutrophils. A shift of both lymphocyte subsets after strenuous exercise could explain such a response. However, the present study revealed an increase in monocytes and eosinophils within the normal range value; this could be as a result of vigorous activity such as galloping in a non-inflammatory environment that can trigger an acute-phase response. This Modification observed in the total blood cell count might be interpreted as normal exercise-induced reactions in the performance of horses such as jumping, endurance, and polo. Bente and Anders (2000) as well as Cuniberti *et al.* (2012) found that neutrophil concentrations rose during and after exercise, while lymphocyte concentrations rose during acute activity and fell below baseline after long-duration exercise [29,30]. The amount of lymphocyte number change in exercise in the horse, according to Malinowski *et al.* (2006) is dependent on age and training. Both splenic contraction, which is regulated by catecholamine, and sweating, which causes fluid loss, may be blamed for the rise in the total number of red blood cells, packed cell volume, and hemoglobin concentration [28,30].

In the current study, the results obtained after exercise revealed that SAA concentrations increased 1.5 times as compared to pre-galloping levels. Exercise has a significant impact on acute phase proteins (APPs) as reported in [31]. The increase in SAA could be attributable to the beginning of the galloping training. Satué *et al.* also reported a significant increase in hematological parameters as a result of splenic contraction which is densely innervated and can normally deposit up to quarter of the total red blood cells in the body, after exercise or haemorrhage; the spleen can release this deposition; therefore, hematocrit PCV value can show up to a fifty percent (50%) increase [32]. The mean PCV and RBC values increased up to 20-30% after exercise. Fan *et al.* (2002)

also reported a significant increase in PCV, RBC, and HB which could be due to its large capacity of sequestering blood cells in the spleen, and WBC in prolonged exercise induced leukocytosis as a result of cortisol release [5]. Our findings confirmed that SAA is perhaps the most responsive APP in horses despite the fact that the event appears to produce a modest reaction when compared to high intensity endurance riding.

## 5. Conclusion

In conclusion, the SAA utilized in the present study could be used as an indicator of post-galloping value in athletic Arabian horses as a result of resistance provided by the sandy terrain which might probably cause glycogen depletion, leading to exhaustion. Individual diversity in response to exercise is evident, with modest increases in SAA levels observed in certain horses. To determine the clinical usefulness of SAA measurement in galloping horses in the study region, more research with repeated post-galloping samplings over a longer period is needed. Our findings back up the idea that galloping exercise in sandy tracts might change SAA, packed cell volume, hemoglobin, and red blood cells. Furthermore, some differential leucocytes count those that have never been reported before such as monocytes and eosinophils to have shown an increase in the current study, but within the normal range values. These early discoveries came about as a result of organized galloping on a controlled number of Arabian horses.

## Authors' Contributions

Lawan Adamu and Muhammed Modu Bukar planned and designed the study. Yakaka Wakil collected the data and carried out the study, while Isa Gulani ran the experiments. Lawan Adamu carried out statistical analysis. All authors read and approved the manuscript.

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## Data Availability Statement

The data supporting the findings of this study are available upon request from the corresponding author.

## Conflicts of Interest

The authors declare that there is no conflicts of interest.

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## Ethical Approval

The study has been approved by the ethical committee of the Faculty of Veterinary Medicine, University of Maiduguri. In

addition, the study also complied with the guidelines of the Declaration of Helsinki.

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# Penetration Enhancers for the Development of Intranasal Formulations for Use in Equines

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## Abstract

The aim of this review is to assess penetration enhancers, such as cyclodextrins, chitosan and their derivatives, surfactants, bile acids, their salts and derivatives, sodium taurodihydrofusidate, and phospholipids used in the development of intranasal formulations with a potential application in horses. In the last few years, the interest in the intranasal administration route in humans has grown because it is bloodless, noninvasive, and painless and represents a direct path toward the central nervous system. However, in equine medicine, the use of this administration route is scarce. Since equines have a nasal cavity with large surface area and blood irrigation, a high bioavailability of intranasal administered drugs is expected. Nowadays, the development of formulations for intranasal administration in equines is a challenge. The present review proposes the assessment of the characteristics and potential effects of the most important penetration enhancers in the development of intranasal formulations for use in equines.

## Keywords

Horses; cyclodextrins; chitosan; surfactants; bile acids; sodium taurodihydrofusidate; phospholipids

## 1. Introduction

Nowadays, the development of formulations for intranasal administration in equines is a challenge. When a drug is administered intranasally, before arriving at the action site, it must face many obstacles that are peculiar to the anatomy of the nasal cavity and the chemical characteristics of the drug [1].

Drug bioavailability can be affected by physiological barriers such as mucociliary clearance, the pathophysiology of the nasal mucosa, nasal metabolism, presence of mucus, and the low permeability of the blood-brain barrier. Moreover, drug bioavailability can be modified by its physicochemical characteristics such as solubility and dissolution ability, molecular size and weight, lipophilicity, pKa, and the pH of the environment. Furthermore, drug bioavailability depends

on features of formulation as drug concentration, doses, osmolality, and dosage form [2,3]. In recent years, different strategies to evade these problems have been studied. A traditional choice is improving drug absorption by adding excipients.

## 2. Anatomy of Equine Nose

The central nervous system (CNS) is directly connected with the environment by only one anatomical site, the nasal cavity. The nasal cavity is divided into two symmetrical halves by the median septum, a central partition bone and cartilage. The dorsal wall of the nasal cavity is formed by the frontal and nasal bones. The lateral walls include the maxillary bone, the premaxilla, and the perpendicular portion of the palatine bone. The nasal cavity and the cranial cavity are connected through the ethmoid lamina, which seems to be a concave



sieve and where it is the olfactory bulb. Inside each nostril, there are three turbinates (the dorsal, the median, and the ventral turbinate) responsible for conditioning the incoming air (**Figure 1**).

The nasal cavity is irrigated by the major palatine artery, sphenopalatine artery, and branches of ethmoids. It is innervated by the trigeminal nerve, whose branches take chemosensory and thermosensory information from oral, ocular, and nasal mucosa [4].

Behind the vestibular region, the nasal cavity is divided into the respiratory region, which warms and moistures the incoming air, and the olfactory region, where smell receptors are located [5].

The respiratory region is covered with nasal respiratory mucosa, whose surface has ciliated pseudostratified columnar epithelia and is reddish because of its high vascularization. Respiratory region is located in a region near the dorsal turbinate and formed by nonciliated and ciliated columnar cells, basal cells, and goblet cells. Respiratory region is also related to seromucosal and intraepithelial glands. Seromucosal glands produce most of the nasal secretions [6–8].

The olfactory region is covered with specializing olfactory mucosa and is located in the roof of the nasal cavity and, in varying extension, in adjacent lateral and medial walls. The olfactory tissue is often yellow in color because of the pigment inside sustentacular cells [5,8].

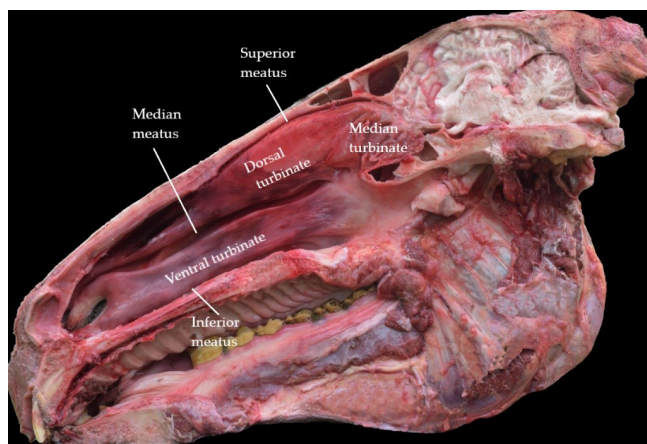
The olfactory region in the nasal cavity has a variable extension, depending on the animal species [5,8].

Lamina propria of the olfactory mucosa is in direct contiguity with periosteum subjacent bone. This conjunctive tissue has considerable blood and lymphatics vessels, unmyelinated olfactory nerves, myelinated nerves, and olfactory glands [8].

The olfactory epithelium, as respiratory one, is pseudostratified, but it contains only three types of cells: the olfactory neural cells, sustentacular cells, and the basal cells, lacking goblet cells [7,8].

The olfactory glands (Bowman's glands) are proper to olfactory mucosa; they are branched serous tubuloalveolar glands that send their protein secretion to the olfactory surface through grooves. The constant flow from glands cleans the mucosa from the rest of odoriferous substances detected, so new smells can be perceived continuously as they emerge [8].

The olfactory system is also formed by accessory organs such as the vomeronasal organ. It is located in both nostrils and in the vomer and is connected to the olfactory bulb with a tube. Its surface is covered with olfactory epithelium and neuronal cells. It has an important function in animal behavior [8].



**Figure 1:** Image of an equine head showing anatomical sites. This figure was originally published in [4].

### 3. Intranasal Administration

Historically, intranasal administration has been limited to topic application with local effects like antihistaminic and corticosteroids drugs; however, in last years, it has received more attention because it is a promising, suitable, and reliable system for brain drug delivery, focusing on drugs that are inefficient by oral administration or must be administrated with injections.

The intranasal route provides a good surface with easy access avoiding first-pass metabolism. Small and lipophilic drug molecules have better bioavailability because the nasal cavity has easy accessibility to blood capillaries. Moreover, absorption and action of the drug are faster and it has a lower risk of overdosing. The nasal mucosa is more permeable to a wide variety of chemical substances than gastrointestinal mucosa because it lacks pancreatic and gastric enzymes and interference of gastrointestinal content. In addition, it is appropriate for patients with long-term therapy because it is not invasive and avoids the blood-brain barrier; therefore, drug bioavailability in the CNS is increased [9–12]. Intranasal administration is one of the most successful approaches for the systemic administration of macromolecules. Its application has been studied in a wide range of indications such as migraine, headache, infection prevention, pain management, hormone replacement therapy, and emergency therapy in seizures. Moreover, small peptides, such as oxytocin (1 kDa), buserelin (1,2 kDa), gonadorelin (1,2 kDa) and nafarelin (1,4 kDa) [11,12], are traded using intranasal formulations.

In equine species, the large surface and irrigation of the nasal cavity allow inferring the viability of this administration route for drug administration [3].

### 4. Mechanisms of Nasal Drug Absorption

It is important to review the pathways and mechanisms of drug passage from the nasal cavity to the systemic circulation and the CNS to advance intranasal formulations.

The properties of the formulation affecting drug absorption depend on the administration route and the type of dosage form selected. Absorption could be affected by the viscosity and osmolarity of the formulation, the nasal blood flow, the enzymatic activity in the nose, and the physical condition of the nasal mucosa. Also, it is advised to maintain the formulation pH between 4.5 and 6.5 because the pKa value of

the drug can influence the amount of ionized and deionized molecules but also prevent the growth of pathogenic bacteria in the nasal passage, avoid nasal irritation, maintain the functionality of excipients, and sustain the normal physiology of ciliary movement [13].

Firstly, when a drug enters the nasal cavity, it suffers mucociliary clearance in the vestibular region. Therefore, drug molecules move towards the posterior region nasal cavity, where there are in close contact with the respiratory and olfactory region. Then, drug delivery to the nervous system takes place by different paths: olfactory nerve, trigeminal nerve, and lymphatic, vascular, and cerebrospinal fluid routes. However, mechanisms and pathways which describe drug delivery from the nasal cavity to CNS are not totally comprised. Drugs would follow one specific route or a combination of the mentioned routes [14–18].

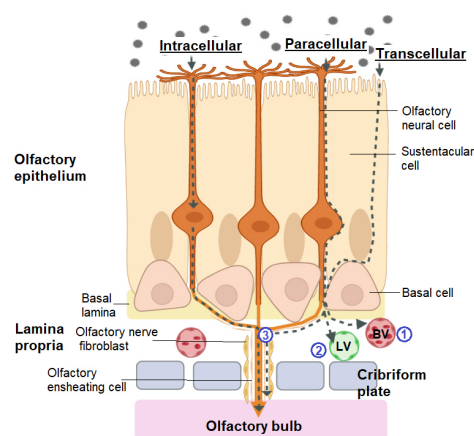
In the olfactory nerve route, the drug interacts with olfactory neural cells and crosses the olfactory epithelium. It arrives at the lamina propria, crosses the cribriform plate, and reaches the olfactory bulb, being finally directed toward the cerebral tissue or cerebrospinal fluid [19–21].

This route is divided into intraneuronal and extraneuronal routes (Figure 2).

In the intraneuronal route, olfactory neural cells catch drug molecules through processes such as endocytosis, passive diffusion, and/or receptor-mediated endocytosis, and the drug reaches the olfactory bulb through axonal transport [3,19].

The intraneuronal route is important but not the predominant route of drug delivery through CNS because the drug needs hours or days to reach the olfactory bulb and the brain. This transport has been reported for gold particles, aluminum salts, and molecules with receptors in olfactory neural cells like wheat germ agglutinin [3,19,22–25].

It has been published that some viruses would use receptor-mediated endocytosis to enter the olfactory epithelium. Many strains of influenza viruses, herpes viruses, and polioviruses use different receptors on the surface of the cilium to enter the olfactory neural cells. Recently, it was published that the SARS-COV-2 virus uses the nose as a gate to the human body. Some articles have shown that the nasal epithelium and olfactory epithelium express angiotensin-converting enzyme II (ACE2), and the virus uses it as an input receptor with cellular serine protease TMPRSS2 for priming protein S. Curiously, loss of smell is an early marker of SARS-COV-2 infection. It is suggested that the damage of sustentacular cells is expressed as nonneural cells and the presence of ACE2 leads to patience with olfactory deficits [11].



**Figure 2:** Drugs can take two different routes to cross the olfactory epithelium (intracellular and extracellular route, involving paracellular and transcellular mechanisms). Once the drug reaches the lamina propria, it can (1) be absorbed into the blood vessels and reach the circulation (BV), (2) be absorbed into the lymphatic vessels and drain to the cervical lymph nodes (LV), and (3) travel to the perineural space between the sheathed olfactory cells and the fibroblasts of the olfactory nerve towards the bulb.

In the extraneuronal route, the drug crosses the epithelium by paracellular route between the olfactory neural cells and the basal cells or by transcellular route through basal cells; then, it is transported to the olfactory bulb (Figure 2). This mechanism is fast and the drug needs only a few minutes or half an hour to reach the olfactory bulb or other areas in CNS [11,19,21,26].

The majority of publications about intranasal administration report fast drug delivery, high concentrations in CNS, and immediate clinical effects, consistent with the extracellular route [3].

In the trigeminal route, a drug is transported through this nerve; although it has been considered a minor mechanism, in recent years, its importance has been recognized [3].

Olfactory and respiratory epitheliums are innervated by the trigeminal nerve, which reach the CNS at the pons with a small portion ending inside the olfactory bulb. A specific characteristic of the trigeminal nerve is that it enters the brain from the respiratory epithelium through the anterior lacerated foramen near pons and the cribriform plate near the olfactory bulb, therefore making entry points, after intranasal administration, in rostral and caudal areas in the brain. Other nerves that innerve the head and face, such as facial nerve or other sensorial structures in the nasal cavity, can provide entry points to CNS after intranasal administration. First-time trigeminal route in intranasal drug administration was demonstrated, I125-labeled insulin growth factor was used. It showed high radioactivity levels on branches from trigeminal nerves, trigeminal ganglion, the pons, and olfactory bulbs; this is consistent with the transport along the olfactory nerve and trigeminal nerve [19].

Furthermore, the nasal septum contains a little aperture near the base to access the vomeronasal-terminal nerve through the vomeronasal organ. Drugs would be absorbed directly to

the brain through the vomeronasal organ and terminal nerve [18,27,28].

When the drug reaches the olfactory bulb, it is transported to other parts on the brain for diffusion using a perivascular bomb driven by arterial pulsation [21,29].

The nasal mucosa has high blood irrigation and permeability, becoming an interesting administration pathway. Its large surface and vascularity contribute to quickly warming and moisturizing the incoming air; these factors predispose it as a formidable drug absorption site [30,31]. Blood vessel density in respiratory mucosa is higher than that in olfactory mucosa, so respiratory mucosa is ideal for systemic drug absorption.

Blood vessels in the respiratory region contain a mix of continuous and fenestrated endothelium, allowing small and large molecules administered intranasally to enter systemic circulation, reaching CNS passing through the blood-brain barrier. It is expected that small lipophilic drugs get into the bloodstream easier than large or hydrophilic drugs such as peptides or proteins. Also, it is possible that instead of being distributed through the systemic circulation, xenobiotics can enter the venous blood supply in nasal passages where they are rapidly distributed to the carotid artery blood supply, which feeds the brain and spinal cord in a process known as countercurrent transfer [19].

## 5. Penetration Enhancers

In the development of intranasal formulations, there is a wide range of strategies for increasing drug bioavailability. A mechanism that allows achieving this objective is adding excipients to improve drug penetration and absorption. Pharmacopeia defines excipient as any substance added intentionally to the formulation of any dosage form, which is different from the active pharmaceutical ingredient. The term absorption enhancer refers to an agent whose function is to increase absorption by enhancing membrane permeation rather than increasing solubility, therefore such agents are sometimes more specifically named permeation enhancers [1,32,33].

Penetration enhancers have been studied to improve the efficacy of peptides, proteins, and other active pharmaceutical ingredients with an inefficient permeability. Penetration enhancers are functional excipients added to formulations to improve the permeation of active pharmaceutical ingredients through biological barriers [34].

An ideal penetration enhancer has some features: at the concentration used, it is pharmacologically inert; its effect must be specific, appropriate with predictable duration, should not cause irritation, allergy, or toxicity, and effective at small concentrations. Also, it should be compatible with the drug and other adjuvants present in the formulation and be able to abide in contact with the nasal mucosa time enough to extend its maximum effect; it should not have an unpleasant smell or taste and should not harm the membrane [1,30,35].

The mechanisms of action of penetration enhancers are not totally understood. However, it is supposed they act by exerting one or both of the following effects [30,36]:

- Physicochemical effects: some penetration enhancers can alter drug physicochemical features by modifying drug

solubility, drug partition coefficient, or weak interactions with the drug. These mechanisms are observed at low concentrations.

- Membrane effects: many penetration enhancers work acting on the surface of the nasal membrane. Although most of these enhancers are not harmful, it is necessary to determine the extension and consequences. In most cases, penetration enhancers have a transitory effect without irreversible consequences.

In the pharmaceutical industry, there is a wide range of penetration enhancers. Behl *et al.* [30] have recognized the difficulty of precisely classifying these agents in a meaningful manner because they often have overlapping chemical properties, and their penetration mechanisms are not wholly understood. In this review, classification proposed by Behl *et al.* [30] is applied.

### 5.1. Cyclodextrins

In the last few years, cyclodextrins have become a focal point of investigation in different areas of applications, with special interests of the pharmaceutical industry. Most intranasal formulations contain penetration enhancers for improving absorption of lipophilic drugs, increasing their aqueous solubility and/or improving their nasal penetration [36–38].

Cyclodextrins are cyclic oligosaccharides of six or more  $\alpha$ -D-glucopyranoses units joined with 1-4 glycoside bonds, produced by the action of enzymes on starch [39,40].

Although they were described in 1891, it was not until 1976 that they were used as part of a pharmaceutical product of Ono Pharmaceutical Co. company [41,42].

The commonest natural cyclodextrins ( $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin, and  $\gamma$ -cyclodextrin) are formed by 6, 7, and 8 glucose units.

Cyclodextrins derivatives are obtained for the substitution of hydroxide group instead of desirable functional groups. Nowadays, more than 1,500 different cyclodextrins derivatives have been described, but only a few are available as pharmaceutical excipients because the analysis of toxicities is costly. It is unlikely that a new derivative would give a wide advantage over the currently employed cyclodextrins; therefore, the availability of new cyclodextrins may increase very slowly. Pharmaceutical products often have methyl, hydroxypropyl, and sulfobutylether derivatives to improve their solubility and capability of inclusion above natural cyclodextrins [42–44].

Cyclodextrins act as a solubilizer and permeation enhancer for nasal drug delivery. Cyclodextrins interact with poorly water-soluble substances increasing their apparent solubility. The mechanism of solubility is given for the formation of an inclusion complex where the guest molecule (drug) and host molecule (cyclodextrin) are in a dynamic balance with the complex [1,38].

As a result of chair conformation glucopyranose units, cyclodextrins take a cone truncated shape (Figure 3(a)). The hydroxyl residues are oriented outside the cone, with the primary hydroxyl groups of the sugar in the narrowest part of the cone and the secondary hydroxyl group in the widest part. The central part of the cavity from the cyclodextrin molecule

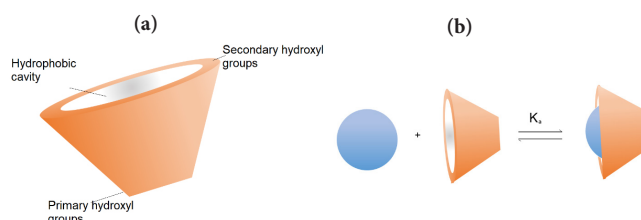
is aligned with the carbon skeleton and glycoside oxygen of glucose. The ends of cyclodextrins that have a hydroxyl group are polar and their interiors are relatively nonpolar [36,38].

In an aqueous solution, the cyclodextrin cavity is slightly nonpolar and is occupied by water molecules that are unfavorably energetic for the formation of a polar–nonpolar interaction, so they can be easily replaced with an appropriate "guest molecule" less polar than water. The dissolved cyclodextrin is the host molecule and part of the driving force of complex formation is the substitution of water molecules with high enthalpy for the adequate guest molecule. Cyclodextrins are attached to guest molecules for the van der Waals bond, hydrogen bond, hydrophobic bond, and covalent bond. One, two, and three cyclodextrins molecules have one or two guest molecules. This mechanism is known as molecular encapsulation (Figure 3(b)). The most frequent and simple situation is when the guest: host relationship is 1:1, although there could be, simultaneously, more complex associations and other balances could exist with a high order. The complex balance depends on cyclodextrin molecular concentration, guest molecule concentration, host molecule concentration, and water concentration. The bond can be broken with changes in pH and solution temperature [45,46].

The mechanism of cyclodextrins as drug penetration enhancers occurs in specific conditions and is not totally elucidated nowadays. It has been reported that methylated  $\beta$ -cyclodextrin increases transcellular and paracellular movements of peptidic drugs, improving transmucosal absorption. Also, it has been shown that tetradecyl- $\beta$ -maltoside-cyclodextrin acts by opening tight junctions between cells [12,34].

Natural cyclodextrins and their derivatives are listed as accepted excipients for FDA (The United States Food and Drug Administration). Cyclodextrins toxicity depends on the administration route. When they are orally administered, they are well tolerated and nontoxic; however, when administered through the intravenous route, they become unsafe since they can crystallize. Cyclodextrins can be corrosive on tissues; however, methylated  $\beta$ -cyclodextrin and dimethylated  $\beta$ -cyclodextrin do not show this local effect and do not produce local nasal toxicity. In fact, the local nasal irritation of methylated  $\beta$ -cyclodextrin is lower than that of benzalkonium chloride [38,43].

Cyclodextrins have been used as intranasal penetration enhancers for calcitonin, insulin, glucagon, and recombinant granulocyte colony-stimulating factor. In the market, there is a commercial cyclodextrin product called KLEPTOSE® HPB (hydroxypropyl- $\beta$ -cyclodextrin), which is proposed as an attractive excipient for intranasal drug administration due to its capacity as a drug solubilizer and low toxicity [34,37].



**Figure 3(a):** Descriptive scheme of molecular structures of cyclodextrins. Cyclodextrins have a truncated shape where their cavity is lipophilic. Hydroxylic groups are outward-oriented, where hydroxyls are in a wider zone and primary hydroxyls in a narrower zone [40]. **(b):** Simplified graphic of molecular encapsulation. The guest lipophilic molecules (circle) interact with cyclodextrin (host molecule) with van der Waals bonds, hydrogen bond, and hydrophobic bond, moving the water, because they are entropically favored for their lipophilic nature.

## 5.2. Chitosan and Derivatives

Chitosan is cationic linear polysaccharides obtained from the deacetylation of chitin, the second most abundant polysaccharide in nature after cellulose; it is present in crustacean shells (shrimp and crabs), the exoskeleton of insects, and fungal cell wall [47,48].

Chitosan is a copolymer composed of 2-acetamido-2-deoxy- $\beta$ -D-glucopyranose and 2-amino-2-deoxy- $\beta$ -D-pyranose units. It has a primary amine group and three free hydroxyls groups for each unit of  $C_6$  (Figure 4). This chemical substance is not usually defined as an exact composition, but it is characterized by the number of sugar units of each polymer molecule and deacetylation grade. These properties impact biological and physical polymer factors [48–50].

Chitosan and derivatives are known as versatile materials because they have diverse activities; they are biocompatible, biodegradable, nontoxic, cheap, and, usually, nonallergenic. Moreover, they have superior physical properties such as large superficial area, porosity, conductivity, and being easily malleable in different shapes, such as films, sponges, fibers, gel, beads, nanoparticles, microparticles, powders, and solutions. These characteristics have led to chitosan and its derivatives being used in numerous agricultural, biomedical, and pharmaceutical applications. Applications in the biomedical industry include wound healing, genetic engineering, water treatment, implant preparation, tissue engineering, obesity treatment, and dietary supplements to lower cholesterol. Moreover, since they have antibacterial activity, they have been used in antibacterial food and textile packaging materials. They also have antiulcer, antioxidant, antifungal, anti-HIV, and antitumor activity. On the other hand, chitosan is used in the pharmaceutical industry as an effective penetration enhancer for hydrophilic drugs with poor absorption and as a drug carrier, not only improving their absorption but also stabilizing their components for targeting and improving their liberation. Absorption improvement through the intestinal and nasal epithelia has been reported for calcitonin,

insulin, and busserelin. Pharmaceutical formulations based on chitosan are presented in **Table 1** [47,49,52,53].

Regarding chitosan derivatives, applications of N-trimethyl chitosan chlorides, esters, and conjugates have been reported.

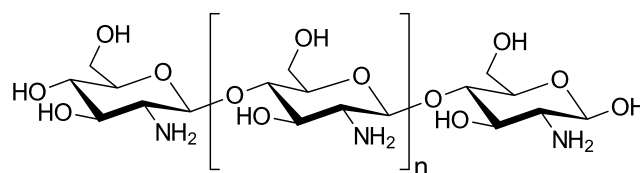
N-trimethyl chitosan chlorides (TMC) are quaternary chitosan derivatives with higher water solubility, intestinal permeability, and absorption over a wide pH range. The polymer of TMC is designated as TMC-20%, TMC-40% and TMC-60% according to its degree of methylation. It has been observed that the higher the substitution degree, the lower its solubility. Hamman *et al.* have shown that quaternization of TMC decreases the transepithelial electrical resistance and thereby influences drug absorption-enhancing properties [54].

Chitosan-derived esters (glutamate, succinate, phthalate) have different solubility profiles. Chitosan-derived esters-based matrix has been successfully used in many formulations, such as sodium diclofenac with a specific delivery to the colon [52].

Lastly, chitosan can be conjugated to bioactive excipients for delivering active ingredients, such as calcitonin. Some chitosan conjugates, such as 5- methylpyrrolidine chitosan and chitosan-4-thiobutylamidine, have shown enhanced penetration and mucoadhesive properties. Guggi and Bernkop conjugated an enzyme inhibitor with chitosan, resulting in a polymer that prevents drug enzymatic degradation by trypsin and chymotrypsin, concluding that these conjugated chitosans have a promising peptide drug delivery as calcitonin [52].

**Table 1:** Formulations based on chitosan (type of system and drugs) [52].

Types of system	Drug
Tablet	Diclofenac sodium theophylline, mesalamine, glipizide
Capsules	Insulin
Microspheres/microparticles	Gentamicin sulfate, hemoglobin, diclofenac, clarithromycin, propranolol- HCl famotidine, bovine serum albumin, clozapine
Nanoparticles	Gadopentetic acid, bovine serum albumin, ascorbic acid, cyclosporin A, doxorubicin
Beads	Bovine serum albumin, insulin
Films	Ofloxacin, paclitaxel
Gel	5-Fluorouracil



**Figure 4:** Scheme of the molecular structure of chitosan. Figure adapted from [51].

It has been reported that chitosan is applicable to the intranasal formulation for proteins delivery. Chitosan has mucoadhesive properties, probably generated for ionic interaction between positively charged amino groups in chitosan and negatively charged sialic acid residues in mucus. It has demonstrated that the strong cationic nature of chitosan promotes interaction with sialic acid present in the mucosa; hence, it opens the tight junctions of nasal epithelial cells. Intranasal formulation with chitosan could improve drug time residence in tissues and cells, showing a sustained drug release, increasing drug bioavailability, and minimizing administration frequency. Also, it has been found that chitosan improves drug penetration through mucosa without damage to the biological system. Illum *et al.* demonstrated that chitosan glutamate can enhance the passage of insulin through rat and sheep nasal mucosa. Histological examination of rat nasal treated with 0.5% (p/v) of chitosan glutamate during 60 minutes revealed slight structural changes [1, 50, 52].

The efficacy of chitosan as a permeation enhancer was confirmed using salmon calcitonin in rats. The absolute bioavailability of calcitonin was higher in animals that were treated with 1% free amine than rats that received 5% of dimethylated  $\beta$ -cyclodextrins. It was found that free amines and chitosan salts produce mild nasal irritation. Based on these results, chitosan seems to be a safe and effective enhancer for intranasal administration [49,50,52,55].

### 5.3. Surfactants

Surfactants are amphiphilic molecules with a hydrophilic head and hydrophobic tail, forming a polar asymmetric structure (**Figure 5**). Surfactants can decrease the surface tension between air and water phases, improving the miscibility of substances allowing their diffusion or mixing as an emulsion in water or other solvents [55,56].

These chemicals have applications in the food, petrochemical, and pharmaceutical industries. In the latter, they are used for drug solubility in an aqueous medium, as emulsion components, for vehicles of formulations for the oral or transdermal route, as a plasticizer in release system for drugs with semisolid form, and as drug penetration enhancer or absorption enhancer [57–59].

When surfactants are added in an aqueous medium, they reduce the surface tension of water. As surfactant concentration increases, surface tension falls to a certain concentration where surfactants molecules form micelles spontaneously. The hydrophobic zone of the molecule is sequestered from an aqueous medium, which is highly polar, through a surrounding layer, and depending on the surfactant, it can take a cylindrical, spherical, hexagonal, laminar cubic, inverted cylindrical, or inverted spherical shape. When these micelles are formed, the addition of an excess of surfactant has no effect on the surface tension of water. This concentration in

which surface tension is constant is known as critical micellar concentration (CMC) [57,60].

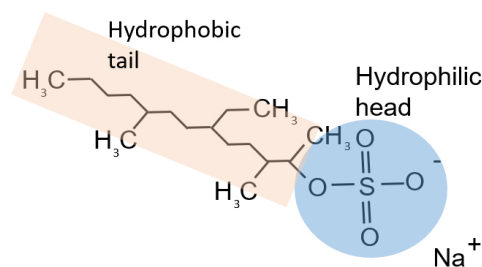
Surfactants can be natural or synthetic, soluble or insoluble, and their chemical structures can be simple or polymeric. Depending on the molecule charge, they are classified into anionic, cationic, nonionic, and zwitterionic surfactant [56].

Anionic surfactants have a negative charge in the hydrophilic region of the molecule. Their hydrophilic groups are classified into phosphate ester (such as aryl ether phosphates and acyl ether phosphates), fatty acid salt type, salt type sulphonate (for example, sodium dioctyl sulfosuccinate or alkylbenzene sulfonates), and carboxylic acid. They can be used as foaming agents, antistatic agents, dispersants, detergents, and emulsifiers (Figure 6(a)) [56,58].

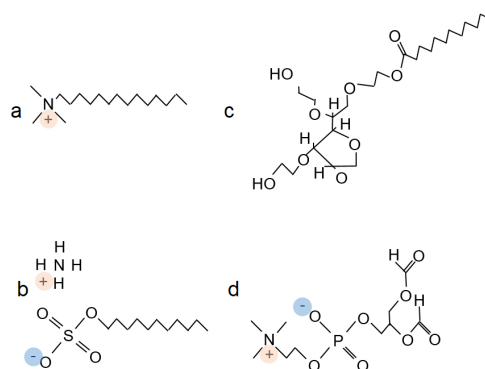
Cationic surfactants have a positive charge in the hydrophilic region of the molecule, for example, an amine with a long chain or a quaternary ammonium salt such as dimethyldioctadecylammonium chloride or benzalkonium chloride. They are classified into open chain cationic surfactants, heterocycle group cationic surfactants, and cationic surfactants with intermediate linkage. They are used in corrosion, oxidation, sterilization, and cleaning skin wounds. They also have bactericidal activity in a wide range of Gram-positive and certain Gram-negative microorganisms; this is the principal reason for their use in the pharmaceutical industry (Figure 6(b)) [57,58].

Nonionic surfactants have no charge on the head of the molecule. They are classified into polyoxyethylene esters, poloxamers, and polyol esters. The latter include glycol, glycol esters, and sorbitan derivatives. Sorbitan fatty acid esters (Spans) and their ethoxylated derivatives (called Tweens) are the most widely used nonionic surfactant. Nonionic surfactants are used in the paper, textile, food, and plastic industry and in the production of glass and pesticides and are widely used in the development of pharmaceutical formulations (Figure 6(c)) [56,58].

Zwitterionic or amphoteric surfactants have a positive and negative charge in their molecule. The most common amphoteric surfactants are betaine derivatives. Depending on the pH of the surrounding medium, surfactants acquire positive, negative, or null charge: in solution with acid pH, they have a positive charge, while if they are in solutions with alkaline pH, they have a negative charge and in solution with a pH in the isoelectric point, both groups have equal ionization. Amphoteric surfactants have minimal toxicity and they have an excellent biodegradation. They are in shower gels, shampoo, cosmetics, industrial softeners, and antistatic agents (Figure 6(d)) [57,58,61].



**Figure 5:** General molecular structure of surfactants. Figure adapted from [57].



**Figure 6(a):** Scheme of the molecular structure of cationic surfactant (cetrimide). They have a positive charge in the hydrophilic region of the molecule. Figure adapted from [62]. **(b):** Scheme of the molecular structure of anionic surfactant (ammonium dodecyl sulfate). They have a negative charge in the hydrophilic region of the molecule. Figure adapted from [63]. **(c):** Scheme of the molecular structure of nonionic surfactant (Tween 20), which carries no charge. Figure adapted from [64]. **(d):** Scheme of the molecular structure of zwitterionic surfactant (egg phospholipids). Figure adapted from [65].

The mechanism of action has a thermodynamic origin. A surfactant (whose name has the origin in surface-active agent) is a substance present in low concentration inside a system and it can be adsorbed onto the surface or interface and alter the free energy of these surfaces or interfaces. The term interface indicates the union between two or more immiscible phases; the term surface denotes an interface where one could be water and the other, air. Free energy is the minimal amount of necessary work to make this interface. Interfacial free energy per area unit is the measure used to determine the surface tension between two areas. Surface tension is a measure of the difference of nature from two phases which are in an interface (or superficies). The higher the difference of the nature between the two phases, the higher the surface (or interfacial) tension [66].

The minimal work required to make an additional amount of interface is calculated as the product between interfacial tension and the increment of the area in this interface. Surfactants are substances that, in low concentration, adsorbed part or the entire interface in the system, changing significantly the amount of work necessary to expand these interfaces [66].

**Table 2** shows some surfactants listed in the FDA's inactive ingredient guide [67].

Surfactants are the most used excipients in intranasal formulations for improving drug permeability through mucosa

[66]. The addition of surfactant to intranasal formulation could modify the permeability of the nasal membrane, facilitating the absorption of drugs. It has been reported that several surfactants improve drug nasal absorption, increasing drug bioavailability significantly. Some surfactants can alter and even dissolve biological membranes [68].

Moreover, surfactants have been widely used in the aerosol nasal formulation. These substances not only reduce surface water tension but also facilitate the atomization of the formulation. In intranasal spray formulations, the formation of a uniform nebulization depends on the device and the formulation, so the use of surfactants is common to achieve an effective dose (by droplet size and nebulization) [58].

As a result, surfactants have been extensively used in the formulation for their potential to improve intranasal drug absorption (Table 3). Li *et al.* studied the potential of some nonionic surfactants as penetration enhancers in the intranasal administration of rats using an *in vitro* perfusion technique. The drug was sumatriptan and nonionic surfactants were sucrose laurate ester, cremophor, and poloxamer 188, being sucrose laurate ester the most effective [68,69].

Velloso *et al.* have recently published an article comparing two surfactants (a cationic surfactant (cetrimide) and a nonionic surfactant (Tween 80)) as penetration enhancers for butorphanol in an intranasal formulation for use in equines. The results showed that, at the concentrations used, Tween 80 was the most suitable for intranasal formulations of butorphanol for equine application [70].

Regarding toxicity, daily exposure to unprotected surfactants is related to a large number of dermatological problems, such as irritation and allergy, in different degrees of severity. Alkyl polyglucosides, zwitterionic surfactant (such as betaines, amido betaines, and isethionates), and various polymeric surfactants are known to be smooth in the skin, while ethoxylated fatty alcohols cause irritation. Long-chain surfactants (greater than 12 carbons) cause maximum skin irritation. Cationic surfactants are more toxic than anionic surfactants, but anionic surfactants are more toxic than nonionic surfactants [61,71].

Surfactants, when used as an enhancer in intranasal formulations, can cause nasal irritation, epithelial toxicity, and ciliated activity [1].

On the other hand, chemically synthesized surfactants are mostly derived from petroleum and are not usually biodegradable, leaving a toxic residue in the environment. In recent years, the research and application of biosurfactants have grown. Biosurfactants are defined as surfactants produced by microorganisms from a wide variety of substances. They have unique properties such as low toxicity, functionality in extreme conditions, and biodegradability. The large diversity of these molecules supports their potential use in the oil industry, medicine, agriculture, food industry, and cosmetics, among others [59,71].

#### 5.4. Bile Acids, Their Salts, and Derivatives

Bile acids are employed in drug formulation for enhancement drug penetration through mucosa, including oral, nasal, ocular, buccal, pulmonary, and rectal, and through the

blood-brain barrier. The mechanism of action as penetration enhancer is based on their capacity to open tight junctions, act over mucolytic activity, and increase the solubility of hydrophobic drugs, increasing the apical and basolateral fluidity and promoting the chemical and enzymatic activity of drugs [12,72–74].

Bile acids are amphiphilic molecules synthesized in the liver from cholesterol; they are the principal substance of bile. They act in the intestine as a biological surfactant on the solubilization and absorption of lipids from dietary fats and endogenous soluble molecules such as cholesterol, fatty acids, and hydrophobic drugs. Beyond their role in digestion, they are also involved in other important physiological processes like glucose homeostasis regulation, lipids metabolism regulation, modulation of inflammatory and immunomodulatory processes, and antibacterial action in the small intestine [72,75,76].

Bile acids have a specific molecular structure due to the presence of a steroid nucleus which is large, rigid, and plane. Its structure consists of four rings, three of them with six carbons and one with five carbons (Figure 7). The concave side ( $\alpha$ ) of steroid skeleton is hydrophilic due to the presence of hydroxyl groups, while the convex side ( $\beta$ ) has angular methyl groups making it hydrophobic. This structure makes bile acids very different from ordinary aliphatic surfactants, which are composed of a polar head and nonpolar tail; as a consequence, they have a high surface activity and in water, they can associate themselves forming polymolecular aggregates, such as micelles or liposomes, as long as, their concentration is above CMC. The facial amphiphilicity of bile acids influences the way they are organized in solution and is presumed to play an important role in their ability to promote membrane penetration of polar drugs. Bile salts are classified into three groups depending on their conjugation with amino acids and the degree of hydroxylation, including trihydroxy conjugates (such as taurocholate and glycolate), dihydroxy conjugate (such as glycodeoxycholate, glycochenodeoxycholate, and taurochenodeoxycholic, and unconjugated forms (such as cholate, deoxycholate, and chenodeoxycholate) [40,72,73].

The micelle formation of bile salts allows facilitating the transcellular drug passage, improving drug absorption. In aqueous solutions, the anions of bile salts are joined together, making simple micelles; moreover, they can form mixed micelles when combined with polar lipids, conventional surfactants, or amphiphilic drugs. Mixed micelles usually have lower CMC and better solubility capacity than the individual components due to synergic interactions. Furthermore, mixed micellar systems can be prepared without the help of organic solvents and have been applied in pharmaceutical formulations to improve the bioavailability of poorly water-soluble drugs, such as oxaprozin, rebamipide, and doxorubicin [73–76,78–80].

Bilosomes are different vesicular systems that contain bile salts and liposomes. Incorporating bile salts into liposomes can stabilize the membrane against the noxious effects of physiological bile acids in the gastrointestinal tract and facilitate the internalization of particles. Since bilosomes are produced from natural lipids are biocompatible, they have been used as oral administration systems for poorly water-

soluble drugs, such as peptide drugs or vaccines, due to greater stability to acid pH, enzymes, and bile salts [73,75].

Polymeric nanoparticles constitute a new generation of the therapeutic delivery platform with several advantages over other lipid-based carriers, such as liposomes or micelles. They show a better circulation time and greater accumulation at the site of action because of their small size and better capacity of cellular penetration, which improve the permeability and retention effect. With this, polymer-lipid hybrids obtain from the conjugation of bile acid and hydrophilic and biodegradable polymer constitutes a promising drug delivery. These amphiphilic hybrids can self-assemble in an aqueous medium to form nano-sized micelles with a unique core/shell structure, which are formed by a nucleus of bile salt and stabilized by the hydrophilic crown. The drug load of hybrids depends on nucleus size and the crown, while the drug entrapment is associated with a higher size in the nucleus. Moreover, the CMC increases as increase the length of the polymer that constitutes the crown. Usually, polyethyleneglycol is the preferential polymer for nanocarrier systems because it improves biocompatibility and hydrophobicity and avoids opsonization [72].

Due to their particular structural properties, bile acids are suitable substances to make supramolecular aggregates, such as the previously mentioned and hyperbranched and hydrogels structures. Furthermore, their good availability, low cost, and simple procedures for derivatization make them an attractive block to design new pharmaceutical formulations for the administration of drugs, vaccines, and biomolecules [72].

The use of hydrophobic bile acids as pharmaceutical excipients has some issues because their accumulation and retention have been linked as one of the main causes of liver damage in cholestasis. Furthermore, they have limited clinical use due to their irreversible mucosal damage and cytotoxicity. Hydrophobicity is decisive in the cytotoxicity of bile acids. Also, it has been reported that bile acids with hydrophobic nature such as deoxycholic acid exert a tumor-promoting effect. Bile acids have the potential to induce cell death acting as nonspecific detergents (membranolytics) and through receptor-mediated interactions. They can promote the generation of reactive oxygen species, which can modify lipids, proteins, and nucleic acids and, eventually, cause apoptosis or cellular necrosis. It was also reported that dihydroxy bile salts are more toxic than trihydroxy bile salts; however, these toxic effects occur specifically when

they are present in supraphysiological concentration. On the other hand, it has been reported that hydrophilic bile acids, such as ursodeoxycholic acid, exert a cytoprotective effect and decrease toxic activity of hydrophobic bile acids. It was demonstrated that phosphatidylcholine prevents bile salt toxicity in epithelia and gastrointestinal membrane, this effect can be linked with the formation of less toxic micelles. The relative cytotoxicity of bile acids depends on their structure and membrane properties such as composition, fluidity, charge, and hydrophobicity. Little structural modification of natural bile salts has led to bile acids derivatives with lower toxicity. Due to the high toxicity of some hydrophobic bile acids, many semisynthetic analogs have been developed with better toxicological and pharmaceutical properties. Colylsacorsin is a nontoxic bile acid derivative that has been investigated as an enhancer excipient. This semisynthetic bile acid increased *in vivo* and *in vitro* the permeability and absorption of peptide drugs, octreotide, and desmopressin, but, to a lesser extent compared with chenodeoxycholic acid. However, for other peptidic drugs, such as calcitonin and parathyroids hormones, it has a more favorable toxicological profile. It has been reported that the substitution of hydroxyl groups with keto groups produces bile salts and significantly fewer surfactants that are less lipophilic with less toxicity in the membrane; in this context, sodium desoxycholate and taurocholate cause mild inhibition of mucociliary clearance [72,73,79].

Although some studies have indicated that bile salts can cause nasal irritation when used in a concentration above 0.3%, the concentration should not be a limiting factor for the research in the nasal formulation because there is vast evidence that they have a positive effect on drug absorption by intranasal administration [1,76].

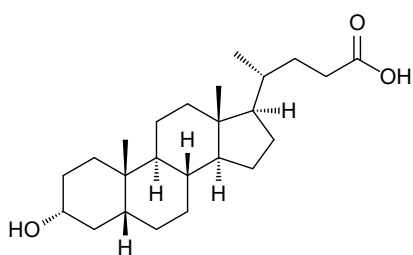
Duchauteu *et al.* studied the effect of six bile salts in the intranasal absorption of gentamicin in rabbits. The enhancers used were cholate, taurocholate, glycocholate, desoxycholate, taurodeoxycholate, and glycodesoxycholate salts. They found that gentamicin without bile salts does not penetrate through the nasal mucosa. Furthermore, they found that the better enhancers were sodium cholate and sodium taurodeoxycholate, and sodium desoxycholate was extremely cytotoxic [81].

Park *et al.* investigated the penetration of acyclovir through nasal mucosa in rats using as enhancer mixed micelles of bile salts-acylcarnitines. The bile salt was sodium glycolate, which increased penetration [82].



**Table 2:** Surfactants listed in the inactive ingredient guide by FDA [67].

Type of surfactant	Surfactant	Route	Dosage form
Anionic surfactants	Ammonium	Topical	Aerosol, emulsion, suspension, shampoo
		Buccal	Tablet, delayed release
		Dental	Gel, paste, dentifrice
		Oral	Capsule, coated pellets, drops, granule, powder (for suspension), chewable, effervescent, film-coated, tablet, orally disintegrating
	Sodium lauryl	Respiratory	Powder
		Sublingual	Tablet
		Topical	Cream, gel, spray, tablet, shampoo, ointment
		Auricular (otic)	Solution, suspension
		Intrarticular	Injection
		Intramuscular	Injection
Cationic surfactants	Benzalkonium	Nasal	Liquid, solution, spray
		Ophthalmic	Drops, gel, solution, suspension
		Respiratory	Spray, solution
		Topical	Cloth, ointment, drops, solution, shampoo
	Quaternium-	Topical	Cream, lotion
		Auricular (otic)	Drops, solution, suspension
		Intramuscular	Injection, powder, suspension
Nonionic surfactants	Polysorbate	Intravenous	Injection, solution, liquid, solution concentrate
		Nasal	Spray, metered
	Ophthalmic	Ophthalmic	Drops, emulsion, solution, gel-forming, extended released
		Oral	Capsule, coated, delayed released, liquid-filled, concentrate, granule for suspension, powder, tablet
		Oral	Suspension
Zwitterionic surfactants	Poloxamer	Topical	Gel, lotion
		Intravenous	Emulsion, injection, powder, lyophilized (for solution)
	Egg	Oral	Tablet
		Oral	Capsule, extended release, suspension, tablet, film-coated
		Lecithin, soybean	Respiratory (inhalation)



**Figure 7:** Molecular structure of bile acids. The steroid nucleus is formed by four rings. The concave side ( $\alpha$ ) is hydrophilic and the convex side ( $\beta$ ) is hydrophobic. Figure adapted from [77].

### 5.5. Sodium Taurodihydrofusidate

Bile salts have limited ability as penetration enhancers through the mucosa, so this fact has stimulated the search

for other structurally related substances, which may further mucosal permeability, exhibit greater protein compatibility, and minimize related toxicity and irritation [83].

The sodium salt of fusidic acid was developed in 1962 by LEO Pharmaceuticals (Denmark) from the fermentation of the fungus *Fusidium coccineum*. The physicochemical properties of the sodium fusidic salts and their glycine and taurine conjugates are similar to free bile acids and their conjugates. Sodium taurodihydrofusidate (STDHF) is a semisynthetic derivative that does not exhibit antibacterial activity and it has a similar structure and micellar characteristics to bile salt taurocholate (Figure 8) [83–85].

STDHF was widely used as an intranasal penetration enhancer for peptide and protein drugs. It was reported that intranasal bioavailability of peptide and protein drugs such as insulin,

calcitonin, human growth hormone, and octreotide were higher when STDHF was used as a penetration enhancer [1].

Deurloo *et al.* reported that 1% of STDHF increases the bioavailability of insulin in rats and rabbits by intranasal administration [87].

Baldwin *et al.* showed that the bioavailability of human growth hormone by intranasal administration increased 11-fold in rats and rabbits and 21-fold in sheep when 0.5% STDHF was used as an excipient [1,88].

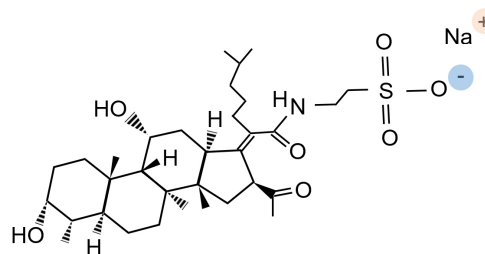
Kissel *et al.* studied absorption improvement and humans' tolerability of octreotide after intranasal administration using STDHF. The bioavailability of formulations with 1.7 and 3% of STDHF were 25.7 % and 29% higher, respectively [89].

It was reported that STDHF, as bile salts, inhibits protease activity and can form transitory pores over lipid membrane [90].

It was reported that STDHF has a mild effect on nasal morphology with a little influence on the ciliary movement. Hermens *et al.* studied the *in vitro* effect of the frequency of ciliary movement of the human nasal mucosa using STDHF as an enhancer [91]. It was evidenced that ciliary movement decreases as the concentration of STDHF increases. When STDHF was used in 0.1 % and 0.2% concentrations, these negative effects decreased, but concentrations of 0.3% or higher induce ciliostasis. However, ciliostasis by STDHF was lower than with laureth-9 (a nonionic detergent) and deoxycholate (a bile salt) [1,90].

**Table 3:** Some of the surfactants evaluated and drugs whose absorption was enhanced [68].

Surfactant	Drug whose absorption is enhanced
1% sodium taurocholate	Scarlet fever toxin
1% sodium glycocholate	Gentamicin
Polyoxyethylene-9-lauryl ether (BL-9, 0,5%) and sodium glycocholate	Hydralazine
1% sodium deoxycholate	Aerosol spray of insulin
Polysorbate 80 (1%)	Progesterone, Testosterone
Carbopol934	Powder form of insulin
Sodium glycocholate (pH 7.6)	Crystalline porcine insulin administered intranasally



**Figure 8:** Molecular structure of taurodihydrofusidate. Figure adapted from [86].

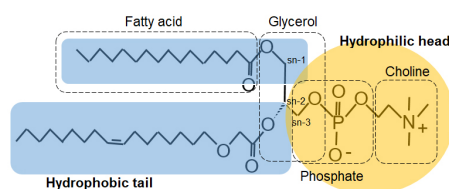
STDHF has two very important limitations. First, the tendency to promote the aggregation of proteins and peptides at pHs below their isoelectric points results in dramatic decreases in protein solubility. This is a result of ion pairing between the negatively charged sulfonic acid moiety on STDHF and basic functionalities on the protein. The second limitation is the wide variation in absorption enhancement for different proteins. These limitations decrease the overall utility of STDHF as a nonspecific mucosal permeation enhancer [82].

### 5.6. Phospholipids

Phospholipids are surfactant compounds found in both animal cells and plant cells and have been explored as nasal permeation enhancers [1].

These substances are lipids that contain phosphorous, a hydrophilic head and a hydrophobic tail. The hydrophilic head and the hydrophobic tail are attached to an alcohol. Depending on the chemical nature of the alcohol, they can be classified into glycerophospholipids and sphingomyelins [92].

Glycerophospholipids have glycerol in their backbone. The sn-1 and sn-2 positions of the glycerol backbone are esterified with fatty acids of varying lengths and degrees of saturation, while the sn-3 position is esterified with phosphoric acid (Figure 9). Variation in the head group leads to different glycerophospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, or phosphatidylserine. On the other hand, in the backbone of glycerophospholipids, there is glycerol; in the backbone of sphingomyelins, there is sphingosine. Furthermore, phospholipids are classified into natural and synthetic origins [90,93].



**Figure 9:** Molecular structure of phospholipids. They are formed by a hydrophilic head and a hydrophobic tail. Glycerophospholipids have glycerol in their backbone and sphingomyelins have sphingosine. The sn-1 and sn-2 positions of the glycerol backbone are esterified with fatty acids with different lengths and degrees of saturation, while the sn-3 position is esterified with phosphoric acid. Variation in the head group leads to different glycerophospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, or phosphatidylserine. Figure adapted from [92].

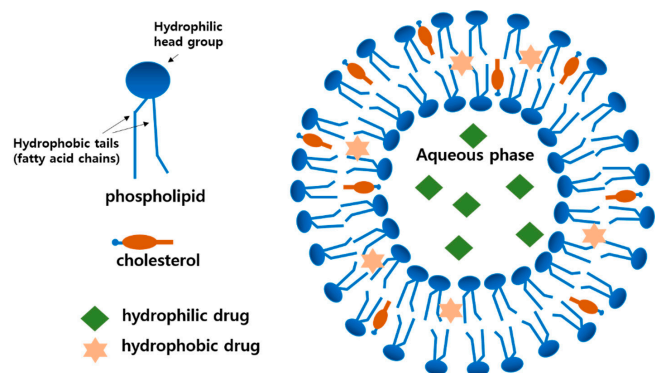
Phospholipids are substances that can self-assemble in an aqueous medium and form colloidal structures, such as liposomes, emulsions, or lipid microspheres. These structures have been widely studied because of their small size and big capacity to take hydrophobic and hydrophilic drugs (**Figure 10**). Also, phospholipids have moisturizing and emulsifying characteristics, which allow them to establish emulsions and cover crystal surfaces to improve the hydrophilicity of hydrophobic drugs. On the other hand, phospholipids are linked to many physiological processes, which provide low toxicity, so they can be used in many administration routes. All these features turn them into potential drug carriers and have been used in many pharmaceutical formulations. In the last few years, various formulations based on phospholipids with clinical applications have been created, such as Doxil<sup>®</sup>, Cleviprex<sup>®</sup>, Valium<sup>®</sup>, and Silybin Phytosome [92–94].

Liposomes have served as carriers for vaccines, antifungals, antitumor drugs, analgesics, and gene therapy drugs. **Table 4** shows some liposome formulations approved for therapeutic use [93].

Regarding the use of phospholipids in intranasal formulations, lysophosphatidylcholine is the most extensively studied penetration enhancer. The absorption of biosynthetic human growth hormone was studied using various permeation enhancers in rats. The relative bioavailability of 25.8% was observed with lysophosphatidylcholine, whereas other permeation enhancers required high concentration to show a similar effect. Drejer *et al.* studied the use of a phospholipid (dodecanoyl-L- $\alpha$ -phosphatidylcholine) as an enhancer for intranasal administration of insulin compared to the subcutaneous route. They found that using this excipient, the intranasal administration was fast, with bioavailability 8.3% higher than subcutaneous

administration and without irritation. Alsarra *et al.* studied liposomes of dipalmitoylphosphatidylcholine and cholesterol as an enhancer for intranasal administration of acyclovir by hydrogel in rabbits. This mucoadhesive-liposomal system showed an improvement on acyclovir permeability by intranasal administration [1,96,97].

Furthermore, in the last few years, nanoparticles have been developed based on phospholipids as a strategy to overcome nasal barriers. Natsheh *et al.* designed the first carrier with vesicles based on phospholipid and modified with ethanol and ethyleneglycol for intranasal administration targeting the brain and systemic circulation. Drugs that used this carrier were efficient for treating diseases such as pain, multiple sclerosis, inflammation, migraine, and insomnia in some animal species. After that, Touitou and Natsheh presented phospholipid magnesosome, a carrier composed of a phospholipid, propylene glycol, magnesium ion, water, and optionally a mucoadhesive polymer such as alginate salt. It was designed to administer peptides, proteins, and small molecules to the brain; experimental evidence shows that it allows a fast and direct route to the brain [98].



**Figure 10:** Scheme of liposome structure. They have a small size and they can take hydrophilic and hydrophobic drugs. Figure was originally published in [95].

**Table 4:** Approved liposomal formulations (commercial name, phospholipids, drug, and year approved [93]).

Product	Phospholipids	Drug	Year approved
Ambisome®	Dihydrogenated Soy Phosphatidylcholine and 1,2-Distearoylphosphatidylglycerol	Amphotericin B	1990 (Europe) 1997 (USA)
Doxil®	Dihydrogenated Soy Phosphatidylcholine and 1,2-distearoyl-sn-glycero-3-phosphorylethanoamine- PEG <sub>2000</sub>	Doxorubicin	1995 1999 2003 (Europe, Canada) 2007
DaunoXome®	Distearoylphosphatidylcholine	Daunorubicin	1996 (Europe) 1996 (USA)
Myocet™	Egg phosphatidylcholine	Doxorubicin	2000 (Europe)
DepoDur™	1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3-phosphorylglycerol	Morphine sulfate	2004
DepoCyt®	1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3-phosphorylglycerol	Cytosine Arabinoside	1999
Lipo-Dox®	Distearoylphosphatidylcholine and 1,2-distearoyl-sn-glycero-3-phosphorylethanoamine-PEG <sub>2000</sub>	Doxorubicin	2001 (Taiwan)
Marqibo®	Egg yolk sphingomyelin	Vincristine	2012 (USA)

## 6. Conclusions

The intranasal administration is recognized as a promising route for the systemic and cerebral administration of drugs.

In veterinary medicine, intranasal administration would be an alternative route to intravenous, subcutaneous, oral, or rectal administration. However, this administration route is not commonly used in horses except for local treatments.

Nevertheless, the drug deals with barriers inherent to the anatomy of the nasal cavity and the chemical nature of the drug before it can reach the site of action, which makes the design of intranasal administration formulations a challenge. This review described the most relevant penetration enhancers that would help us overcome this challenge.

On the other hand, nowadays, there is not enough information about the development of intranasal formulations for equines, and considering the extensive advantages of this route, it is expected that, in the future, studies in this area will increase.

## Authors' Contributions

Concept – F. L.; Design – M. I. V., F. L.; Supervision – F. L.; Literature Search – M. I. V.; Writing – M. I. V.; Critical Reviews – F. L.

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## Conflicts of Interest

The authors declare that there is no conflicts of interest.

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