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EVALUATION OF ALTERNATIVE ROUTES OF INTRAVENOUS ADMINISTRATION TO THE METHOD OF ADMINISTRATION SUBSTANCES BY PENILE VEIN IN THE *IN VIVO* ANTIGENICITY TEST IN GUINEA PIGS

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Abstract

Guinea pigs are rodents still used for scientific purposes in experimental models for tuberculosis, diphtheria, vitamin C deficiency, etc. One of the tests to which the guinea pig is widely used is the in vivo test of antigenicity. In this test, the substances are inoculated intravenously onto a previously sensitized guinea pig and analyzed whether the products tested cause anaphylactic shock. Intravenous inoculation is done, according to procedures, 2 times at 7 days in the intravenous penile vein, therefore only males are used. The purpose of the study was to evaluate intravenous inoculation methodology and welfare of guinea pigs by assessing the reaction produced after 3-way intravenous injection, penile vein, auricular vein and safenous vein, in the antigenicity test. The article describes the intravenous inoculation methodology for each route, the clinical health status assessment and the histological evaluation of the inoculated veins. The results obtained show that the safenous vein represents a safe way of intravenous inoculation, both as a technique and as side effects and histopathological lesions, and can be an alternative to the current method.

Key words*: guinea pigs, histological evaluation, intravenous inoculation, inoculation technique.*

INTRODUCTION

Guinea pigs are a rodent, still used in experimental studies, due in particular to their ability to replicate infectious diseases (Padilla-Carlin et al., 2008). Of that is used as an experimental model in tuberculosis and diphtheria in particular (McMurray, 1994). From their guinea pigs that do not synthesize vitamin C are used in metabolic studies respect in deficiency or excess of this vitamin (Frikke-Schmidt et al., 2011). The guinea pigs are used in toxicological studies of antigenicity by verifying that the products tested produce anaphylactic shock (Kouchi et al., 1989; Kawano et al., 1990) and also the guinea pigs has been the animal of choice for predictive sensitization tests for several decades (International Organization for Standardization, 2010).

Guinea pigs also becoming a loved and increasingly popular pet due to the fact that he is small, easy to care and docile (Meredith, 2015). So the morphology of these veins must be known by clinicians (Stan, 2014).

In the tests listed above, but also in some cases of administration of different treatments, intravenous injections are required. This type of injections is quite difficult to do on guinea-pigs because the veins are superficial and fragile. If innovation has made many treatments to be administered subcutaneously, remain the antigenicity tests which test substances is administered intravenously and is the standard with no alternatives at this moment. The most useful method is injection into the penile vein, therefore only the male are used in this test (Takahashi et al., 1986). Other inoculation techniques described in the literature are inoculation into the auricular, saphenous and tarsian vein (Decad & Birnbaum, 1981; Hochman & Blanchard, 1983; Carraway & Gray, 1989). However, there is no analysis of the effects of inoculation on these veins and on

animals, more frequently describing techniques for blood collection from these mentioned or others veins (Hem at al., 1998; Williams & Kendall, 2015; Rodrigues et al., 2017).

This study aims was to simulate the antigenicity test by performing 2 repeated inoculations of the penile, auricular and saphenous veins of a substance undergoing the antigenicity test and the normal saline solution. The effect of inoculations was monitored by assessing animal health status, body weight evolution, necropsy examinations of animals and histopathology of inoculated veins.

MATERIALS AND METHODS

Etics statement

The study was performed in animal facility of Cantacuzino Institute, a veterinary authority accredited facility, in accordance with:

a. Protocol study approved by the Institutional Ethics Committee of the Cantacuzino Institute and the Bucharest Veterinary Authority.

b. Law no. 43 of 11 April 2014 on protection animals used on scientific purposes (Romanian Parliament, 2014).

c. International Standard ISO 10993 Second Edition: 2006-07-15, "Biological Evaluation of Medical Devices - Part 2: Animal Welfare Requirements". Reference Number: ISO 10993-2: 2006 (E) (International Organization for Standardization, 2006).

Animals

Guinea pigs (*Cavia porcellus*), Dunkin Hartley strain, was provided from the animal farm of Cantacuzino Institute. 30 guinea-pigs, males and females were used, youth adult, 10–11 weeks old, weight at study initiation was between 310–335 grams. Animal identification has been marked with a permanent marker on animal fur. Animals were housed under standard laboratory conditions in an environmentally–controlled, air–conditioned room with adequate air supply (12 to 15 air changes per hour), room temperature 19°C to 22°C and relative humidity 45% to 65% with 12 hours of fluorescent light and 12 hours of dark cycle. The temperature and relative humidity were recorded once a day. Animals were housed individually in a standard polypropylene cage with a stainless steel mesh top grill having facilities for holding pelleted feed and drinking water in a water bottle fitted with a stainless steel sipper tube. Clean wood shavings were provided as bedding material.

Guinea pigs were fed with pellet diets manufactured by Cantacuzino Institute. Food was provided *ad libitum* throughout the acclimatization and experimental period. Water was delivered in plastic water bottles with stainless steel sipper tubes ad *libitum* throughout the acclimatization and experimental period.

Acclimatization

Healthy young adult animals were acclimatized for a period of 6 days to laboratory conditions prior to beginning study and were observed for clinical signs once daily. Veterinary examination of all the animals was performed on the day of receipt and on 5th day of acclimatization.

Grouping

The animals were weighed and arranged in ascending order of their body weight. These weight-stratified guinea pigs were distributed to all experimental groups using the Microsoft Excel Spreadsheet, so that the body weight variation of the animals selected for the experiment did not exceed ± 20% (+ 2.96% to 2.29%) of the mean body weight. The grouping was done 2 days before the initiation of study. The body weights of the animals were analyzed statistically for mean body weight to exclude the statistically significant difference between the groups. There were formed 6 groups, 2 consisting of male and 4 of female, each group consisting of 5 animals.

Study Design

The study was composed of 6 groups, G1, G2, G3, G4, G5 and G6 as it is shown in Table 1.

The study included an intravenous injection on day 1 and then on day 8. The dose was 0.2 ml / per animal/per injection. The solvent 0.9% w/v sodium chloride for injection (normal saline) was from Helvetica Profarm SA and control substance was AFLUTOP from Biotehnos Company, substance that have include in his production the antigenicity in the control test. The purpose of the study was to evaluate the intravenous inoculation methodology in guinea pigs and the welfare of the guinea pigs by assessing the reaction produced after 3-way intravenous injection by successive doses at 7 days. The reason for using both test substance and physiological serum was to quantify the lesions produced by the substance separately

from those determined by the inoculation technique and the chosen vein.

Table 1. Study groups

Groups no	Sex	Injected Substance	Intravenous veins
1	Male	Control substances	Penile vein
2	Male	Normal saline	Penile vein
3	Female	Control substances	Ear vein
4	Female	Normal saline	Ear vein
5	Female	Control substances	Saphenous vein
6	Female	Normal saline	Saphenous vein

Preparation of Animals

Intravenous injection was performed on an awake and total anesthetized animal. For anesthesia it was used a cocktail of 45 mg/kg Ketamine (Romvac Company) and 5 mg/kg Acepromazine (Romvac Company) by intra-peritoneal injection because on intramuscular injection necrosis occurs on muscular masses.

Techniques of inoculation

The following inoculation techniques were used:

The guinea pigs in lot 1 and 2 were inoculated intravenously into the penile vein as follows:

- The operator's support keeps the guinea pig upright and presses gently over the genital area to reveal the penis, thus ensuring the stasis;
- The veterinarian pulls the penis out of the foreskin and highlights the lateral penis vein;
- Inserted the needle into the vein and aspire a little;
- Inoculated the solution.
- After inoculation, removed the needle completely and make a hemostasis for at least 15 seconds;
- The needles and syringes that were used were 27G;

The guinea pigs in lots 3 and 4 were inoculated intravenously into the auricular vein, as follows:

- The auricular vein is approached by pulling hair from the side or central ear vein;
- Local anesthesia was applied by applying an anesthetic spray (Lidocaine 10%, 1 shot, Egis Pharmaceuticals LTD);
- Applied an alcohol swab and warm the ear;
- Inserted the needle, aspirate a little, and then inject the substance;
- After inoculation needle is withdrawn completely and make hemostasis for at least 45 seconds;
- The needles and syringes that were used were 27G.

The guinea pigs in lots 5 and 6 were inoculated in the saphenous vena as follows:

- Stretched the leg and approach the saphenous vein by trimming and raised the external and internal area of the foot;
- Disinfected the place of inoculation with alcohol-soaked cotton wool and apply a stasis with a garage or hand over the place of inoculation;
- Disinfected the place of inoculation with alcohol-soaked cotton wool;
- Inserted the needle into the vein and aspire a little;
- Stacked up and inoculated;
- After inoculation, removed the needle completely and make a hemostasis for at least 45 seconds;
- The needles and syringes that were used were 27G;

Monitoring and evaluation

All animals were observed once daily for clinical signs of toxicity and twice daily for mortality. Body weight was recorded prior to initiation of the study (day 1) and at termination. After the completion of the 3 day observation period after the last inoculation, all animals were sacrificed under excessive anesthesia and subjected to necropsy and complete histopathological examination of the inoculated veins.

Histopathological examination was performed on the vein collected from all animals. The veins were collected, trimmed off any adherent tissue, as appropriate. All the mentioned veins below, all from animals, were preserved in 10% v / v Neutral Buffered formalin and embedded in paraffin wax, sectioned at 4 to 5 micro-meters, stained with haematoxylin and eosin and further subjected for histopathological examination. The biological response of

inoculation, which were assessed and recorded as appropriate:
- The extent of fibrosis/fibrous capsule (layer in micrometers) and inflammation;
- The degeneration as determined by changes in tissue morphology;
- The number and distribution of the inflammatory cell types namely polymorph nuclear neutrophil leucocytes, lymphocytes, plasma cells, eosinophils, macrophages and multinucleated cells;
- The presence, extent and type of necrosis;
- Other tissue alterations such as vascularization, fatty infiltration and granuloma formation;

Statistics
Data was subjected to statistical analysis. The computer printout of the data (in the form of appendix) was verified with the original raw data. After verification, the data was subjected to statistical analysis using SPSS Software version 22.
Body weight and percent change in body weight were subjected to statistical analysis. Comparison of means between test item treatment group and predicate sample group was done using 't' test. All analysis and comparisons were evaluated at the 95% level of confidence ($P<0.05$).

RESULTS AND DISCUSSIONS

Inoculation method
Inoculation was performed on each animal corresponding vein group. The inoculation was performed by 3 people, beginner on experiment with animals but experienced in the pet clinic. Inoculation was first performed on vigilant animals, which were then total anesthetized. For inoculation, the lateral veins of the penis, both ears and medial and lateral saphenous veins on both legs from each animal were used. The results show that the inoculation technique was learned by each practitioner after 2-3 inoculations, irrespective of the vein. Total inoculation of the inoculums was 100% in the anesthetized animal, regardless of the vein.
As inoculation features we can say:
Inoculation in the penile vein is the easiest. The vein is turgescent and has a large volume. Anesthesia ensure prolapsing penis and inoculation is easy. No lesions were observed at 7 days.
Inoculation in the auricular vein is the most complicated. The vein is on the surface, fragile, breaks gently, and the inoculated substance can penetrate subcutaneously.
As a rule, per vascular tissue is persistent. Inoculation is easier on white ear guinea-pigs, with the vein grazing slightly, but it can also be done with colored ear specimens if the hair from the ear is well removed.
The veins are not completely restored in 7 days and the alternative use of the ears is recommended.
Inoculation in saphenous vena was done in the medial and lateral vein after hair trimming and shaving of the animal's leg. The vein gets harder, even if given with alcohol.
After identifying the vein, the inoculation should take into account that the skin in the foot area is thickened and puncture the needle can penetrate the vein and a hematoma appears. Inoculation can also be done subcutaneously if it does not penetrate the vein that is sinuous.
At 7 days no injuries to the inoculated vein were observed, and the inoculation could be repeated in the same foot.
After Day 1 inoculation, hematomas were observed in the inoculated areas in 45% of guinea pigs inoculated in the auricular vein and 15% in the guinea pigs in the saphenous vena, and at day 8, 40% in the ear vein and 5% in the saphenous ven.
Figures 1-4 exemplify the above mentioned inoculation techniques.

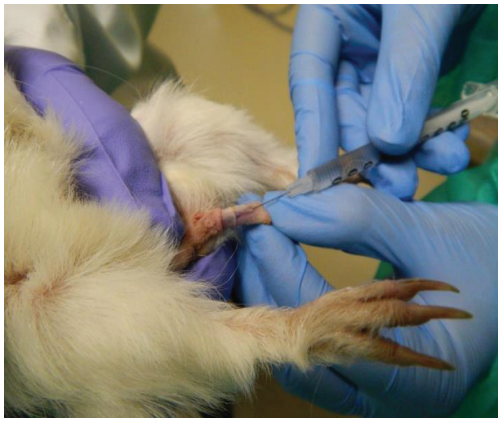

Figure 1. Guinea pig inoculated in penile vein (original photo)

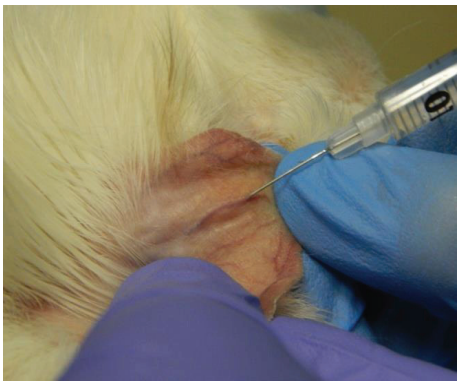

Figure 2. Guinea pig inoculated in ear vein (original photo)

Figure 3. Guinea pig inoculated in lateral saphenous vein (original photo)

Figure 4. Guinea pig inoculated in medial saphenous vein (original photo)

Clinical Signs of Toxicity and Mortality

No clinical signs of toxicity and mortality were observed in any of the animals in all groups.

Body Weight

No inoculation related changes in body weight and percentage change in body weight with respect to day 1 were observed. All animals showed a normal physiological increase in body weights (Table 2).

Table 2 - Body weight

Group, Sex & Treatment	Injected vein	Body Weight (g) on Days		Percent Change in Body Weight with Respect to Day 1 to 7
		1	8	
		Mean	Mean	
		± SD	± SD	
G1, Male & Control substances	Penile vein	339.04	375.07	10.62
		5.93	4.14	2.05
G2, Male & normal saline	Penile vein	343.09	378.16	10.22
		4.22	0.96	4.50
G3, Female & Control substances	Ear vein	340.13	369.28	8.57
		4.50	4.22	0.96
G4, Female & Normal saline	Ear vein	333.56	368.27	11.04
		3.45	3.23	3.29
G5, Female & Control substances	Saphen vein	339.77	376.12	10.69
		5.05	5.59	0.85
G6, Female & Normal saline	Saphen vein	342.80	389.93	11.37
		4.29	5.10	0.56

Necropsy

There were no gross pathological changes observed in any of the animals.

Histological observation

The histopathological findings are presented in Table 4 and exemplified in Figures 5-10.

The lesions produced in groups 1, 3 and 5 shows that the lesions are a consequence of the action of the substance that is irritating to the veins. If the physiological serum is inoculated only in the ear veins, histopathological lesions

of inflammatory and hemorrhagic nature have been observed, which means that the auricular vein is too thin for inoculation or that the inoculation should be done with a much smaller needle. But then the inoculation time will increase.

Table 4 - Histological observation

Group, Sex & Treatment	Inoculated vein	Lesion	Animal no
G1, Male & Control substances	Penile vein	Hyperemia with inflammatory reaction	3
		Crystals in the blood vessels	1
		Presence of crystals and perivascular reaction with mixed cellularity	1
G2, Male & normal saline	Penile vein	No lesion	5
G3, Female & Control substances	Ear vein	Hyperemia with inflammatory reaction	4
		Crystals in the blood vessels	2
G4, Female & Normal saline	Ear vein	Hemorrhage , inflammatory type reactive , vessels wall interrupted	4
G5, Female & Control substances	Saphene vein	Hyperemia with inflammatory reaction	2
G6, Female & Normal saline	Saphene vein	No lesion	5

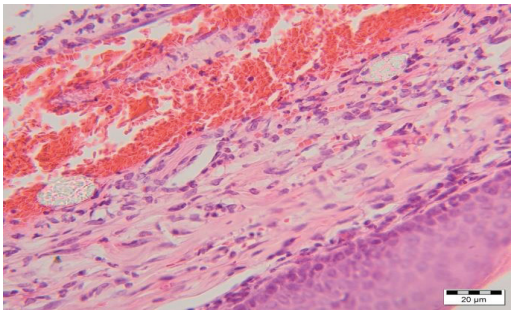

Figure 5 - Penis; guinea pig inoculated in penile vein with test substance; necrosis, secondary infection, altered vessel, perivascular inflammatory, eosinophilic, allergic reactive cells, crystals in the vessel (400×, HE)

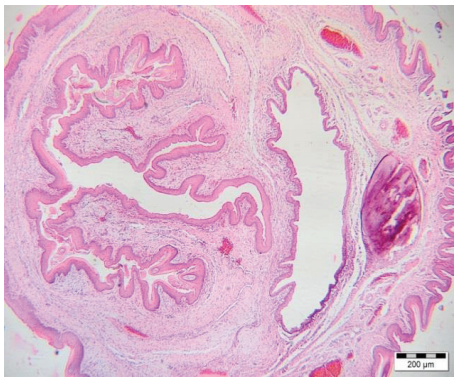

Figure 6 - Penis; guinea pig inoculated in penile vein with normal saline solution; no lesions (40×, HE)

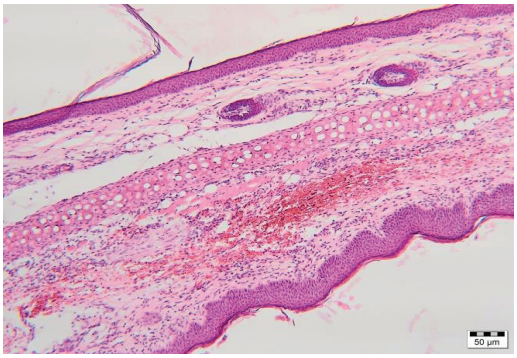

Figure 7 - Ear; Guinea pig inoculated in ear vein with normal saline solution; guinea pig inoculated in penile vein with test substance, perivascular haemorrhagic aspect with disruption of wall integrity (100×, HE)

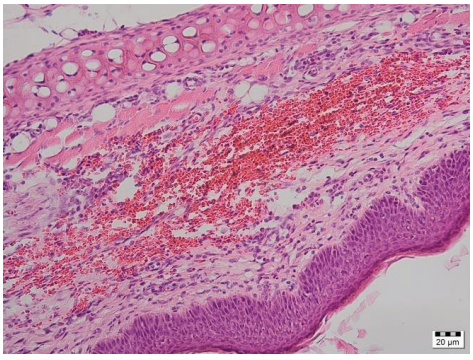

Figure 8 - Ear; Guinea pig inoculated in ear vein with test substance; broken-vessel vessel detail, hemorrhage and acute inflammatory reaction (neutrophil and active local macrophages) (200×, HE)

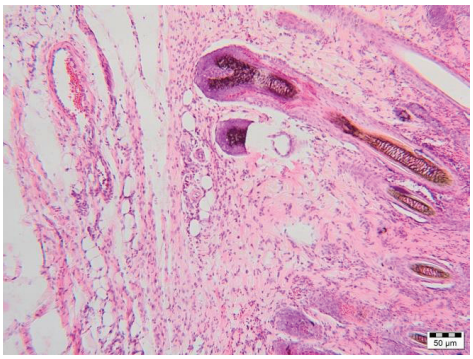

Figure 9 - Saphenous vein; Guinea pig inoculated in saphenous vein with normal saline solution; no lesions (100×, HE)

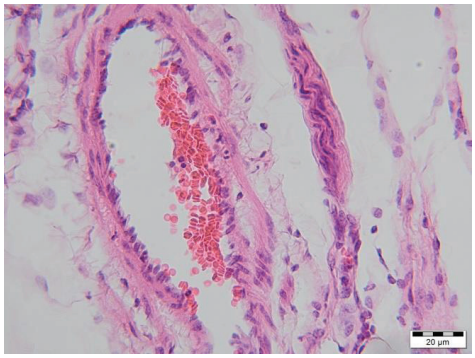

Figure 10 - Saphenous vein; Guinea pig inoculated in saphenous vein with test substance; no lesions (400×, HE)

The corroborated results show that intravenous inoculation technique in the veins accessible to guinea pigs (penile, auricular and saphenous) can be learned relatively easily. Inoculation is better for the anesthetized animal, but a firm contention can provide easy access to the inoculated veins in vigilante animal also. In case of repeated inoculations there were no changes in the weight evolution or pathological clinical signs, except for hematomas in the inoculated veins (auricular and saphenous.) Histologically, for the test substance, injuries to all veins were observed over time when inoculation with saline alone in the ear veins, lesions was observed.

Based on these results, we believe that the safenous vein may be an alternative to penile vein inoculation in antigenicity tests, so females can also be used, and animals can be relocated for other studies after the test. The safenous vena can also be used in the case of guinea pigs used as pets for intravenous treatments.

CONCLUSIONS

In this study, clinical and histopathological effects were analyzed on 3 techniques of intravenous inoculation into the penile, auricular and saphenous vein, and the evaluation of these methods on the veins in an attempt to find an alternative to penile vein inoculation that is now practiced in the antigenicity tests.

The obtained results show that as technique and taking into account the lack of histological lesions, the saphenous vein may be an alternative to the penile vein. In this way, females can also be used in studies.

It remains that the saphenous inoculation method to be validated in the drug control laboratories to enter in the current testing.

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