avogadro LS

Set-up and validation of a paw inflammation model with kaolin in cats

CONFIDENTIAL

1. INTRODUCTION

The aim of this study was to set-up and validate a feline model of reversible paw inflammation induced by Kaolin injections as previously described and validated in the literature [1] with Meloxicam as drug of reference at the dose 0.3 mg/kg. Meloxicam was selected as a test NSAID, because it is a well-established NSAID in cat [2] and also used in dog [3] and man.

The set-up was carried out in the same conditions than the aforementioned validation [3]. This model would be useful in effectiveness assessments of anti-inflammatory and analgesic drugs targeted to cats as the primary species [4] [5].

2. MATERIAL AND METHODS

2.1. STUDY DESIGN

The study plan was favourably assessed by the Avogadro LS Animal Ethics Committee.

Animal housing and care comply with the recommendations of Directive 2010/63/EU. The animal facilities at Avogadro LS have the authorisation number D 31 188 01 obtained from the French Veterinary Authorities and the animal care and use program is AAALAC accredited.

The trial was designed to involve 8 cats for the comparison of results in a two-sequence, two-period cross-over study. In each period, animals received by subcutaneous route either Meloxicam at 0.3 mg/kg (Metacam solution injectable®) as reference item or 0.9 % saline solution (Placebo) at the same volume that reference item.

Time zero (abbreviated ‘T0’) was defined, for a given animal, as the end time of kaolin injection.

2.2. ANIMAL TRAINING AND SELECTION

All included animals were acclimatized in the same conditions and trained for:

- Tunnel Creeping Time (TCT).
- The peak vertical force (PVF) of the paw when the animal walks along the force plate with a harness and a lead.

Training took place in 20 european short hair male cats over around 4 weeks in order to get 9 ready. Animals were considered as fully trained when repeated consecutive values of TCT showed no further improvement and when their walk on the Force Plate became regular. All cats were also acclimatized to the rectal temperature measurements by measuring their rectal temperature for the whole acclimatization period.

Within one week prior to inflammation induction, 8 animals were selected based on results obtained during training sessions. One remaining animal was also selected to be considered spare. Spare animal was definitely removed from the study after the last of animals was successfully induced with Kaolin.

During the washout period; animals were trained the last week before the second paw inflammation induction, once a day.

2.3. HOUSING

The animals were collectively housed in pens throughout the in-life phase, except from the moment of induction to the last outcome measure of the period, when they were individually housed.

2.4. PAW INFLAMMATION INDUCTION

A reversible Kaolin inflammation model [1] was used to induce an experimental acute inflammation of hind paws.

A Kaolin suspension was prepared at a concentration of approximately 250 mg/g It was administered in the right paw (for all the cats) on Period 1 and in the left paw on Period 2 by subcutaneous injection in sterile conditions under general Medetomidine at 80µg/kg, by intramuscular (IM) administration and ketamine at 5 mg/kg (IM administration) was used.

At the end of the Kaolin injection, medetomidine effects were reversed by IM administration of 200 µg/kg of atipamezole.

The induced inflammatory process lasted on average about 4 days, but it depended on individuals.

2.5. ADMINISTRATION OF REFERENCE ITEM AND PLACEBO

Cats were dosed the reference and placebo after T26h assessment by subcutaneous route.

2.6. OBSERVATION OF ANIMALS

Each animal was observed at least once daily and abnormal findings were recorded.

2.7. EFFICACY ASSESSMENT

To assess the analgesic efficacy of the reference item, four outcome measurements were obtained by blinded operators in the following order:

1. Rectal temperature
2. Tunnel Creeping Time. (TCT)
3. Peak vertical force (PVF, expressed in kilograms) applied to the ground for the induced hind limb measured with a force plate.
4. Lameness Score (LSc)

The efficacy assessment was performed two times per day; on one day within the 4 days preceding the inflammation induction (before inflammation and dosing, baseline) and then at T24h, T26.5h, T29h, T31.5h, T34h, T36.5h, T39h, T48h, T50.5h, T53h, T55.5h, T58h, T60.5h, T72h, T75h, T78h, T81h T96h, T99h, T102h, T105h, T120h, T124h, T128h, T144h after induction with an allowed variation of ±10 minutes. After T144h, one assessment per day was performed until signs were completely disappeared on each of the cats.

2.7.1. MEASUREMENT OF RECTAL TEMPERATURE

Rectal temperatures were measured (in Celsius degrees) once for each event using a digital medical thermometer.

The baseline corresponds to the mean of the rectal temperature measured during 4 days of the training period.

2.7.2. LAMENESS SCORE (LSC)

Lameness of the limb receiving the Kaolin injection was assessed watching the cats walking for one minute, using the following scale [1]:

Observation whilst walking	**Score**
Full weight bearing, no lameness at all	0
Barely detectable lameness over the most of the observation period (lameness with substantial weight bearing can be observed on a few strides)	1
Mild lameness (substantial weight bearing)	2
Moderate lameness (minimal weight bearing)	3
Severe lameness, uses paw (walking movement initiated and/or touches lightly the ground) but does not bear weight	4
Could not be more lame (reluctant to rise and/or avoidance of any contact of the affected paw with the ground)	5

2.7.3. MEASUREMENTS OF TUNNEL CREEPING TEST (TCT)

Creeping time through a tunnel was measured (in seconds) five times per event in order to obtain the 3 most homogenous values.

2.7.4. MEASUREMENTS OF THE PEAK VERTICAL FORCE EXERTED BY THE HIND LIMB ON A FORCE PLATE

The peak vertical force (PVF, expressed in kilograms) applied to the ground for the induced hind limb was measured with a force plate in order to allow simultaneous measurement of the PVFs exerted by the limbs of the cats during walking.

The force plate SATEL (SATEL-Patrick Savet, Blagnac, France) was connected to a computer equipped with a digital analogical acquisition card and a signal processing software (Satel Véto, ENV Toulouse, France). The force plate was inserted in a path which the cats were trained to walk on a lead at a constant and similar speed.

Cats passed at least 5 times in the path of the force plate in order to obtain 3 interpretable values for the hind limb.

2.8. ANIMAL FATE

After the end of the in-life phase, animals were returned to the Avogadro LS' stock.

The spare animals were removed from the study and returned to stock on Day 0, once successful Kaolin induction of the study animals was completed.

3. RESULTS

The results obtained in this study are presented below and are detailed for each parameter of efficacy assessment: presentation of data obtained during in-life phase correlated with corresponding statistical analysis results.

The statistical analysis consisted on validating the inflammation model by evaluating firstly the group homogeneity between the Placebo and the Reference groups and the kaolin effect to confirm the inflammation was correctly installed.

Then a validation to confirm that the model is applicable to the test of anti-inflammatory and analgesic drugs, the effect of the meloxicam on each of the studied parameters was studied through the post-inflammation period.

Signs of inflammation of hinds paws were observed until Day 14 for both periods, therefore, the efficacy assessments were performed until signs disappeared.

3.1. CLINICAL SIGNS

Some vomits were observed in two animals, not related to the treatment, but possibly with hairballs (transparent and foamy).

As a secondary effect of the Kaolin injection, some suppuration and a white spot were observed in some of the injection sites of two animals, around 32 days after the inductions. This is a normal reaction of the tissues to eject extraneous substances and no further consequences were observed, therefore, it was not considered as an adverse event.

3.2. RECTAL TEMPERATURE

Kaolin efficacy for Rectal Temperatures:

- This parameter could not be validated for Period 1 (p-values > 0.01 between baseline-T24h and Baseline-T26.5h) inductions but only for Period 2.

Efficacy of reference item:

- A non-statistical comparison of both treatments was performed by using a ratio of recovery to normalize all results taking into account the individual variability. The means of efficacy by groups is presented below in Figure 1, where 0% represents no recovery at all, comparing to the mean of the results obtained between T24 h and T26.5 and 100% a return to the baseline. All calculations were stored with the Study Data.

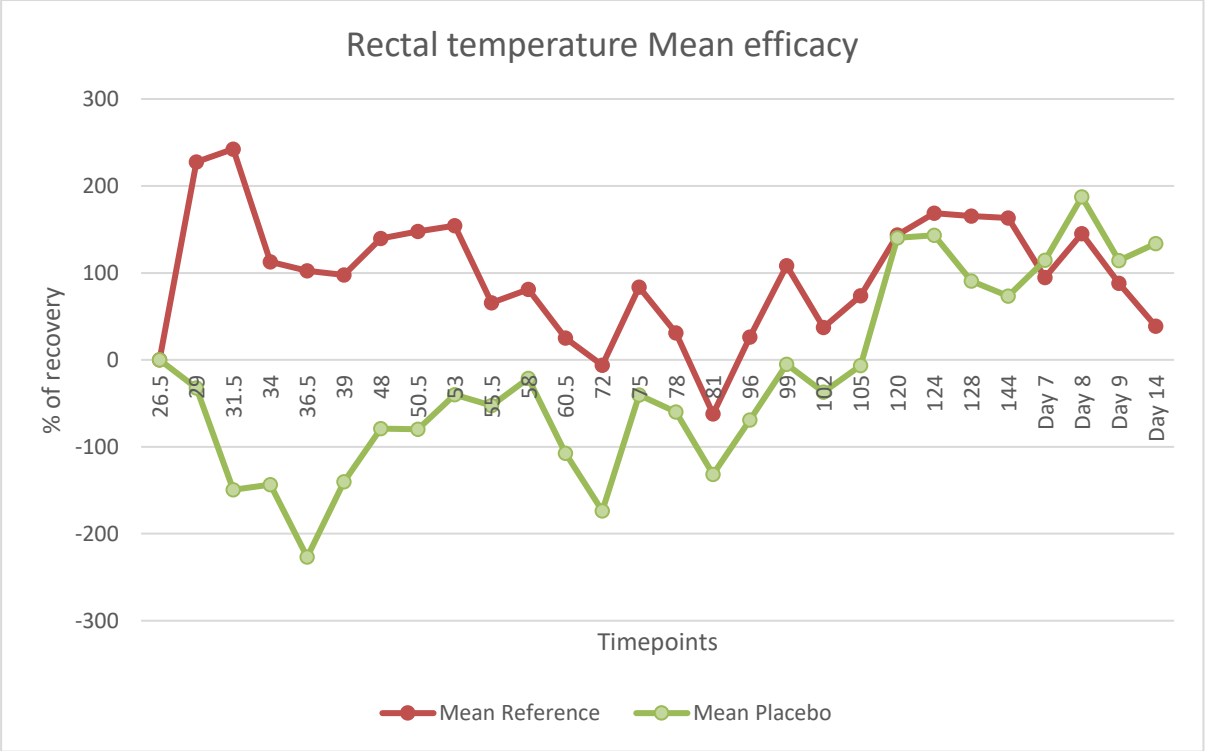

Figure 1: **Mean % of recovery of Rectal Temperature.**

Reference and Placebo Items were administered after T26.5 h assessments. The first assessment was performed 2.5 hours after dosing, when the efficacy of the Reference Item is expected to be installed.

Based on Figure 1, animals having received the Reference Item showed an improvement (return to baseline or exceed the baseline temperatures) after dosing and up to 31.5 h, *i.e* 5 hours after dosing.

- Statistical analysis confirmed the significant difference between Reference and Placebo group from T29 h to T48 h. (p-values <0.001 for all timepoints except at T48 h p-value 0.011)

3.3. LAMENESS SCORE

Kaolin efficacy for Lameness Score:

- This parameter was validated for both periods (p-values <0.001 between baseline-T24 h and Baseline-T26.5 h, highly significant)

Efficacy of reference item:

- A non-statistical comparison of both treatments was performed by using a ratio of recovery to normalize all results taking into account the individual variability. The means of efficacy by groups is presented below in Figure 2, where 0% represents no recovery at all, comparing to the mean of the results obtained between T24 h and T26.5 and 100% a return to the baseline. All calculations were stored with the Study Data.

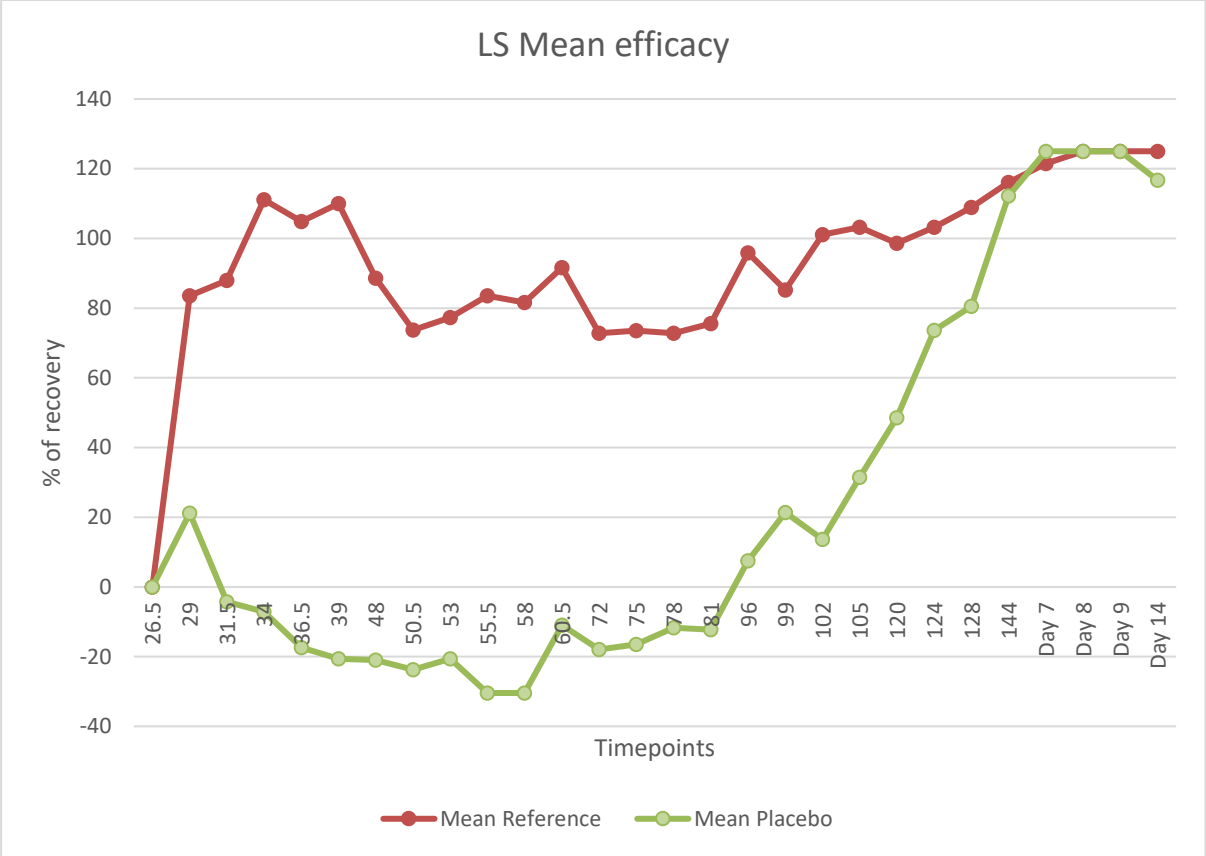

Figure 2: **Mean % of recovery of Lameness Score.**

Reference and Placebo Items were administered after 26.5 h assessments. The first assessment after dosing was performed 2.5 hours later (29 h), when the efficacy of the Reference Item was expected to be installed.

An improvement of more than 80% in average was already noticed during the first assessment after dosing (T29 h) in animals treated with the Reference Item. This improvement remained increasing over the following four assessments until T39 h. After that, improvements were variable increasing or decreasing depending on time points, but were never under 70%. Efficacy of the Reference Item could be considered present until the T81 h assessment, when the animals having received the Placebo seemed to begin their spontaneous recovery, while animals from Reference group continued to show the improvement already seen, even more evident (pass from 75 % to more than 100% from T102 h. Placebo animals showed some slight recovery at T29 h (20%) but were always under the baseline from T31.5 to T81 h, when the spontaneous recovery started.

- Statistical analysis showed a group effect confirming a significant difference between Reference and Placebo group (p-value <0.001).

3.4. MEASUREMENTS OF TUNNEL CREEPING TIME (TCT)

Measured Tunnel Creeping Times were noted for each time point and the mean of the three more homogeneous values was calculated to be used for to the statistical analysis.

Kaolin efficacy for Tunnel Creeping Time:

- This parameter was validated for both periods (p-values <0.001 between baseline-T24 h and Baseline-T26.5 h, highly significant)

Efficacy of reference item:

- A non-statistical comparison of both treatments was performed by using a ratio of recovery to normalize all results taking into account the individual variability. The means of efficacy by groups is presented below in Figure 3, where 0% represents no recovery at all, comparing to the mean of the results obtained between T24 h and T26.5 h and 100% a return to the baseline.

  All calculations were stored with the Study Data.

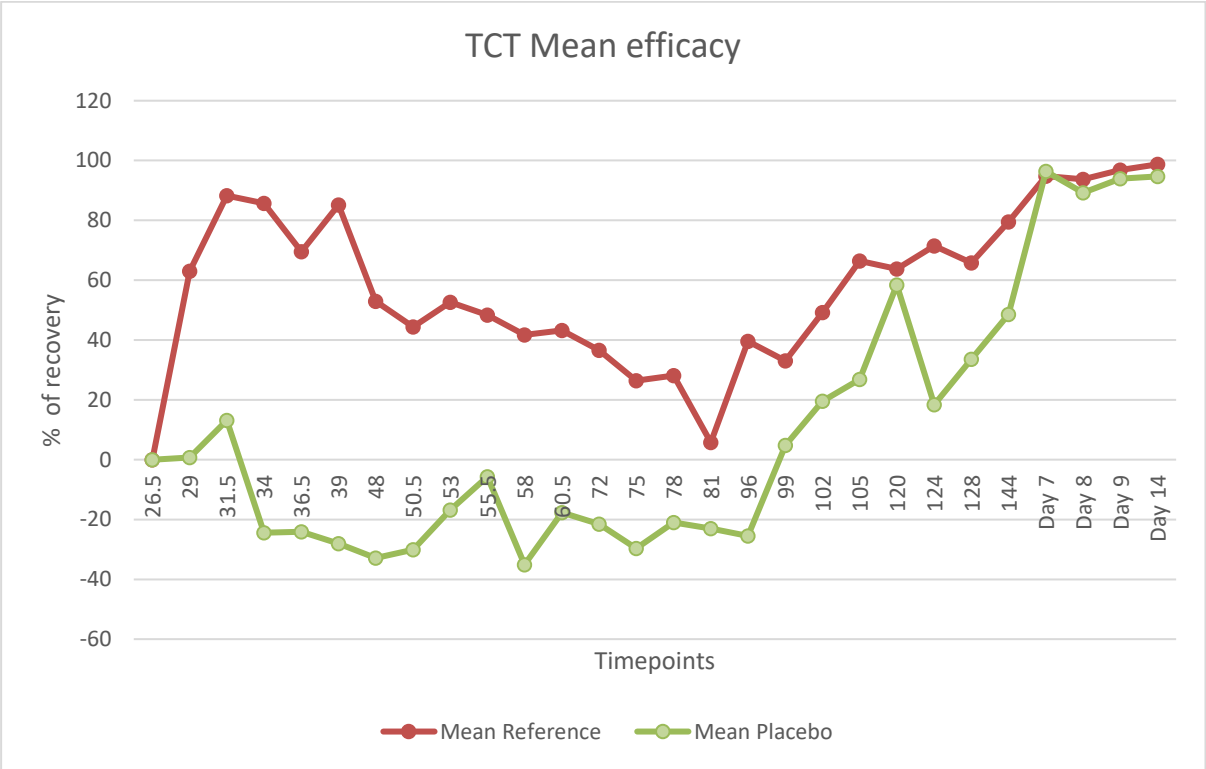

Figure 3: **Mean % of recovery for Tunnel Creeping Time**

Reference and Placebo Items were administered after T26.5 h assessments. The first assessment after dosing was performed 2.5 hours later (T29 h), when the efficacy of the Reference Item is expected to be installed.

Animals having received the Reference Item showed efficacy doing animals recover up to 88% at T31.5 h (five hours post-dosing) and then decreasing gradually, with some slight ups and downs, until 81 h, when the spontaneous recovery seems to start, for both groups: Reference and Placebo. Placebo animals showed some slight recovery at T29 h (15%) but were always under the baseline from T31.5 to T99 h, coinciding with the spontaneous recovery seen in the Lameness Score efficacy.

- Statistical analysis confirmed the significant differences between both groups from T29 h to T60.5 h (p-values ≤ 0.001 until T53 h, then <0.01).

3.5. MEASUREMENTS OF THE PEAK OF VERTICAL FORCE (EXERTED BY THE HIND LIMB ON A FORCE PLATE)

Three interpretable values of the Peak of Vertical Force were issued from the force plate software and used to calculate a mean that was used for the statistical analysis.

Kaolin efficacy for Peak of Vertical Force:

- This parameter was validated for both periods (p-values <0.001 between baseline-T24h and Baseline-T26.5h, highly significant).

Efficacy of reference item:

- A non-statistical comparison of both treatments was performed by using a ratio of recovery to normalize all results taking into account the individual variability. The means of efficacy by groups is presented below in Figure 4, where 0% represents no recovery at all, comparing to the mean of the results obtained between T24 h and T26.5 and 100% a return to the baseline.

  All calculations were stored with the Study Data.

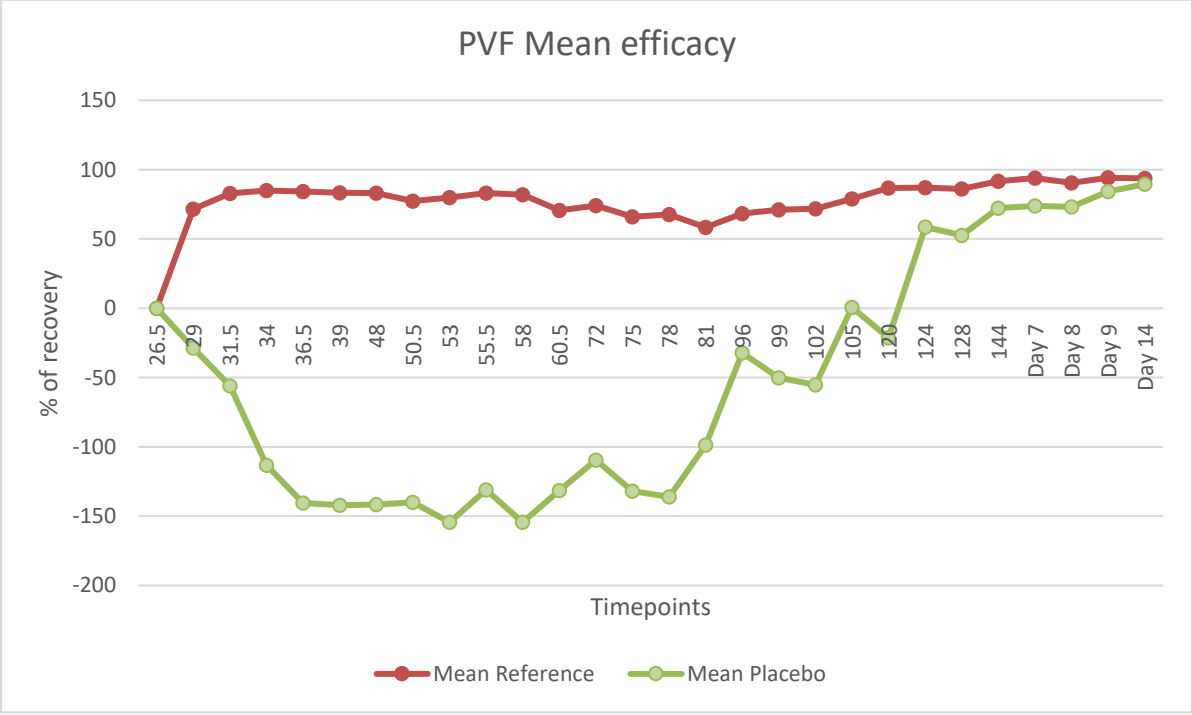

Figure 4: **Mean % of recovery of Peak of Vertical Force.**

Reference and Placebo Items were administered after T26.5 h assessments. The first assessment after dosing was performed 2.5 hours later (T29 h), when the efficacy of the Reference Item was expected to be installed.

Peak of Vertical Force in animals having received Reference Item showed an improvement at all studied time points comparing to baseline, but never at 100% of recovery. For this parameter, recovery due to the Reference Item administration was completely merged with spontaneous recovery. Results of efficacy in animals from Placebo group showed a worsening from the first assessment, arriving to be at less than -150% at T53 h. After this time point, recovery started to improve, little by little, with some up and downs until T120 h, when a real improvement started to reach 95% on Day 14.

- Statistical analysis confirmed the significant differences between both groups from T29 h to T120 h (p-values ≤ 0.001 until T105 h, then =0.002)

3.6. SAMPLE SIZE

A power and sample size statistical evaluation was performed to find out the best sample size to apply in this kind of study.

Results of this analysis revealed different sample size to reach a power of 80% for each studied parameter assuming different scenarios on the expected difference between tested compound and placebo and the expected variability.

In general, to obtain the minimum Standard deviation possible and an "average" difference between tested compound and placebo, the sample size was between 4 and 8 animals. The summary of all obtained results is presented below:

Rectal temperature	**Sigma/Delta**	**1.0°C**	**0.6°C**	**0.5°**
	SD=0.5	N=4	N=8	N=16
	SD=0.8	N=12	N=28	N=40

Tunnel creeping time	**Sigma/Delta**	**20 sec**	**15 sec**	**10 sec**
	SD=10	N=4	N=8	N=16
	SD=12	N=6	N=11	N=24

Peak of Vertical Force	**Sigma/Delta**	**1.5 kg**	**1.0 kg**	**0.6 kg**
	SD=0.5	N=4	N=4	N=10
	SD=0.7	N=4	N=8	N=22

Lameness score	Effect profiles	Strong reference effect vs. Placebo	Intermediate effect Reference vs. Placebo	Weak Reference effect vs. Placebo
		N=8	N=8	N=16

4. CONCLUSION

There were no clinical signs of pathology observed during the in-life phase of this study. Some vomits were observed in two animals, not related to the treatment, but possibly with hairballs (transparent and foamy).

As a secondary effect of the Kaolin injection, some suppuration and a white spot were observed in some of the injection sites of two animals, around 32 days after the inductions. This is a normal reaction of the tissues to eject extraneous substances and no further consequences were observed, therefore, it was not considered as an adverse event.

- **Kaolin efficacy and validation of the model**

  Due to the high individual variability and low number of individuals in the study, a lack of homogeneity was revealed for almost all studied parameters. However, statistical test performed proved differences by time compared to baseline, allowing to validate the model for all parameters except Rectal Temperature on Period 1.

- **Rectal temperature** could not be validated during Period 1 but for Period 2, showing differences until T48 h.

- **Lameness score** showed a group effect in the homogeneity evaluation. However, results in baseline comparisons revealed significant differences, allowing to validate that this outcome measurement is useful in this model, as effects of the kaolin and study items are visible from T26.5h to almost Day 7.

- **Tunnel Creeping Time** was validated with measurable effects from T24 h to T60 h.

- **Peak of vertical force** was validated from T 24h to T 120h. In addition, this parameter is judged as one of the most valuable to be studied in this model because of the objectivity of results, not subjected to the operator's eyes.

- **Use of this model for NSAIDs evaluation**

  The model showed its utility to be used on Proof of Concept studies for analgesic and anti-inflammatory drugs, as the statistical and non-statistical comparisons showed. The principal particularity of this model is the possibility for evaluating long lasting drugs (efficacy to be tested from 24 h to, at least 7 days after) The non-statistical comparisons of the percentage of recovery seemed to be a good complementary method of data analysis to avoid the impact of the lack of homogeneity and allow to evaluate the efficacy of anti-inflammatory or analgesic drugs.

- **Sample size**

  To obtain the minimum Standard deviation possible and an "average" difference between tested compound and placebo, the sample size was between 4 and 8 animals. The sample size should be adapted to the aims of each study and the expected differences.

5. REFERENCES

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