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**Faculty of Veterinary Medicine
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Department of Clinical Sciences

The Pharmacodynamics of Zuclopenthixol Acetate in Horses

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The Pharmacodynamics of Zuclopenthixol Acetate in Horses

De farmakodynamiska egenskaperna av zuklopentixol acetat hos häst

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SUMMARY

Stress is an important homeostatic function which may not always be entirely positive for the individual. It has been found that stress both has the ability to amplify and reduce pain. Also, pain may induce stress and fear. An important element of pain assessment in horses is the observation of certain pain-induced changes in the horses' behaviour. When pain is accompanied with stress and fear, behavioural pain assessments may therefore be flawed. Long-acting neuroleptics (LANs) have been used empirically to reduce stress during handling and transportation of wild animals. Zuclopenthixol acetate (ZUP) is a LAN that has shown valuable properties within this area. Acepromazine, a neuroleptic commonly used in horses today, has a pronounced sedative effect which is not desirable for long term treatment or for observations of behaviour. It would therefore be advantageous if ZUP had the same fear reducing effect on horses as in wild animals without sedative acting as acepromazine. Studies on the basic pharmacodynamics and pharmacokinetic properties of ZUP have not been published for other species than man. Doses and treatment regimens used in wildlife species have thus been established entirely on a trial and error basis. The objective of this study was to establish a dose of ZUP that would reduce fear or stress in horses, but not induce side effects by testing certain pharmacodynamic properties of selected ZUP doses extrapolated from earlier wildlife studies. Four horses were included in two dose-range finding pilot studies and four other horses were included in a pharmacodynamic study. In the two dose-range finding pilot studies the horses were given either 1 mg/kg, 0.5 mg/kg, 0.25 mg/kg or 0.1 mg/kg as a single intramuscular injection of ZUP (Cisordinol-Acutard[®] or Clopixol-Acutard[®]). Based on the findings in the pilot studies, the dose 0.25 mg/kg was used in a pharmacodynamic observer blinded, placebo controlled, randomised, cross over designed study. That study was conducted in two six-day study sessions with an eight days wash out period in between. Fear behaviour was tested via a Novel Object Test (NOT), sedation and ataxia was scored using two descriptive scales. These experiments were repeated in each study session. Blood sampling was performed for the pharmacokinetic profile presented in the thesis of Belfrage (2016). Two horses scored a higher reactivity score in the NOT when given placebo compared to ZUP. One of these horses also had a slightly elevated sedation score on Day 1 and 3 and showed signs of mild ataxia on Day 3. None of the other horses were scored as sedated or ataxic at any occasion. Overall, the heartrate elevation was larger in the NOT that was performed in Day 2 compared to Day 4 each study session ($P=0.027$) which indicates a habitual effect to the NOT. The effect of ZUP seems to be highly individual since there were no noticeable effects in two horses administered 0.25 mg/kg, while a third horse had side effects from that same dose. Side effects that were documented for doses 0.25 mg/kg, 0.5 mg/kg and 1 mg/kg and comprised extrapyramidal symptoms, muscle fasciculations, inappetence, aggressive behaviour, tachycardia, colic and submandibular edema. The dose 1 mg/kg was chosen because it is recommended for use in several wild life studies including ruminants. No empiric recommendations exist for equids. However the dose of 0.25 mg/kg also caused some side effects which indicates that horses may be more sensitive to this substance than several other species. Since the pharmacodynamic study included only four horses it is not possible to draw any valid conclusion about the drugs' potential anxiolytic effect in horses. The substance may though have an anxiolytic or stress reducing effect in horses since reduction of fear related behaviour to a quite scary novel object was observed in two out of four horses administered 0.25 mg/kg. A safe and at the same time effective dose recommendation of ZUP for horses could therefore not be established in this study. Since horses have shown high sensitivity to ZUP and the drug has a long acting time, the future use of ZUP and the formulations of the drug should be thoroughly considered when proceeding with research.

SAMMANFATTNING

Stress är en viktig homeostatisk funktion som inte enbart är positiv för individen. Studier har visat att stress både kan amplifiera och reducera smärta. Smärta kan också inducera stress och rädsla. En viktig del av smärtbedömning hos häst är att observera speciella smärtinducerade förändringar i hästarnas beteende, men när smärta åtföljs av stress och rädsla kan dessa beteendebestämningar bli bristfälliga. Långtidsverkande neuroleptikum (LAN) har använts empiriskt för att reducera stress vid hantering och transport av vilda djur. Zuploentixol acetat (ZUP) är ett LAN som har visats ha värdefulla egenskaper inom detta område. Acepromazin, ett neuroleptikum som ofta används till häst idag, har en uttalad sederande effekt. Denna effekt är dock inte önskvärd vid långtidsbehandling eller vid beteendeobservationer. Det vore därför fördelaktigt om ZUP visade samma anxiolytiska effekt hos häst som hos vilda djur utan att verka sederande som acepromazin. Studier på ZUPs basala farmakodynamiska och farmakokinetiska egenskaper har inte publicerats för andra djurslag än människa. De doser som använts vid hantering av vilda djur har enbart fastställts genom "trial and error".

Målet med denna studie var att fastställa en dos ZUP som reducerar stress och rädsla hos hästar utan att inducera biverkningar genom att testa specifika farmakodynamiska egenskaper hos utvalda ZUP-doser, extrapolerade från tidigare studier med vilda djur. Fyra hästar inkluderades i två pilotstudier för att finna lämplig dos och fyra andra hästar inkluderades i en farmakodynamisk studie. I de två pilotstudierna gavs hästarna antingen 1 mg/kg, 0,5 mg/kg, 0,25 mg/kg eller 0,1 mg/kg som en enkel intramuskulär injektion av ZUP (Cisordinol-Acutard[®] eller Clopixol-Acutard[®]). Baserat på resultaten från pilotstudien användes dosen 0,25 mg/kg i en farmakodynamisk observerarblindad, placebokontrollerad, randomiserad, cross over-studie. Studien delades upp i två sexdagarssessioner med åtta dagars "wash out" mellan. Rädslereaktioner utvärderades med hjälp av ett Novel Object Test (NOT), sedering och ataxi bedömdes utifrån två deskriptiva skalor. Dessa experiment upprepades under varje studiesession. Blodprover togs för att få fram en farmakokinetisk profil som presenteras i en uppsats av Belfrage (2016).

Två hästar bedömdes ha en högre reaktionsgrad vid NOT då de administrerats placebo jämfört med ZUP. En av dessa hästar bedömdes också milt sederad dag 1 och dag 3 och visade tecken på mild ataxi dag 3. Ingen av de andra hästarna bedömdes vara sederade eller visa tecken på ataxi vid något tillfälle. Hjärtfrekvensstegringen var generellt högre i det NOT som utfördes dag 2 jämfört med dag 4 i båda studiesessionerna ($P=0,027$), vilket indikerar en habitueringseffekt. Doseringen verkar vara mycket individuell då två hästar inte uppvisade någon effekt av 0,25 mg/kg, medan en annan fick biverkningar av samma dos. Biverkningar dokumenterades vid administrering av doserna 0,25 mg/kg, 0,5 mg/kg och 1 mg/kg och innefattade extrapyramidala symptom, muskelfascikulationer, inappetens, aggressivitet, takykardi, kolik och submandibulärt ödem. Dosen 1 mg/kg valdes då den rekommenderas i flera studier med vilda djur inklusive idisslare. Inga empiriska rekommendationer finns för hästdjur. Dosen på 0,25 mg/kg gav, som tidigare nämnts, också upphov till biverkningar vilket indikerar att häst är mer känslig mot substansen än flera andra djurslag. Eftersom den farmakodynamiska studien endast inkluderade en population av fyra hästar försvårar det möjligheten att dra några slutsatser kring substansens potentiella anxiolytiska effekt hos häst. Substansen kan dock ha en anxiolytisk eller stressreducerande effekt hos hästar då reduktion av rädslerelaterat beteende mot ett skrämmande föremål observerades hos två av fyra hästar doserade 0,25mg/kg. En säker och samtidigt effektiv dos kunde inte fastställas i den här studien och hästarna har uppvisat hög känslighet för substansen, vilket bör tas i noga beaktning vid eventuellt fortskridande av denna forskning.

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INTRODUCTION

“The stress response represents an animal’s attempt to reestablish the body’s homeostasis after injury, intense physical activity, or psychological strain.” (Wagner, 2010). Studies have shown that stress has the ability to both inhibit and enhance pain (Jennings *et al.*, 2014; Butler & Finn, 2009). It is suggested that different types of stressors has this property, where anxiety is thought to increase pain and acute stress is linked to inhibiting pain (Rhudy & Meagher, 2000). It can be difficult to distinguish anxiety from fear since they are overlapping states. Fear is usually defined as being induced by a direct threat and anxiety by an expectation of a threat (Öhman, 2008). Stress is an important bodily function to prepare the body for fight or flight and its acute pain inhibiting function may help an injured animal escape a predator despite injury.

Due to rapid and complex biological responses stress is difficult to measure outside the laboratory (Moberg, 1987). Since horses are prey animals they often mask their pain (Weary *et al.*, 2009). Stress may also mask horses’ pain behaviour, thus making it more difficult for veterinarians to detect and treat pain (Lerche, 2009). It is therefore important to evaluate and manage stress in painful or potentially painful situations.

Under certain circumstances, for example under hospitalisation procedures and especially during pain research, it would therefore be desirable to inhibit/modulate horses’ anxiety. This modulation should, however, not act sedating or impair the horses’ behaviour or ability to perform basic needs such as eating and drinking. If the anxiety of a horse could be reduced without the induction of sedation, behaviourally based assessments of for example pain probably would be more accurate.

To decrease stress when handling and transporting wild animals the long-acting neuroleptic zuclopenthixol acetate (ZUP) has been used empirically and been found to have valuable properties (Read & McCorkell, 2002; Read *et al.*, 2000; Holz & Barnett, 1996). Treated animals were easier to handle, showed less increase in physiological parameters indicating stress and acclimatized faster to their new environment. Acepromazine, a neurolept drug commonly used in horses today, has a pronounced sedative effect which is not desirable for long term treatment and the sedated behaviour hampers pain assessment and the use of a neurolept drug with less sedative effect is therefore warranted. However, no studies on the basic properties of ZUP have been published for other species than man. Doses for use in wildlife species have been established entirely on an empiric basis, as have a significant part of the estimations of the wild animals body weight. Reports of both wanted and adverse effects from these wildlife field studies could thus be suspected to be inaccurate and, given the circumstances in the field, also sparse. Therefore, the overall aim of this study was to investigate clinically relevant pharmacodynamic properties of ZUP in horses. The research questions were: which ZUP doses are relevant and safe for horses, whether ZUP has the ability to reduce fear of a novel and frightening object and whether ZUP has sedative properties, including ataxia, or other unwanted effects.

The hypotheses of the study were that doses of ZUP relevant for horses were comparable to those in wildlife and that such ZUP doses would reduce behaviour indicative of fear without inducing ataxia or side effects. The objective of the study was to test different doses of ZUP in horses, extrapolated from earlier wildlife studies, to establish a relevant anxiolytic dose that

would not induce side effects and use this dose to perform a pharmacodynamics study in four horses.

The study was conducted as two dose-range finding pilot studies with four horses and an observer-blinded placebo controlled randomised cross over study of four other horses during two six-day periods. Fear behaviour was tested via a Novel Object Test (NOT), sedation and ataxia was scored using two descriptive scales.

This study was performed simultaneously with the study of pharmacokinetic properties of ZUP (Belfrage, 2016), Parts of the Material and Methods section will therefore be similar between these two studies. Also pharmacokinetic data was made available and will followingly be cited where relevant.

LITERATURE REVIEW

Zuclopenthixol acetate

ZUP is a neuroleptic in the thioxantene class. The effect is probably exerted mainly by blocking Dopamine receptors (Jayakody *et al.*, 2012). The formulation of Cisordinol-Acutard[®] and Clopixol-Acutard[®], used in this study, is oil based and thus providing a slow release of the substance and is effective from one to three days. The formulation is considered very tissue friendly.

ZUP is used for schizophrenia and to reduce psychoses in human medicine. As it has anxiolytic properties it is also used in veterinary medicine, mainly when translocating and handling wild ungulates. In this area, it has been shown to have advantageous properties. Treated animals have shown less incidents of captive myopathy, been easier to handle and have acclimatized faster to their new environment (Read & McCorkell, 2002; Read *et al.*, 2000; Holz & Barnett, 1996). However, the drug has not shown the same positive qualities in all species. In cheetahs, side effects such as ataxia, inappetence, akathisia and extrapyramidal reactions were seen and the authors therefore concluded that zuclopenthixol acetate was not to be used for cheetahs (Huber *et al.*, 2001).

The doses have varied widely among different species, several wildlife studies in ungulates present desirable effects of 1 mg/kg ZUP (Read & McCorkell, 2002; Read *et al.*, 2000; Diverio *et al.*, 1996). In another study red necked wallabies were treated with up to 15 mg/kg with no observed side effects (Holz & Barnett, 1996). Recommended dosing for human varies between 50 and 150 mg per person (Lundbeck, 2011). The substance has not previously been tested in equides.

Stress and pain in horses

The International Association for the Study of Pain (IASP) define pain as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” (IASP, 2012). Pain is an individual experience and studies have shown that the presence of stress both can inhibit pain, so called “stress-induced analgesia”, and enhance pain, also known as “stress-induced hyperalgesia” (Jennings *et al.*, 2014; Butler & Finn, 2009). A study of Marntell *et al.* (2006) showed that horses castrated in a foreign environment (in an animal hospital) needed more anaesthetics than horses who underwent the same surgical procedure in its home environment.

When an animal is exposed to a stressful situation the sympathetic nervous system together with the endocrine system prepares the animal for an emergency. This leads to increased excretion of epinephrine and norepinephrine, increased heart rate, vasoconstriction, reduced motility of intestines, dilation of bronchioles, pupil dilation amongst other things. During stress corticotrophin-releasing hormone (CRH) is also released, which makes the hypothalamus release ACTH. This in turn causes the adrenal cortex to release glucocorticoids such as cortisol (Sjaastad *et al.*, 2010b; Sjaastad *et al.*, 2010a). This reaction is also called the “hypothalamic pituitary adrenocortical axis” or HPA-axis. This axis is often the focus when measuring stress, although it is important to not only investigate this parameter since the response is more complex (Novak *et al.*, 2013).

Physiological parameters used to measure both stress and pain in horses are for instance beta-endorphin, cortisol, heart rate, abdominal sounds and catecholamines (dopamine, adrenaline, noradrenaline) (Raekallio *et al.*, 1997). Since several of the physiological parameters mentioned above increase in both stressful and painful situations it is also important to observe the horses’ behaviour to separate the two states. Behaviours that indicate pain are for instance: head-lowering, flaring of nostrils, kicking towards the abdomen, aggression, pawing, sweating, flight behaviours, restlessness, rolling, reluctance to be handled, teeth-grinding and rigid posture (Bufalari *et al.*, 2007; Raekallio *et al.*, 1997). Another way to evaluate pain in horses is by observing their facial expressions (Gleerup *et al.*, 2015).

Behavioural analysis

In order to measure, compare and quantify horses' behaviour and temperamental traits in a standardized manner, one can use a so-called Novel Object Test (Visser *et al.*, 2001). The Novel Object Test (NOT) is a behavioural test used in several species including horses. The test involves exposing the animal to a novel, objectively harmless, but frightening object, whereupon the animals’ reactions are observed. This test has for instance been used as an objective tool to describe horses’ “personalities” (Visser *et al.*, 2001). This test has also been used in a study similar to this where the behavioural effects of L-tryptophan in horses was investigated (Noble *et al.*, 2008).

Several different objects have been used as novel objects and some objects tend to be more frightening than others (Bulens *et al.*, 2015). The horses also appeared to react more to some colors than others (Christensen *et al.*, 2008; Hall & Cassaday, 2006).

To make the NOT more accurate, several previous studies have habituated the horses by accustom them to the test arena and handling/testing procedure without performing the actual NOT before the test (Christensen *et al.*, 2005).

MATERIAL AND METHODS

This study was approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Dose range finding studies

Two dose range-finding pilot studies were performed. The pilot studies included four warmblooded trotters, one gelding and three mares, aged 4, 5, 5 and 6 years. Based on earlier studies two horses were dosed either 1 mg/kg or 0.5 mg/kg of ZUP (Cisordinol-Acutard[®] or Clopixol-Acutard[®], 50 mg/ml) intramuscular in the neck (Read & McCorkell, 2002; Read *et al.*, 2000; Diverio *et al.*, 1996). Blood was collected for analysis of ZUP concentration, according to expected pharmacokinetic data from the jugular vein. On the basis of these data a second study was performed where two other horses were dosed either 0.25 mg/kg or 0.1 mg/kg and blood sampling schemes were adjusted. Different types of NOT were conducted in order to establish the most reliable method for the pharmacodynamic study.

Study design of the pharmacodynamic study

Four horses were included in an observer blinded, placebo controlled, randomised, cross over designed study. Each horse was dosed either ZUP or saline in the same volume as it would have been if given ZUP in the first session, and the opposite in the second session (see Table 1). The substances were given as a intramuscular injection in the neck on the opposite side of the permanent cannula (see below) by a veterinarian otherwise unrelated to the project and without presence of the research students.

Table 1. *Order in which the horses were given ZUP and placebo respectively.*

	Study session 1	Study session 2
Horse 1	ZUP	Placebo
Horse 2	Placebo	ZUP
Horse 3	Placebo	ZUP
Horse 4	ZUP	Placebo

Animals, housing, handling and management

Included in this study were two warmblooded trotters, a gelding aged 2 and a mare aged 11, one “Svensk Ridponny” gelding aged 23 and one Swedish warmblood mare aged 25. The horses belonged to the Swedish University of Agricultural Sciences (SLU) teaching herd, and were housed and cared for at SLU according to the guidelines for horse keeping (Jordbruksverket, 2014). They were stabled on shavings in individual loose boxes in a compartment of the stable, separated from the other part of the teaching herd stables. The horses were let out during the day (except on Day 1 each study session) approximately between 07.00 and 15.00 hours. The horses were fed grass hay four times a day, water ad libitum and a small amount of soaked sugar beet with some paraffin oil between Day 1 and Day 4 to 5 each study session to avoid constipation. Each box was equipped with a salt lick. The experiments were carried out between 17 October 2015 and 11 November 2015. One day before the first injection a complete clinical examination was performed and all clinical findings were noted. Daily from Day 1 (day of injection) to Day 7 general condition, heart rate, respiratory rate, capillary refill time, appearance of oral mucosa, temperature and bowel sounds (right dorsal and ventral and left dorsal and ventral) were noted.

Blood sampling

A permanent cannula was inserted in the right jugular vein (first study session) or left jugular vein (second study session) prior injection of the substance and was used for blood sampling within the first 24 hours. After removing the permanent cannula a Vacutainer[®] with a 20 G needle was used. Blood sampling was performed at 0 hours (just before injection of substance or placebo) and at 2, 4, 8, 16, 24, 36, 48, 72, 96, 120, 144, 168 and 192 hours post injection using Vacuette[®] EDTA tubes. After blood sampling, the tubes were immediately centrifuged at 3000 rpm for 10 minutes and the EDTA plasma was then separated and collected in a Cryo Tube[™] or Sarstedt micro tubes. The EDTA plasma was frozen to -20 degrees C immediately after centrifugation.

When all blood samples had been collected the tubes were transported in a Styrofoam box at -20 degrees C to Lundbeck A/S, Copenhagen, Denmark, for analysis. These data are reported in a thesis of Belfrage (2016).

Novel object test (NOT)

Test arena

The NOTs were performed in the indoor riding arena of SLU, located approximately 200 meters from the stables where the horses were kept. The riding arena measured 27 x 21 meters with 2.2 meter high walls. The underlay of the arena consisted of sand and small gravel. The colour of the underlay was brown/beige and the arena was illuminated by strip light during the tests.

The novel object consisted of a radio-controlled electric car (Vortex A969 Truck RTR 2.4GHz) measuring 29 x 17 x 6 cm with the body wrapped in black self-adhesive bandage to give it a uniform colour. On top of the car two balloons of the same colour, measuring approximately 25 cm in height and 18 cm in width, were attached. They stood for the overall impression of the novel object (see Figure 1). The car was connected to different corners of the arena every NOT by a 3-meter string with a shock absorber made out of a Penrose drain (elastic string) in the end to ensure the same travel distance in all NOTs. The top speed of the car was 40 km/h. The RC-car had a distinct motor sound.



Figure 1. *RC-car with balloons used as novel object.*

Three dummies mimicking the car were made out of test tube racks, wrapped in the same self-adhesive bandage measuring 25 x 15 x 5 cm, see figure 2. The dummies were also equipped with two balloons each, with the same size as those attached to the car. The colours of the balloons were green, blue, yellow and pink/red.



Figure 2. *Dummy mimicking the novel object.*

In each corner (below named 1, 2, 3, 4) of the testing arena, the novel object (RC-car) or a dummy was placed 50 cm from the wall. Between each NOT session both the colour of the balloons in respective corner, and in which corner the novel object was placed, was changed (see Table 2). The setup for the first NOT session was randomly selected and the following setups were balanced.

Table 2. *The colour of the balloons attached to the dummies and the RC-car relative to the corners. The colour of the balloons on the RC-car followed by “*” which indicates from which corner the RC-car is coming from in each NOT.*

	Corner 1	Corner 2	Corner 3	Corner 4
Novel object test 1	Pink/Red	Blue	Green*	Yellow
Novel object test 2	Yellow*	Green	Blue	Pink/Red
Novel object test 3	Blue	Pink/Red*	Yellow	Green
Novel object test 4	Green	Yellow	Pink/Red	Blue*

In the middle of the arena, 17 m from the corners, a bucket with oats was placed. Figure 3 shows a sketch of the arena.

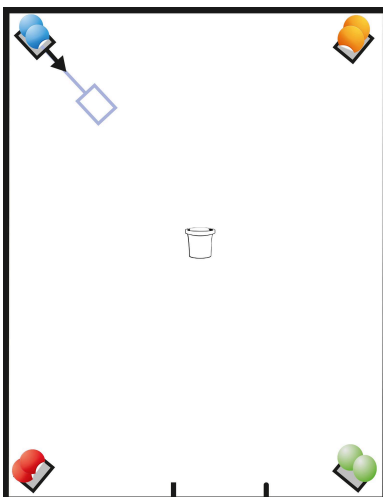


Figure 3. *Sketch of the test arena. The model with the blue balloons symbolizes the RC-car in its original position and with its end position marked. The other models with balloons symbolize the dummies. The food bucket is marked in the middle. The sketch is not made to scale.*

Testing procedure

Prior to the testing, the horses were habituated to the test arena at least two times the two days before the test. The habituation consisted of leading the horse two laps around the arena and then letting it eat oats from the food bucket for a couple of seconds. After that the horse was led a few meters from the bucket and released. The handler then stepped out of the arena. The horse was left loose in the arena for maximum ten minutes or until the habituation criteria, which was: eating continuously from the food bucket for minimum 20 seconds, was met. The habituation procedure was adapted from Christensen *et al.* (2005). All horses met the habituation criteria before the NOT was conducted.

Heart rate was measured by a Polar RCX3, an electrode belt (see Figure 4) transmitting to a wrist-watch receiver attached to the halter. The data was stored in the receiver and later transferred to a computer by Polar DataLink. Before the NOT the pulse imaging equipment was attached to the horse inside the stable. The heart rate was recorded during walking to the arena and as the horse was released the handler started a new lap on the monitor to mark this time and relate it to the heart rate during the NOT. During the habituation process the horses wore the pulse imaging equipment once.



Figure 4. *Horse equipped with the electrode belt for pulse imaging.*

The NOT was performed in the arena at Day 2 and Day 4 of each study session, 30-31.5 and 78-79.5 hours post injection, respectively. Thus each horse was subjected to the NOT a total of four times. Before the horse was released the habituation procedure was performed. The handler then stepped out of the arena. While the horse ate from the food bucket the RC-car was run towards the horse in full speed to the end of the attached string (3 meters).

The NOT was video recorded by a Go Pro[®] Hero 3+. To synchronize the recordings the observer started the video recording at the same time as the handler started a new lap on the pulse monitor. The videos were cut in a videoediting program (iMovie[®]) to show 19-48 seconds of the NOT and the clips were then blinded and randomized for the observers. The horses' reactions were later classified by the observers Linda Keeling (professor at the department of Animal Environment and Health, SLU) and Karina B Glerup (DVM, Ph.d., Assistant Professor, University of Copenhagen), using a protocol adapted from von Borstel *et al.* (2010) and Christensen *et al.* (2006) (see Table 3).

Table 3. *Reactivity scores used to classify the horses' reaction during the NOT.*

Score	Reaction
0	None. The horse may or may not direct its attention (turn ear and/or eye) to the stimulus but does not stop eating/chewing or lift the head in response to the stimulus.
1	Head up. The horse throws its head up, stops eating but does not move away.
2	Alert. The horse quivers and may take up to five steps away from stimulus.
3	Away. The horse jumps to the side or back and trots or gallops away for more than five but less than ten strides.
4	Flight. The horse jumps to the side and or back and gallops or trots for more than ten but less than fifteen strides.
5	Run off. The horse jumps to the side and or back and gallops or trots for more than fifteen strides.

The time before the horse returned to feeding after the appearance of the Novel Object was noted. The test ended either when the horse returned to feeding or when the horses had been inside the arena for a maximum of 10 minutes. If a horse did not return to feeding within the maximum time the time was set to 60 seconds.



Figure 5. *Horse eating from the food bucket in the NOT.*



Figure 6. *Horse reacting on the novel object which is marked with an arrow.*

Sedation scoring and head position in stable

On Day 1 and 3 at 16.30-17.30 hours, which correspond to 5.5 – 6.5 and 53.5 – 54.5 hours post injection, respectively, the horses were video recorded using a Canon Ixus digital pocket camera in their boxes. Before each video session the horses' compartment of the stable was left quiet for a minimum of 30 minutes.

The horses were filmed for a minimum of 3 minutes at each occasion. The session started by filming the horse from a distance, if possible without disturbing the horse. The horse was then approached and its face filmed in profile and from an approximately 60-degree oblique angle visualizing both nostrils from a short distance. After that the observer interacted with the horse and presented with a bucket of oats. The assessment of depth of sedation was performed directly by the author and later by Pia Haubro Andersen (professor at the department of Clinical Sciences, SLU) based on a sedation scale used by Taylor *et al.* (2014), see Table 4.

Table 4. *Scoring system for sedation.*

Score	Signs
0	No sedation. Animal is alert, with normal posture and response to contact with assessor.
1	Mild sedation. Low head carriage, relaxed facial muscles and pendulous lower lip.
2	Moderate sedation. Head lowered towards ground and swaying of hind legs.
3	Marked sedation. Nearly becomes, or becomes, recumbent.

While filming, if possible before the horse was disturbed, its head position was noted and scored by the author while video recording and later the clips were evaluated by Pia Haubro Andersen according to Table 5.

Table 5. *Scoring system for head position.*

Score	Position of muzzle
0	Muzzle above shoulder.
1	Muzzle between shoulder and carpus.
2	Muzzle between carpus and fetlock.
3	Muzzle below fetlock.

Ataxia scoring

To detect any ataxia the horses were led both up and down a curb four times and in figure of eights for four times. These gait assessment tasks were adapted from Olsen *et al.*, 2014. This test was performed at Day 1, 3 and 6 at 17.00-18.00 hours, which corresponds to 6-7, 54-55 and 126-127 hours post injection. Ataxia was assessed directly by one of the research students according to Table 6.

Table 6. *Scoring system for ataxia.*

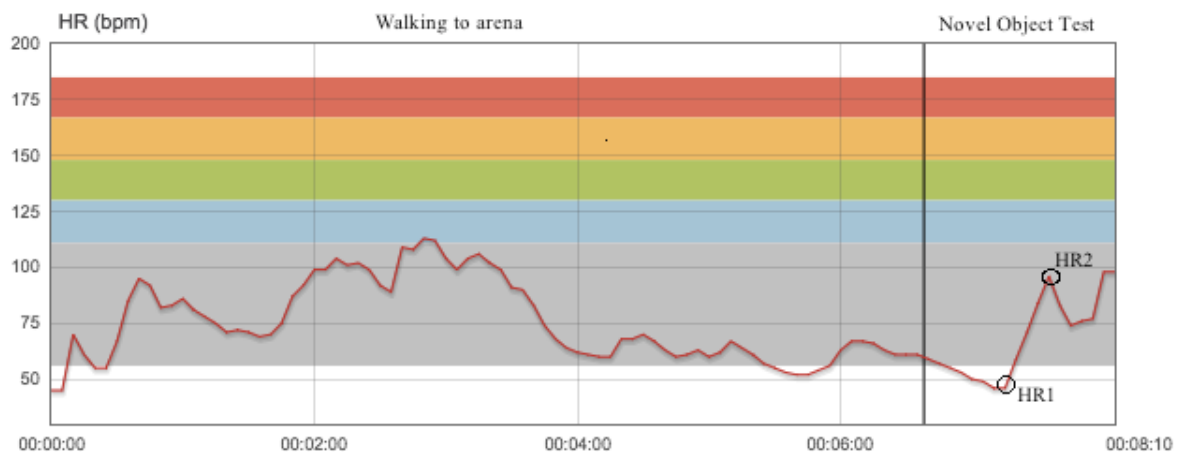
Score	Degree of ataxia
0	No ataxia. The horse stands and walks normally; is able to turn tightly.
1	Mild ataxia. The horse is able to walk, but has some lack of limb control.
2	Moderate to marked ataxia. The horse can walk only with support or is unable to walk without danger of falling.

Statistical analysis

No statistical analysis was performed on the pilot studies. In the pharmacodynamic study all data were entered in an Excel[®] sheet and descriptive graphs and tables were made of raw data, where possible. Minitab[®] 17 statistical software was used for statistical analysis.

From the Polar data sheet, the heart rate when the novel object appeared (HR1) and the first turning point (peak or bottom) after the appearance of the novel object (HR2) was extracted. See Table 7.

Table 7. *Polar data sheet showing heart rate when walking to arena and during NOT. HR1 and HR2 are marked with circles.*



The following hypotheses were tested:

- Is the difference between HR1 and HR2 smaller when given ZUP instead of placebo?
- Do the horses, when given ZUP, return to feeding faster than when given placebo?
- Do the horses score a lower reaction-score when given ZUP instead of placebo?

The above-mentioned hypotheses were also tested including only the first NOT conducted each study session (NOT 1 and 3) and by comparing NOT 1 and 3 to NOT 2 and 4.

RESULTS

Novel object test

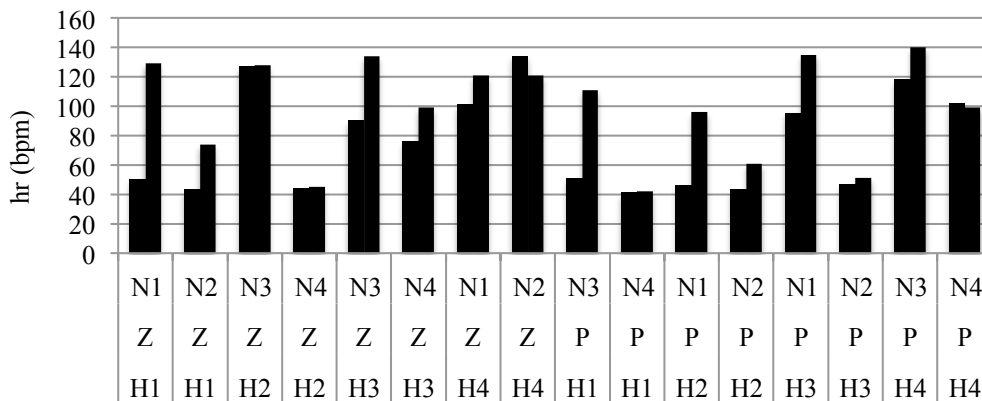
The scorings made of both evaluators are presented in Table 8 and the evaluators are named Evaluator 1 and 2.

Table 8. *Scorings NOT. E1 = Evaluator 1, E2 = Evaluator 2. *Treated with ZUP.*

Study session	Day of NOT	NOT score							
		Horse 1		Horse 2		Horse 3		Horse 4	
		E1	E2	E1	E2	E1	E2	E1	E2
1	2	3*	5*	3	4	5	5	1*	1*
	4	2*	3*	2	2	1	1	1*	1*
2	2	3	3	1*	1*	3*	5*	3	5
	4	1	1	0*	3*	3*	5*	2	2

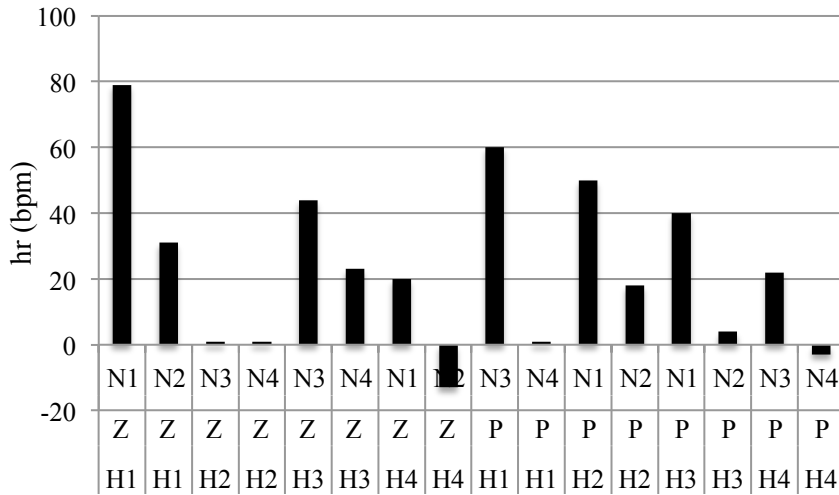
The heart rate when the novel object appeared (HR1) and the first turning point (peak or bottom) after the appearance of the novel object (HR2) for each horse and NOT are presented in Table 9.

Table 9. *HR1 and HR2 in each NOT for each horse. N = Novel Object Test, Z= ZUP, P=Placebo, H=horse.*



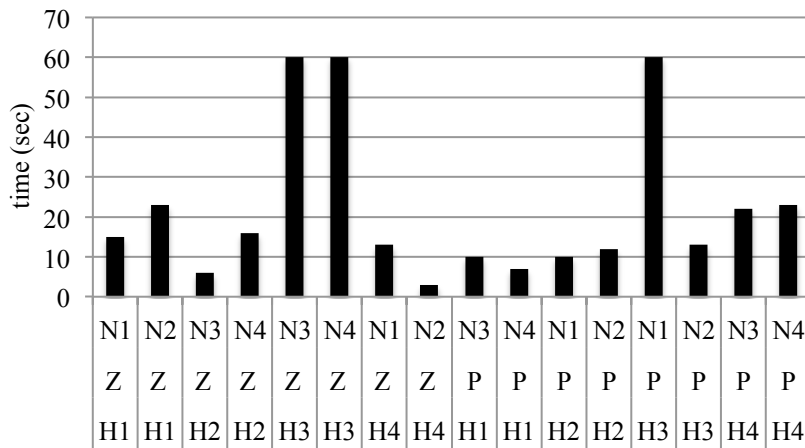
The difference between HR1 and HR2 for each horse and NOT are presented in Table 10.

Table 10. *Difference between HR1 and HR2 in each NOT for each horse. N = Novel Object Test, Z= ZUP, P=Placebo, H=horse.*



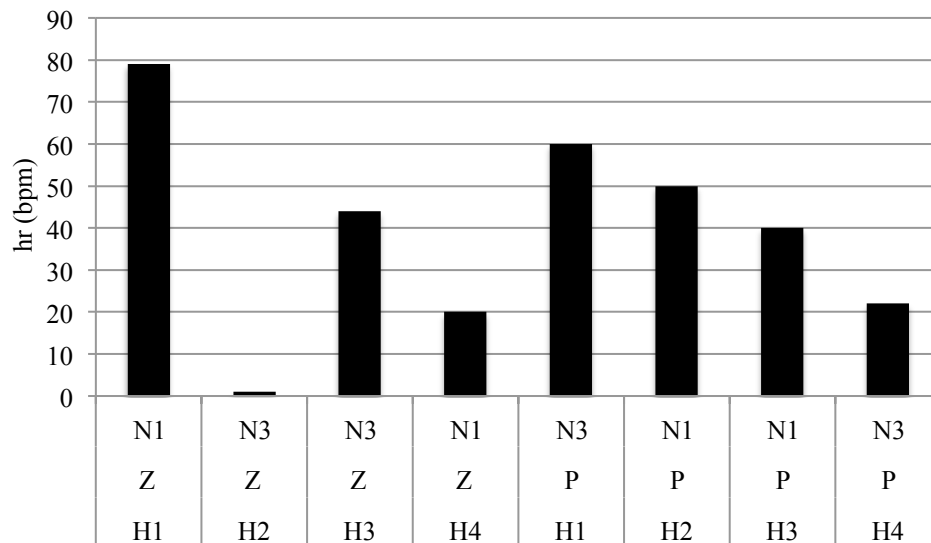
Time before the horse returned to feeding after the appearance of the Novel Object is presented in Table 11. On three occasions (in NOT 1, 3 and 4) Horse 3 did not return to feeding within the maximum time and the time was therefore set to 60 seconds.

Table 11. *Time to return to feeding in each NOT for each horse. N = Novel Object Test, Z= ZUP, P=Placebo, H=horse.*



The difference between HR1 and HR2 for each horse in NOT 1 and 3 are presented in Table 12.

Table 12. *Difference between HR1 and HR2 in NOT 1 and 3 for each horse. N = Novel Object Test, Z= Zuclopenthixol, P=Placebo, H=horse.*



There were no significant differences between ZUP and placebo in any of the tested parameters. The difference between HR1 and HR2 were significantly greater in NOT 1 and 3 compared to NOT 2 and 4 ($P=0,027$, paired t-test).

Sedation scoring

The sedation and head position scores are shown in Table 13 and 14. One horse (Horse 4) was rated as mildly sedated and had a lower head position (muzzle between shoulder and carpus) on Day 1 and Day 3 when given ZUP.

Table 13. *Sedation score. *Treated with ZUP.*

Study session	Day of scoring	Sedation score			
		Horse 1	Horse 2	Horse 3	Horse 4
1	1	0*	0	0	1*
	3	0*	0	0	1*
2	1	0	0*	0*	0
	3	0	0*	0*	0

Table 14. *Head position score. *Treated with ZUP.*

Study session	Day of scoring	Head position score			
		Horse 1	Horse 2	Horse 3	Horse 4
1	1	0*	0	0	1*
	3	0*	0	0	1*
2	1	0	0*	0*	0
	3	0	0*	0*	0

Ataxia scoring

Ataxia scores of all horses are shown in Table 15. One horse had mild ataxia on Day 3, two days post injection.

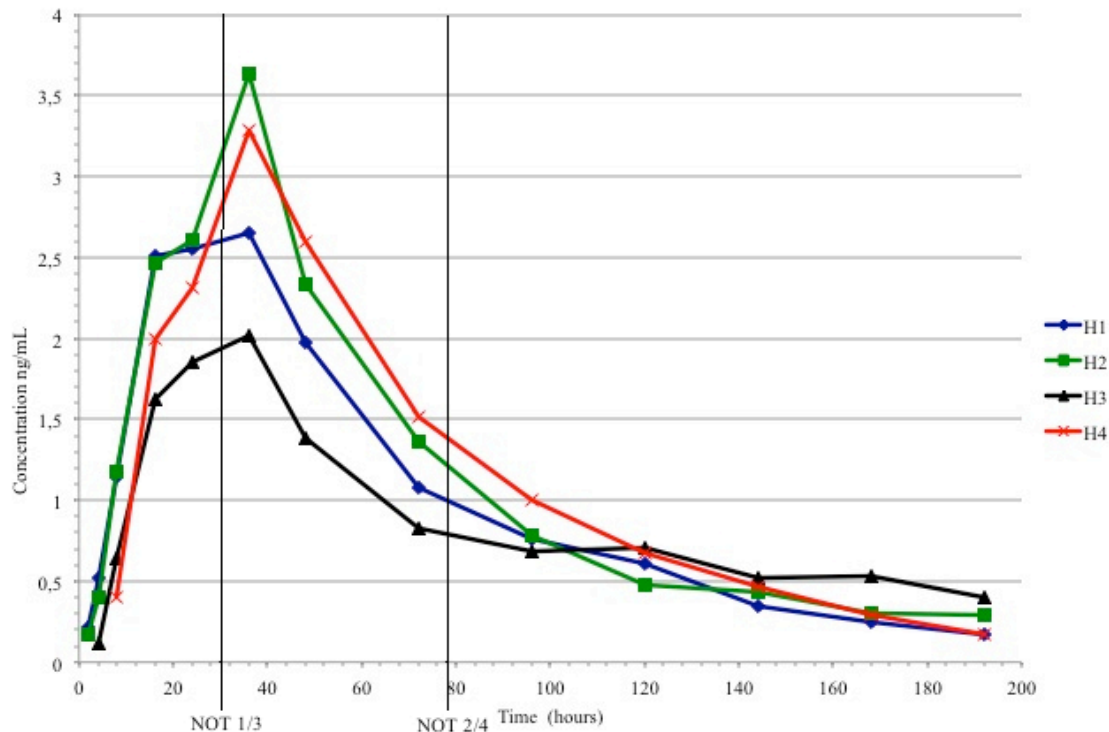
Table 15. *Ataxia scores. *Treated with ZUP.*

Study session	Day of scoring	Ataxia score			
		Horse 1	Horse 2	Horse 3	Horse 4
1	1	0*	0	0	0*
	3	0*	0	0	1*
	6	0*	0	0	0*
2	1	0	0*	0*	0
	3	0	0*	0*	0
	6	0	0*	0*	0

Pharmacokinetics

The plasma concentration of ZUP was about twice as high when performing the NOT on Day 2 (NOT 1/3) compared to Day 4 (NOT 2/4), see Table 16. The pharmacokinetics of the study are investigated in the thesis of Belfrage (2016).

Table 16. *Time of NOT relative to plasma concentration in all horses. H=horse.*



Side effects

One horse given 1 mg/kg obtained severe extrapyramidal symptoms including rhythmic head tossing in a snakelike manner, pawing, compulsive circling, sweating, backing up against the walls and kneeling on both carpi. This horse also developed intermittent tachycardia, muscle fasciculations, edema in the submandibular area and later signs of colic.

Another horse given 0.25 mg/kg also presented with colic symptoms and was diagnosed with constipation in flexura pelvina. Muscle fasciculations were also seen in another horse given 0.25 mg/kg.

Two horses, one given 0.5 mg/kg and one given 0.25 mg/kg showed aggressive behaviour towards humans when handled. One horse given 0.25 mg/kg became mildly inappetent. No adverse tissue reaction at the injection site were seen.

Other observations

One horse included in the pilot study, who was given 1 mg/kg, resented (mostly by powerful attempts to escape) when handling its permanent cannula during blood sampling before and just after the injection of ZUP. At the second blood sampling, 4 hours post injection, and henceforth the horse stood completely still when handling its permanent cannula.

DISCUSSION

This study aimed to investigate whether ZUP decreases fear and anxiety in horses. As fear and anxiety are emotions which cannot be communicated directly between man and horse, current knowledge of horses behaviour and reaction to frightening experiences was used to design test systems that could be used for this. Parameters that were used was: elevation of heart rate, time to return to feed and reaction to a novel object. Since only four horses were included in the study, statistically validated conclusions could not be drawn. Therefore this study should be regarded as an initial pilot study. In the following, the performance of the above mentioned methods will be discussed.

Novel object test

Reduction of fear related behaviour was observed in two out of four horses administered 0.25 mg/kg. One of these horses was scored as less reactive on the NOT in the first study session, when treated, compared to the second study session when given placebo, indicating that despite any habitual effect to the NOT ZUP did seem to have an anxiolytic or stress reducing effect in some horses. The plasma concentration of ZUP was about twice as high when performing NOT on Day 2 (NOT 1/3) compared to Day 4 (NOT 2/4). Therefore the test values from NOT 1 and 3 were analysed separately.

A habitual effect to the NOT was demonstrated as a significantly greater difference between HR1 and HR2 in the NOT performed Day 2 (NOT 1/3) compared to Day 4 (NOT 2/4). Habituation may hamper this test and to reduce the habitual effect, the spatial location of the moving object (corner from which the novel object came from), and the colour of the balloons attached to the moving object, was varied in a balanced matter for each NOT. The colours used for the balloons in the NOT were green, blue, yellow and pink/red. As previously shown by Smith & Goldman (1999) horses can discriminate these colours from grey. To avoid the habitual effect completely the NOT should have been performed only once but then the horses could not have been their own controls. Without the horses being their own control such a small number of horses would carry the risk to not give any relevant information since the horses' reactions to the NOT are highly individual (Visser *et al.*, 2001). Another alternative would have been to include a large number of horses and treat half of them with ZUP and the other half with placebo before the NOT. In that way the individual variation would become less significant and the effect of habituation could have been ruled out.

It is important to note that this study only included measuring heart rate elevation and behavioral parameters. There is, as mentioned above, several other parameters to measure stress that could be used to gather more information. At the same time, taking for example blood samples at the time of NOT could be stressful in itself and therefore bias the results.

To avoid "novelty effect" of the surroundings and thus make the NOT more specific, the horses were habituated to the arena with all the equipment needed for the NOT. The habituation procedure was repeated until the horse could stand still and focus on eating for 20 seconds. In this way the only novel object was the moving RC-car and not the entire arena. Besides the habitual effect the practical performance of the NOT worked well and the horses displayed large variation in reactivity both between individuals and test sessions. Another novel object that was tested in the pilot study, an umbrella lowered from the ceiling (also used in Visser *et al.* (2002) and Visser *et al.* (2001)), barely caused any observable reaction in any of the horses and was therefore not suitable for this study. The fact that the novel object used in this study differed quite a lot from the novel objects used in several previous studies and

also made sounds makes the results less comparable to these studies although the principle is the same.

The scorings of the NOTs were consistent between the evaluators in most cases though one evaluator systematically scored the reactions lower than the other. This may be due to lack of sufficiently clear instructions, borderline cases, subjective definitions of what counts as a step or difficulty to count the amount of steps. The ethogram used had been proven useful in other studies (von Borstel *et al.*, 2010; Christensen *et al.*, 2006).

Ataxia and sedation

In contrast to the NOT scoring there was a high degree of agreement between the raters of ataxia, probably due to the simplicity of the scale. One out of four horses became mildly ataxic two days post injection. Ataxia was also seen as a side effect in cheetahs treated with ZUP (Huber *et al.*, 2001). The same horse was also assessed as mildly sedated on Day 1 and 3. This means that sedation can not be ruled out as a side effect of a first dose of ZUP. In humans, sedation is reported as a side effect during the first doses of ZUP, and the sedative effects then resides with subsequent doses (FASS, 2014). We did not test further doses and whether sedation would disappear with repeated dosing in horses is therefore not known.

Establishment of a relevant dose of ZUP

A ZUP dose which was safe and clinically effective for all horses could not be established in this study. The fact that the dose of 0.25 mg/kg gave no noticeable fear reducing effect in two horses and side effects in a third horse indicates that the effect may be highly individual. This is surprising since the drug has been used extensively during the last 30 years for wild life handling. The recommended dose varies considerably depending on the species. Horses thus seem much more sensitive to the side effects of ZUP than other species, and this is important to note before any further use of the drug is initiated. Parallels to such species differences is also seen in other drugs, for example the $\alpha 2$ antagonist xylazine where cattle doses are around 10% of effective horse doses (Greene & Thurmon, 1988). Since both the horses in this study and the cheetahs in the study of Huber *et al.* (2001) were sensitive to the substance this species variation should be considered in further research of the substance in other species. The reason for this species differences can only be speculative, but dopamine receptor profiles, numbers of receptors and the neuroanatomy of the extrapyramidal system may be involved.

As mentioned above fear is usually defined as being induced by a direct threat and anxiety by an expectation of a threat (Öhman, 2008). Since the novel object is perceived as a threat the NOT causes fear. The NOT was performed four times with each horse in this study and therefore the horses may have had an expectation of the threat the last three times and due to that they may have felt anxious as well. As a consequence this study has the potential to induce both fear and anxiety. It would therefore be interesting to design a study focusing only on whether the substance reduces anxiety since this mainly is thought to amplify pain sensation. It is though difficult to determine what kind of stress an animal is experiencing.

Side effects

The extrapyramidal adverse signs seen in one of the horses from the pilot study were very similar to the side effects described in horses treated with the neuroleptic fluphenazine decanoate (Baird *et al.*, 2006). Extrapyramidal signs have been observed in humans and white-tailed deer treated with zuclopenthixol acetate (Hassler *et al.*, 2014; Read & McCorkell, 2002).

Since two horses in the pilot studies presented with obstipation of the large colon all horses in the pharmacodynamic study were prophylactically administered oral paraffin oil together with soaked sugar beet once a day from Day 1 to Day 4-5 of each study session. The concrete reason for the colic is not known but the horses had been subjected to several colic predisposing factors such as relocation, change of forage, feeding routines etcetera before this study and it is therefore not possible to determine whether or not the substance was the cause of colic (Cohen *et al.*, 1999). However, constipation is listed among common human side effects, though as a long term effect (FASS, 2014). One disadvantage of the slow release drug formulation is that it can not be reversed if side effects appear. The formulation in visceo oil was therefore not suited for this research purpose. The formula is otherwise advantageous since it reduces the number of injections. The side effects are discussed more in detail in the thesis of Belfrage (2016).

Aggression

In this study two horses showed aggression towards humans during handling at the time of peaking blood concentrations. The theories behind the causes of this are obscure but interesting. Most horses today are trained with negative reinforcement (McGreevy & McLean, 2011). A study by Sankey *et al.* (2010) showed that horses trained with negative reinforcement instead of positive reinforcement sought less contact with humans and showed “an increased emotional state” during training. Wildlife studies suggest that the animals treated with ZUP became less afraid of humans than the controls (Read & McCorkell, 2002; Read *et al.*, 2000; Holz & Barnett, 1996). We speculate if it is possible that the reason for this aggression is of a similar manner as seen in these wildlife studies. Could aggression be a form of expression for a loss of boundaries set by training with negative reinforcement? Aggressive behaviour has also been observed in dogs treated with ZUP during research. The dogs were usually kept two and two in cages but had to be separated due to fighting during the treatment of ZUP (Højelse, 2016).

Ethics

Since it is impossible to know exactly how an animal perceives its surroundings, it is likewise problematic and difficult to evaluate the possible effect of a drug that may act through change of perception. If the drug would reduce fear and anxiety during unpleasant situations of horses animal welfare might eventhough be increased. One can speculate whether the altered perception was experienced as unpleasant. No reports of unpleasant body feelings have been reported from the use in humans with psychosis. Since the horses were healthy and seemed much more sensitive to the drug, this information may not be valid for horses. Two horses in this study were reluctant to enter their boxes the days following the injection of ZUP but not before or after treatment. This may indicate that ZUP in these doses inflicted discomfort in these horses, though the extent of the negative aspects of these experiences remain uncertain. With this in mind more research is needed in this area and it is crucial that the risks and benefits of using these drugs are carefully analysed to ensure that the positive aspects outweigh the negatives. The use of ZUP may be of interest in relation to research, for example in pain research when elimination of stress related behaviour is needed, but other drug formulations that allowed rapid adjustment of the dose would be preferable to avoid side effects.

CONCLUSION

The response to ZUP seems to be highly individual and horses seem to be far more sensitive to the drug than other species. The doses 0.5 and 1 mg/kg gave rise to moderate and severe side effects in two horses. A dose of 0.25 mg/kg caused mild side effects in one out of four horses. A dose of 0.1 mg/kg did not have any observable effect in one horse. ZUP did not give rise to pronounced ataxia or sedation in the dose 0.25 mg/kg. The substance may have an anxiolytic or stress reducing effect in horses since reduction of fear related behaviour was observed in two out of four horses administered 0.25 mg/kg. However, a safe and at the same time effective dose recommendation of ZUP for horses could not be established in this study.

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